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ORIGINAL ARTICLE

Immunophenotype of Blood Lymphocytes in Neuroblastoma-Associated Opsoclonus-Myoclonus

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Objective: To determine whether the distribution of peripheral blood mononuclear cells (PBMCs) is altered in paraneoplastic opsoclonus-myoclonus (POM).

Methods: PBMCs from 17 children with POM, 17 children with OM but no tumor, and 17 controls were immunophenotyped using a comprehensive panel of surface markers by dual-laser flow cytometry. All groups were matched for age and gender; POM and OM patients were matched for treatment.

Results: In the POM patients, the CD4+ T-cell subset was smaller in both relative size (−18%, $P = 0.02$) and absolute size (−41%, $P = 0.03$) compared with controls. The CD4/CD8 ratio also was less (−29% to −44%) and was related to POM duration ($P = 0.03$). The absolute but not relative size of the γδ T-cell subset was reduced (−44%, $P = 0.02$). There were no significant abnormalities of CD19+ B-cells, CD3− or CD3+ NK cells, HLA-DR+ or CD25+ T-cells, or CD45RA+ or CD45RO+ T-cells. Prior tumor chemotherapy, which was associated with a higher percentage but not number of CD8+ T-cells, did not restore the CD4+ T-cell subset. When the POM and OM groups, which were not significantly different, were combined, chemotherapy decreased both the relative and absolute size of the CD19+ B-cell pool and had small effects on other lymphocyte subsets.

Conclusions: POM is characterized by T-cell abnormalities of PBMCs, the most robust of which is reduction of the CD4+ T-cell subset and the CD4/CD8 ratio. Although this reduction was found previously in cerebrospinal fluid in POM patients, PBMC subsets did not otherwise reflect cerebrospinal fluid abnormalities. Longitudinal studies will be necessary to determine whether PBMC abnormalities could serve as treatment markers.

Key Words: ACTH, Kinsbourne syndrome, IVIG, myoclonus, neuroblastoma, paraneoplastic syndrome

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Lymphocyte immunophenotyping by flow cytometry is now the preferred method of delineating lymphocyte distribution in human immunologic disorders. The presence of flow cytometers in most large medical facilities and the rapid return of test results make immunophenotyping an ideal tool when clinical decisions must be made promptly.

Paraneoplastic opsoclonus-myoclonus syndrome (POM) is one such putative immunologic disorder in need of attention.[1] Although the presence of the paraneoplastic syndrome is associated with enhanced survival from the underlying tumor,[2] which in children is usually neuroblastoma,[3] outcome is marred by persistent and serious neuropsychological problems. Immunologic modeling in POM has been put forth,[4] drawing on autoantibody data and other clues,[5] but much of the mechanism by which an onconeural antigen elicits the neurologic syndrome remains to be elucidated. Autoantibodies continue to be a focus of research in POM,[6,7] but potential T-cell involvement, which is so important in many other autoimmune disorders, has been understudied.

We have been searching for biomarkers of disease activity, and the evolving story of lymphocyte involvement in POM led us to study lymphocyte subsets in peripheral blood. Recently, we found both T-cell and B-cell abnormalities in cerebrospinal fluid (CSF) in POM,[8] and we now set out to determine whether less intrusively obtained information from circulating peripheral blood mononuclear cells (PBMCs) is clinically useful in the disorder.

PATIENTS AND METHODS

Patients

Seventeen children with neuroblastoma and POM were recruited through the National Pediatric Myoclonus Center for screening by the principal investigator (M.R.P.) to establish eligibility and obtain consent for this institutional review board-approved study. The clinical characteristics are shown in Table 1.

