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Investigating Lymphocytic Involvement in Minimal Change Nephrotic Syndrome

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Summary

Minimal change nephrotic syndrome, also known as minimal change disease (MCD) or minimal change nephrosis, is a subcategory of non-inflammatory glomerulopathy – a degenerative disorder of the main functional unit of the kidney. Neither the cause nor the pathogenesis of minimal change nephrotic syndrome is known. A destructive epithelial disorder would explain the clinical signs of MCD such as proteinuria (excessive protein in the urine), hypoalbuminemia (low serum albumin), and hyperlipidemia (high serum lipids). As a result of the association between the diagnosis of minimal change disease (MCD) and a history of heightened immune responses, it is believed that MCD may be caused by a disorder of T lymphocytes. It is suspected that T-lymphocytic deregulation may release lymphokines toxic to the glomerular basement membrane, which in turn leads to increased ultrastructural permeability (thus leading to symptoms of MCD). We investigated the hypothesis that minimal change nephrotic syndrome is caused by abnormal T lymphocytes, specifically CD4 T cells, as they are known to be involved in many microscopic degenerative kidney disorders involving increased membranous permeability [1]. To test this hypothesis, we measured T-cell counts in peripheral blood samples by flow cytometry. We found that eight of ten patients had decreased CD4:CD8 ratio, thus strengthening the immune involvement postulate of MCD. More specifically, we found the number of CD4 cells to be abnormally low; the results in this study can be used to further investigate whether or not MCD is a true autoimmune disorder. Understanding T-lymphocyte abnormalities in MCD can lead to further research efforts in finding a cause for MCD.

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Introduction

Researchers have proposed several different causes for MCD. It is postulated that T cell deregulation causes damage on the molecular level, [10] specifically ultrastructural degradation of the basement membrane, which in turn leads to greater permeability within the nephron. These changes could explain why proteinuria and hypoalbuminemia are amongst the most common findings in MCD. Others [9] have studied the involvement of CD4 and CD8 lymphocytes in MCD, with disparate results. Lama et al. found that CD8 cells were elevated in the relapse period in patients with chronic renal lesions [9]. Shimada et al. found that CD4 cells were slightly elevated in comparison to other T cell subgroups therefore there is currently no consensus on T-cell involvement in MCD. As such, this study used the CD4:CD8 ratio to identify a precise deregulation in one of the two T-cell subpopulations. We tested our hypothesis via analysis of CD4 and CD8 in the scope that results would elucidate distinct immuno-histological alterations, namely alterations in CD4 or CD8 cells that may cause a decrease in CD4:CD8 ratio. To make quantitative measurements, our approach was to use flow cytometric and indirect immunofluorescence techniques, which are reliable, accurate, and accessible. Our results supported our hypothesis in that absolute numbers of CD4 cells were lower on average in the MCD patient group when compared to the control group. This study supports the theory that autoimmune disease can correlate with renal illness and therefore the results can be used as a "pilot study" for future work.

Results

To test whether T-cells contribute to MCD pathogenesis, we collected peripheral blood samples from ten patients in the experimental group and from five healthy volunteers and used flow cytometry to measure CD4 and CD8 T-cell counts. Proteinuria and hypoalbuminemia (measurable signs) are typical indicators of minimal change nephrotic syndrome in children; quantitative measures of these two values aided in confirmatory diagnosis of MCD. We measured urine protein, via urinalysis, for proteinuria in the experimental and control group. Similarly, we analyzed serum albumin via serum protein electrophoresis in both groups. In addition, we used immunofluorescence and quantitative flow cytometric analysis to quantify the numbers of CD4 and CD8 T-lymphocyte subpopulations in samples from both MCD patients and healthy controls.

