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T-lymphocyte subsets in nephrotic syndrome

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T-lymphocyte subsets in nephrotic syndrome. T-lymphocyte subsets when measured in steroid responsive nephrotic syndrome (SRNS) have demonstrated significant variance from normal values. T-cell subsets were studied by using two-color flow cytometric analysis in 32 children (9.2 ± 5 years of age) with SRNS. The children were divided into four groups: a) SRNS in acute relapse, on prednisone; b) SRNS in acute relapse, off prednisone; c) SRNS in long-term remission, off prednisone (nephrotic controls); d) patients in remission on long-term prednisone therapy; and e) 15 age-matched normal controls. Children suffering an acute relapse of SRNS showed an increase in Leu2a+/DR+ (CD8) activated lymphocytes ($P < 0.05$), a decrease in Leu4a+ total T-lymphocytes ($P = 0.01$) and a decrease in Leu3a+ (CD_4) helper T-cells ($P < 0.05$) when compared to normal controls and nephrotic controls. Though some subset changes may represent a prednisone effect and the functional role of these lymphocytes in the disease process is unknown, this study provides additional evidence to support a role for abnormal T-cell subsets in the etiology of SRNS.

Steroid responsive nephrotic syndrome (SRNS) is a glomerular disease characterized by proteinuria, hypoalbuminemia, generalized edema, and hypercholesterolemia. The etiology of SRNS remains unclear, but ample reason exists to suspect a role for abnormal lymphocyte populations and function. The onset of the nephrotic syndrome is often associated with prior upper respiratory tract infections and other immunogenic stimuli such as bee stings or contact dermatitis [1–3]. Remission of SRNS has been known to be associated with measles infection and Hodgkins's disease [4, 5], both of which cause a depression of cell-mediated immunity.

Shalhoub first hypothesized the existence of a circulating basement membrane-damaging lymphokine produced by an abnormal lymphocyte subset in SRNS patients [4, 6]. More specific evidence for lymphocyte involvement includes the finding that T-lymphocytes from patients with symptomatic SRNS demonstrate markedly reduced responses to mitogenic stimulation, an effect which may be related to a circulating humoral factor or lymphokine [6–10]. One previous study using monoclonal antibodies (MAb's) to study lymphocyte subpopulations found no significant differences between SRNS patients and normal control [11]. However, Kerpen, et al earlier demonstrated that SRNS patients had an abnormal lymphocyte population bearing surface markers characteristic of both B and T lymphocytes [12]. Using the relatively new technique of flow cytometry, one can more directly quantitate and examine lymphocyte subsets. We used flow cytometric analysis to study T-lymphocyte subsets in 32 children with SRNS. The use of two-color flow cytometry allowed us to distinguish activated from non-activated T-lymphocytes. Using this methodology, we found distinct abnormalities in the T-lymphocyte subsets of children with relapsing SRNS.

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Methods

Patient population

Thirty-two children with steroid responsive nephrotic syndrome were studied, all of whom are followed in the Pediatric Renal Clinic at Arkansas Children's Hospital (ACH). The diagnosis of SRNS was made from clinical responsiveness to steroids and was not confirmed by renal biopsy. These patients ranged in age from seventeen months to 18 years (mean 9.2 ± 5.2 years). None of the patients were taking or had been given immunosuppressive drugs (such as cyclophosphamide). For study purposes, the SRNS patients were divided into one of three subgroups: (1a) SRNS on prednisone therapy but suffering an acute nephrotic relapse, defined by sudden onset of proteinuria of three days' duration with 3+ protein by urine dipstick; (1b) SRNS patients not receiving prednisone and suffering the same symptoms as Group 1a, including two patients at initial diagnosis; (2) SRNS in long-term remission, defined as being free of both prednisone therapy and proteinuria for at least six months; (3) patients whose disease was in a state of remission maintained by prednisone therapy for at least six months. Thirteen patients were suffering acute relapse symptoms, six in the prednisone-free group and seven receiving prednisone. Eight patients were in long-term untreated remission, and the remaining eleven were in a prednisone-maintained remission. Fifteen normal control patients also participated in the study. These children were enlisted from among outpatients seen at the Children's Care Center, ACH or from children having elective surgery. Only those patients who demonstrated no reasons for suspecting immunological abnormalities or renal dysfunction were selected as normal controls. An attempt was made to match normal patients to the nephrotic patients by age and sex. These normal control patients ranged in age from ten months to fifteen years (mean 10.1 + 4.8 years). The patients in long-term untreated remission served as a nephrotic control group. Twenty-one females and twenty-four males participated.