All the patients had symptoms of active disease at the time of evaluation despite tumor resection. None had evidence

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TABLE 1. Clinical Information

Parameter	POM			OM/No Tumor			Controls
	All	No Chemo	Chemo	All	No Chemo	Chemo	
Number of cases	17	6	11	17	8	9	17
Gender							
Male	5	1	4	6	0	6	6
Female	12	5	7	11	8	3	11
Age at testing (yr)	3.1 ± 0.5	3.4 ± 1.2	2.9 ± 0.3	3.3 ± 0.4	2.9 ± 0.8	3.6 ± 0.5	3.8 ± 0.5
Age at OM onset (yr)	1.4 ± 0.2	1.5 ± 0.1	1.4 ± 0.3	1.9 ± 0.3	1.9 ± 0.4	1.9 ± 0.5	—
OM duration (yr)	1.7 ± 0.5	1.9 ± 1.2	1.5 ± 0.4	1.4 ± 0.3	1.1 ± 0.5	1.7 ± 0.2	—
Tumor stage (INSS)							
I	13	5	8	—	—	—	—
II	3	1	2	—	—	—	—
III	1	0	1	—	—	—	—
IV	0	0	0	—	—	—	—
Tumor location							
Thoracic	9	2	7	—	—	—	—
Abdominal	7	4	3	—	—	—	
Pelvic	1	0	1	—	—	—	—
Tumor type							
Neuroblastoma	13	5	8	—	—	—	—
Ganglioneuroblastoma	4	1	3	—	—	—	—
Tumor treatment							
Cyclophosphamide	8	—	8	6	—	6	—
Other chemotherapy*	3	—	3	3	—	3	—
Surgical resection	17	6	11	—	—	—	—
Radiation therapy	1	0	1	—	—	—	—
Neurologic severity†	16.7 ± 2.6	20.0 ± 4.1	14.8 ± 3.3	12.8 ± 2.5	17.8 ± 3.6	8.3 ± 2.7	—
Current immunotherapy‡							
None	4	3	1	4	2	2	—
Monotherapy	3	1	2	5	3	2	—
Two or more agents	10	2	8	8	3	5	—

*For POM: carboplatin, doxorubicin, etoposide; or doxorubicin; or doxorubicin, cisplatin, etoposide. For OM: 6-mercaptopurine.
†Total score on the OM Evaluation Scale: mild, 0–12; moderate, 13–24; severe, 25–36.
‡ACTH (Acthar gel); steroids included oral prednisone, IV pulse methylprednisolone; IVIG (infused monthly).

of recurrent neuroblastoma using neuroimaging and blood and urine tumor markers. For those who had received chemotherapy, the mean length of time from completion of therapy was 10.3 ± 4.1 (SEM) months. Due to the referral pattern to our national center for opsoclonus-myoclonus (OM), which accepts children at any stage in the course of their disorder, this was a cross-sectional study.

Controls

Two different kinds of control groups were used. Seventeen age- and gender-matched children with nonimmunologic and noninfectious neurologic disorders served as neurologic controls: they had neither OM nor neuroblastoma. Many had myoclonus or ataxia of other etiologies, controlling for these aspects of POM, and none of the children had ever received immunotherapy.

The second control group was 17 age- and gender-matched children with OM in whom no tumor was found. They were matched with the POM patients with respect to age,

TABLE 2. Monoclonal Antibodies*

Cell Type	MA6 and Source
αβ T-cell	CD3†
Helper/inducer T-cell	CD4†
Suppressor/cytotoxic T-cell	CD8†
Monocyte	CD14†
NK cell	CD16,56‡
B-cell	CD19†
IL-2 receptor, activated αβ T-cells	CD25†
Pan leukocyte	CD45†
Naïve T-cell	CD45RA‡
Memory T-cell	CD45RO‡
γδ T-cell	TCR-γδ‡
Activated αβ T-cell	HLA-DR‡

*Antibody panels were 3/4/8/45, 3/16, 56/19/45, 5/14/19/45, 4/TCR/8/3, 25/DR/3/45, 45RA/TCR/3/45, 45RO/TCR/3/45, G1/G1/3/45.
†Beckman-Coulter.
‡Immunotech.