Patient	Urine Protein (mg/dL)	Serum Albumin (g/dL)	Absolute CD4 Count
M1	28	2.9	370
M2	39	2.3	240
M3	54	3.8	168
M4	47	2.1	273
M5	63	2.2	270
F1	26	4.1	279
F2	33	2.1	266
F3	32	3.9	300
F4	49	2.8	500
F5	57	5	258
C1	15	3.7	500
C2	10	3.9	670
C3	17	4.3	580
C4	9	4.7	554
C5	11	4.8	665

Table 1: Patient-control laboratory findings. Patients are identified by gender: M (male) and F (female). The accepted range in our laboratory is <20mg/dL. Serum albumin range is 3.4-5.4 g/dL. CD4:CD8 ratio is normal between the values of 1 and 4. All patients (100%) exhibited proteinuria; six (60%) presented with hypoalbuminemia; eight (80%) had decreased CD4:CD8 ratios. No control group patients exhibited proteinuria or hypoalbuminemia. CD4 and CD8 approximate absolute numbers are recorded. CD4:CD8 ratio in healthy controls was normal, with a range from 2.5 to 3.5. p <0.03, Student's t-test.

Once flow cytometry was completed, we calculated the ratio between CD4 and CD8 cells. The lymphocyte ratios were derived by comparing the mean absolute number of CD4 cells to the mean absolute number of CD8 cells. Since T cell subpopulations in a single sample can be quite large, (>1.0 x 10^3 cells per sample) CD4:CD8 ratios were simplified to an *X*:1 ratio, where *X* = number of CD4 cells to one CD8 cell. Once ratios were complied, data interpretation was carried out via comparison of the MCD patient population to the healthy control group. A student's t-test was used to assess statistical significance; the p value was <0.003, therefore the data has a strong statistical relevance.CD4:CD8 quantitative analysis showed markedly decreased ratios in experimental group samples. With the exception of two patients, the experimental group showed a ratio range of 0.4:1 – 0.9:1. Only two patients in the experimental group, M1 and F4, had normal ranges, with values of 3.7:1 and 2.3:1, respectively (**Table 1**). Healthy controls exhibited ratios from 2.5 to 3.5, all within the range previously described [10]. Graphic representation of the ratios (**Figures 1 and 2**) demonstrates the decreased experimental group ratios as compared to controls, specifically decreased CD4 cell counts. These results are promising and are consistent with the theory that MCD is a primary effect of T-lymphocyte deregulation.

Discussion

Neither the cause nor the evolution of minimal change nephrotic syndrome in children is known. The most commonly cited theories include the involvement of the immune system in the pathogenesis of MCD, predominantly the abnormal numbers of T lymphocytes [9,10]. CD4 cell subpopulation correlations were established as the specific "target cell" in our MCD patient population, The results of this experiment do indeed provide evidence supporting the immune hypothesis of disease. Due to the fact that the immunologic theory of MCD does not pinpoint specific T-cell subpopulations, our CD4-specific results can be considered relevant in the development of this theory. The CD4:CD8 ratio is a useful metric as it accurately quantifies the subpopulations of T-cells, thereby allowing for specific identification of abnormalities (decreased CD4 cells in this study). A speculative explanation for this finding lies within the possible helper T-cell release of glomerulo-toxic lymphokine, which degrades the nephron by biochemically altering the permeability of basement membrane cells of the kidney parenchyma. Although we conducted our experiments in a highly controlled environment, our methods varied from that of previous studies [10]. More specifically, laboratory values for serum albumin, as well as urinalysis values, can vary due to collection methods. For example, heparinized blood samples (samples with anti-clotting medium) may potentially dilute the specimen, while non-heparinized collection methods will not. In this study, we used heparinized collection methods in order to keep the blood specimens from coagulating.

Ultimately, further studies are needed to determine whether lower CD4 cell counts are causative of or merely correlate with the development of MCD. Such studies should include comprehensive immunoglobulin testing (gamma globulin M and G has been found to have a correlation with the podocyte process destruction typical of MCD) [8]. This would provide evidence for this classification of MCD as an autoimmune disease. Genetic testing for hereditary traits correlating specifically to

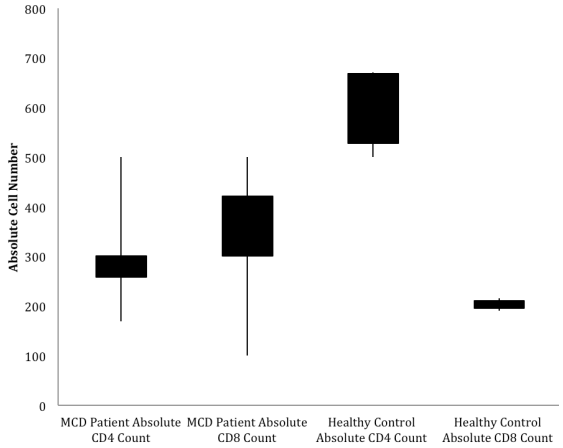

Figure 1: Comparison of absolute CD4 and CD8 cell numbers. On average, MCD patients demonstrated a slightly higher CD8 count (although not clinically relevant as figures were in the normal range) as well as a noticeably decreased CD4 count, when compared to healthy controls.