Informed consent was obtained from all patients and controls (Table 1).

Sample collection and treatment

Peripheral venous blood was collected and treated with a battery of monoclonal antibodies for flow cytometric analysis. Blood was collected in acid citrate dextrose (ACD) tubes and was submitted to monoclonal antibody staining within 48 hours of collection. In most, but not all, an additional sample was obtained for white blood cells (WBC) counts performed by a Coulter Counter and peripheral blood smears.

Monoclonal antibodies (Becton-Dickinson Immunochemistry, Mountain View, California, USA) were labelled with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE), except for the anti-Leu4a/HLA-DR Simultest reagent, which contains both FITC-conjugated anti-Leu4a and PE-conjugated anti-HLA-DR. The MAb's used are defined as follows: anti-Leu4a/HLA-DR Simultest (CD3, total T-lymphocytes); anti-Leu3a (CD4, T-helper); anti-Leu2a (CD8, T-suppressor and cytotoxic); anti-HLA-DR (B-lymphocytes and activated T-lymphocytes), anti-transferrin receptor (proliferating T-lymphocytes), and anti-IL2 receptor (activated T-lymphocytes).

An aliquot of each sample was stained with both an anti-T cell (CD3, CD4, or CD8) and an activation marker for the determination of the percentage of those T-cells that were activated as evidence by staining with that particular activation marker.

The cells were stained using standard whole blood staining techniques as outlined in the package insert. Briefly, the cells were incubated for 30 minutes at room temperature in the dark and the RBC's then lysed with FACS Lysing Solution (Becton Dickinson). The cells were then washed with PBS and fixed with 1% paraformaldehyde. The fixed cells were stored at 4°C and analyzed within 24 hours of staining. Cells were analyzed with a Coulter EPICS 751 flow cytometer (Coulter Corp., EPICS Division, Hialeah, Florida, USA). Parametrized two-color data was collected on the samples after gating on the lymphocyte population using forward and 90° angle light scatter. The threshold for positivity was determined by utilizing isotypic IgG PE and/or FITC as a negative control peak with 1% positive staining occurring outside this peak interval. Percentages of double and single-stained cells were determined using Coulter Quatstats analysis program, on the EPICS 751. This program allows division of a two-parameter histogram into four quadrants, as shown in the sample histogram (Fig. 1). Unstained, or negative cells are boxed into quadrant one and stain with PE only (the activation marker), while cells in quadrant four stain with FITC only (the anti-T-cell). Those cells falling in quadrant two are the double-stained cells positive for both FITC and PE. The percent double positive activated lymphocytes and single positive anti-T-cells but were used to calculate absolute lymphocyte counts for each T-cell subset.

Statistical methods

The mean and standard deviation were determined for each variable in all patient groups. Significance of differences were determined by analysis of variance (ANOVA).

Results

The percent of each T-cell subset obtained by our methodology is given for each diagnostic group in Table 2. Absolute lymphocyte counts for each group are given in Table 3. Not every patient received a complete blood count (CBC) at the time of flow cytometry, therefore, the number of patients with absolute lymphocyte counts is slightly smaller than the total patient population. Patients in acute untreated relapse, defined by 3+ or greater proteinuria, demonstrated a significant decrease in the percentage of total T-cells or Leu4a+ cells, when compared with normal controls ($P < 0.05$). Total lymphocyte counts were also decreased in this group (Fig. 2). There were no differences in percentile or absolute lymphocyte counts between the normal, non-nephrotic control patients and the patients who were in long-term, untreated remission from their proteinuria (nephrotic controls). These two groups seemed similar in all the aspects investigated in this paper.