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gender, and treatment, thereby controlling for the effects of neuroblastoma. For those who had received chemotherapy, the mean length of time from completion of therapy was 4.2 ± 2.0 (SEM) months, which was not significantly different from the POM group.

Scoring of Neurologic Status

Each child with POM or OM/no tumor was videotaped. A trained observer (E.D.T.) unaware of the child's treatment status rated motor impairment using the OM Evaluation Scale, which we devised and validated.[9] Each item of the 12-item scale was rated from 0 to 3 as an index of increasing neurologic severity or impairment. Total score was calculated as the sum of subscores, with a score of 36 indicating maximum abnormality.

Flow Cytometry

Whole peripheral blood samples (100 μL/tube) were stained with various panels of directly conjugated monoclonal antibodies (Table 2). We have implemented a broad panel of lymphocyte markers, designed previously to explore different potential mechanisms of pathophysiology, including γδ cells, an "atypical" T-cell, and NK cells, important to host tumor defenses. They were further incubated for 10 minutes with 100 μL/tube Optilyse B (Immunotech). One milliliter of water was subsequently added to each sample, and it was incubated for a final 10 minutes at room temperature. Appropriately labeled isotypes (IgG1) were used as internal controls.

Samples were acquired and analyzed by flow cytometry on a FACSCalibur cytometer equipped with a 488-nm argon/633-nm HeNe laser (Becton-Dickinson, San Jose, CA). For each antibody combination, 10,000 events were acquired and analyzed with CellQuest (Becton-Dickinson). Instrument calibration was monitored daily with Calibrite beads (Becton-Dickinson). Data were plotted as log versus log fluorescence.

Blood Lymphocyte Counts

Because the relative size of lymphocyte subsets is not always consistent with a change in the absolute size of the