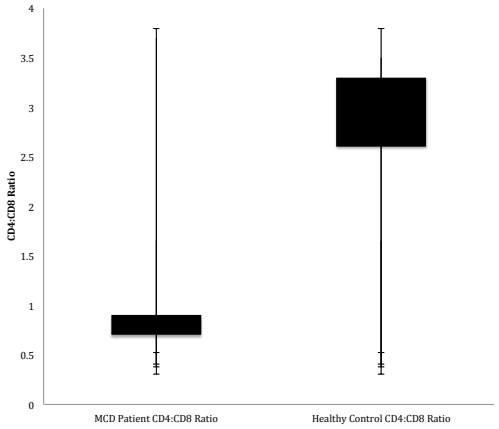

Figure 2: CD4:CD8 ratio comparison. CD4:CD8 ratios were, on average, lower in MCD patient population when compared to healthy control group. $P < .003$, One tailed-Student's t-test.

CD4 deregulation should also be completed. In all, this study can act as a pilot study and as a precedent to future studies with the aim of concluding the cause and pathogenesis of pediatric MCD.

Materials and Methods

Before any samples were taken, parental consent was obtained. All procedures were approved by the hospital review board.

We evaluated ten patients with MCD aged 4 years old. Of these ten, five were male and five were female. All ten patients (100%) had a confirmatory diagnosis of MCD based on their abnormal blood work. All (100%) presented with proteinuria (urine protein concentrations ranging from 26 mg/dL to 63 mg/dL); six (60%) presented with hypoalbuminemia (serum albumin ranges from 2.1 g/dL to 2.9 g/dL) and all (100%) have previously responded to initial prednisone treatments. Corticosteroid therapy prior to experimentation included an initial dosage of 5 mg/kg/day of prednisone until proteinuria dissipated, followed by 1mg/kg/day for the next month. Corticosteroids were discontinued a period of two weeks prior to the study. Five healthy control samples were used (three males, two females), following the same protocol as the experimental group. Our control group was smaller than the experimental group due to a limited healthy patient population that willingly consented to the study. Despite this, all control group subjects were age-matched. Venipuncture and urine collection was carried out according to lab specifications. Following blood collection, immunofluorescent staining was completed along with flow cytometry to gather both qualitative and quantitative results. The immunofluorescent staining procedure was as follows: 10µL of anti-human CD4/CD8 primary antibody pellets (BD Biosciences) were added to 100µL of heparinized whole blood. Samples were incubated at 25 ºC for twenty minutes. To complete lymphocyte analysis, erythrocytes were lysed with 2mL of red blood cell lysis buffer (BD Biosciences) (10:1 dilution, lysis buffer to deionized water) and incubated at 25 ºC for an additional twenty minutes. Samples were then centrifuged at 350 X g for ten minutes and supernatant material was discarded. After this, primary antibodies were conjugated with 10 µL of anti-goat fluorochrome Immunoglobulin g secondary antibody (BD Biosciences). Samples were washed with 1mL staining buffer and re-suspended in 0.25 mL of 2% paraformaldehyde-PBS fixation buffer. Cells were observed under the microscope for any qualitative lymphocytic abnormality. Flow cytometric analysis was carried out to measure, quantitatively, the subpopulations of T-cells and to generate ratio of CD4:CD8 lymphocytes via cell percentages through flow gates . BD FACSCALIBUR flow cytometer as well as De Novo FCS Express 5 flow cytometry software were used in this study. Ratios were recorded and statistical significance was determined using a Student's t-test. CD4:CD8 ratio ranges from the experimental group were compared with ratios from the control group as well as normal laboratory values.

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