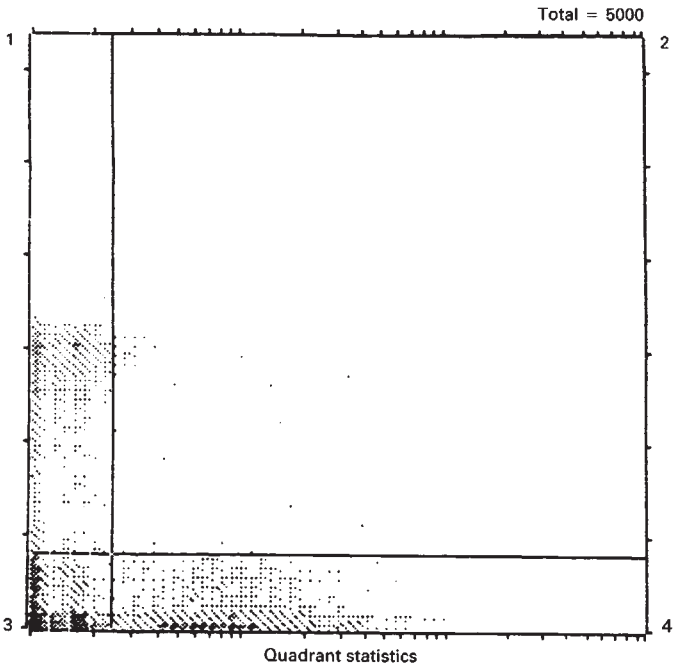

Fig. 1. *Quatstat for a representative double-stained sample.*

There was an increase in the percent of activated Leu2a+DR+ cells (Fig. 3) in children with acute proteinuria when compared to those with minimal proteinuria, that is, those patients treated and untreated in remission for their nephrotic syndrome ($P < 0.05$).

The percentage and absolute numbers of Leu3a+, or total T-helper cells, was found to be significantly decreased in the patients in relapse, both treated and untreated, when compared to normal controls and nephrotic controls ($P < 0.05$). This decrease in Leu3a+ cells may be an effect due to prednisone.

Table 1. Patient population of children with SRNS

	No. pts.	Age	Sex	
			Male	Female
Normal control	15	7.9 ± 5.1	8	7
Untreated relapse	6	7.8 ± 4.1	3	3
Treated relapse	7	8.0 ± 4.1	2	5
Untreated remission	11	9.6 ± 3.2	6	5
Treated remission	8	8.9 ± 3.5	5	3

Table 2. Percent T-lymphocyte subsets in children with SRNS

	No. pts.	Leu_4+	Leu_4+DR+	Leu_3+	Leu_3+DR+	Leu_2+	Leu_2+DR+
Normal control	15	61.3 ± 9.9	1.5 ± 0.8	39.6 ± 8.6	0.9 ± 0.5	22.0 ± 7.7	0.78 ± 0.42
Untreated relapse	6	49.1 ± 20.5[a]	1.6 ± 0.7	29.3 ± 13.7[b]	1.1 ± 2.0	21.5 ± 11.3	1.6 ± 2.1[c]
Treated relapse	7	63.5 ± 16.1	2.3 ± 1.8	35.2 ± 9.8[b]	1.7 ± 1.5	18.3 ± 15.4	2.1 ± 2.0[c]
Treated remission	11	52.6 ± 13.3	1.5 ± 0.9	26.8 ± 11.0[b]	0.8 ± 0.6	20.9 ± 7.6	0.75 ± 0.41
Untreated remission (nephrotic controls)	8	70.1 ± 4.9[a]	1.6 ± 0.7	40.1 ± 6.5	0.9 ± 0.4	27.4 ± 4.0	1.0 ± 0.1

[a] $P < 0.05$ compared to normal controls
[b] $P < 0.05$ compared to normal controls or to untreated remission
[c] $P < 0.05$ compared to normal controls, treated or untreated remission

Table 3. T-lymphocyte subset in children with SRNS: Absolute cell counts (cells/mm^3)

	No. pts.	Total lymphocytes	Leu_4a+DR+	Leu_3aDR+	Leu_2aDR+
Normal control	15	4409 ± 2889	58 ± 37	36 ± 25	27 ± 23
Untreated relapse	4	2270 ± 144[a]	46 ± 62	18 ± 15[b]	33 ± 45
Treated relapse	5	2590 ± 1056[a]	37 ± 29	37 ± 56	26 ± 13
Treated remission	8	2539 ± 880[a]	42 ± 40	23 ± 22[b]	22 ± 13
Untreated remission (nephrotic control)	4	4192 ± 194	84 ± 50	46 ± 27	50 ± 40

[a] $P < 0.05$ compared to normal control or to treated remission
[b] $P < 0.05$ compared to treated remission

However, a comparison of Leu3a+ values of normal controls with those of relapsing patients not on prednisone also showed a significant difference (Fig. 4). The demonstrated decrease in the Leu3+ subset is probably largely responsible for the decrease in total T-cells (Leu4a+ cells) seen in patients with acute proteinuria. More evidence that the decreased numbers of Leu3a+ cells was due to prednisone treatment can be drawn from a comparison of patients in remission maintained by prednisone with untreated patients in long-term remission (nephrotic controls), all of whom had been off prednisone for at least six months. Compared to those in a long prednisone-free remission, patients in prednisone-maintained remission demonstrated a significant decrease in Leu3a+ cells ($P < 0.05$). Leu3a+DR+ cells were neither significantly increased nor decreased among the groups. Comparison of the group in acute relapse to the group maintained in remission by prednisone yielded no significant values for any of the variables. Staining with anti-transferrin receptor (mean 1.8 ± 2.7) and anti-IL2 receptor (mean 0.3 ± 0.3) to test for lymphocyte activation yielded no significant differences between groups.

Discussion

Both CD4 (Leu3a+) and CD8 (Leu2a+) cells seem to demonstrate abnormalities in SRNS. CD4 cells comprise the T-helper functional subset, important in the initiation of an immune response. CD8 lymphocytes are more diverse in function. These cells can posses either suppressor activity, crucial for regulation of an immune response, or cytotoxic activity, known to be important in tumor immunity and in viral infections.

Patients in acute relapse demonstrated an increase in the percentage of Leu2a+DR+ cells and a significant decrease in the percentage and absolute numbers of Leu3a+ cells when compared to normal controls. The decrease in Leu3a+ cells in these patients is probably due to their prednisone therapy, since a comparison of Leu3a+ values in controls and relapse patients free of prednisone yielded no significant differences. Also, patients maintained in remission by prednisone had lower Leu3a+ values than patients enjoying a long-term remission free of prednisone treatment. It is important to note, however, that the increases in Leu2a+DR+ cells in relapsing patients cannot be merely an artifact secondary to the prednisone-induced drop in the Leu3a+ percentage because the increase in Leu2a+/DR+ cells was still significant in the patients not on prednisone therapy at time of relapse.

T-cells which have been activated by IL-2 express surface antigens which are not seen on non-activated T-cells. These activation markers include HLA-DR, a receptor for transferrin, and a receptor for IL-2. Transferrin and IL-2 are usually elevated in an inflammatory reaction. The absence of changes in transferrin or IL-2 receptors during acute episodes of nephrotic syndrome or during prednisone therapy would suggest the

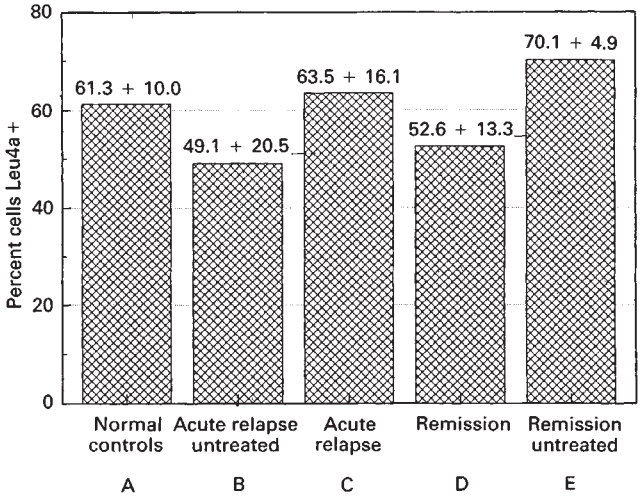

Fig. 2. *Percent cells Leu4a+DR+.* *$P = 0.01$ for B vs. E.

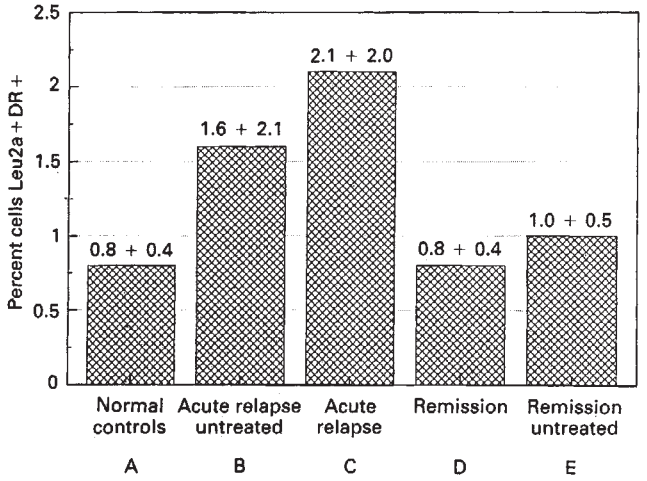

Fig. 3. *Percent cells Leu2a+DR+.* $*P < 0.05$ for A, D and E vs. C; $P < 0.05$ for A, D and E vs. B.

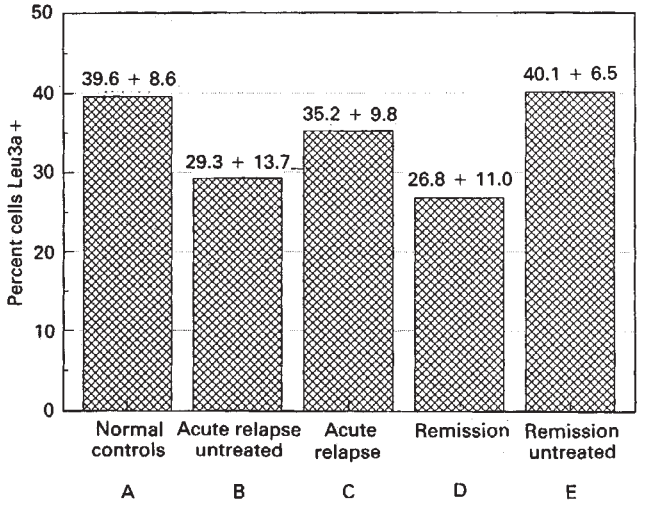

Fig. 4. *Percent cells Leu3a+.* $P < 0.05$ A vs. B and D; E vs. B and D.

absence of inflammatory reactions associated with these markers.

A two-color flow cytometry study of lymphocyte abnormalities in SRNS performed by Yamada et al reported a similar decrease in the percentage of Leu3a+ cells in patients receiving prednisolone (PSL) coupled with an increase in the percentage of Leu2a+ cells [13]. The two-color approach used by Yamada's group did not assay lymphocyte activation. Rather, they used second markers to more specifically define subsets, such as suppressor-inducer cells (Leu3a+Leu8+) and killer T cells (Leu2a+Leu15+). Interestingly, in nephrotic patients not receiving prednisolone, Yamada's group found no significant difference from normal control values for any of the T-lymphocyte subsets. Presently there seems to be ample reason to suspect a role for abnormal lymphocyte function in the etiology and pathogenesis of SRNS in children. Yamada's findings and the results of our study lend credence to the basic hypothesis that an abnormality of T-lymphocyte subsets and function may be involved in the pathogenesis of SRNS, though the data is somewhat inconsistent. More convincing evidence might be obtained from a prospective longitudinal study of patients with SRNS. If an abnormal T-cell subset is consistently identified, it should be further assayed for functional activity and production of Shalhoub's hypothesized lymphokine [4, 9].

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