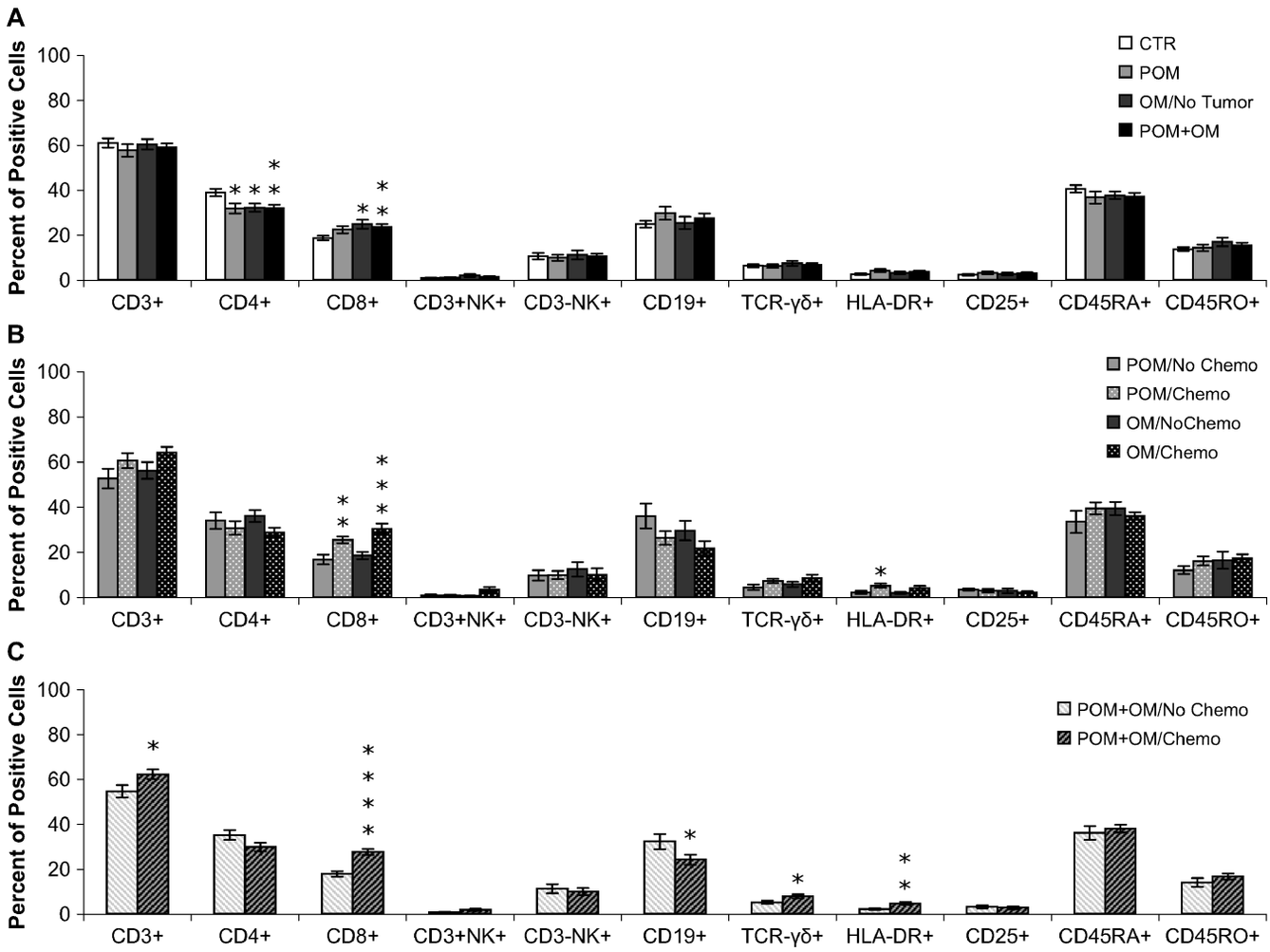

FIGURE 1. The relative size of PBMC subsets. Data are means ± SEM. A, In both the POM and OM groups, the percentage of CD4+ T-cells was significantly lower than controls (CTR). B, In the subgroup of POM and OM patients who had received chemotherapy as tumor therapy, the percentage of CD8+ T-cells was higher. C, When the POM and OM groups were combined, the chemotherapy group showed significant increases in multiple lymphocyte subsets. Asterisk indicates statistical significance by *t* tests: *$0.01 \leq P < 0.05$, **$0.001 \leq P < 0.01$, ***$0.0001 \leq P < 0.001$, ****$0.00001 \leq P < 0.0001$.

subset,[10] lymphocytes were counted in a separate blood sample on a Sysmex 9500 automated counter in the clinical laboratory. Counts were expressed as K/mm^3. Data from our controls compared favorably with the literature.[10–12]

Statistical Analysis

Data were analyzed statistically by *t* tests or one-way analysis of variance (ANOVA) using the Statistical Analysis System.[13] Because the role of chemotherapy in POM is controversial—most tumors are low-stage and survival is excellent without it—we identified prior tumor chemotherapy for secondary analysis.

RESULTS

Lymphocyte Immunophenotype

The relative size of the CD4+ T-cell subset (Fig. 1) was significantly less in the POM patients (−18%) compared with controls ($P = 0.02$, *t* test). The CD4/CD8 ratio (Fig. 2) was 29% lower in the POM patients ($P = 0.013$). There was a trend toward a higher percentage of HLA-DR+ T-cells (+68%, $P = 0.06$) and CD8+ T-cells (+20%, $P = 0.056$). There were no significant changes in CD19+ B-cells, CD3− or CD3+ NK cells, γδ T-cells, CD25+ T-cells, CD45RA+ T-cells, or CD45RO+ T-cells.

The absolute size of lymphocyte subsets in the POM patients (Fig. 3) showed a significant decrease in the number of CD4+ T-cells (−41%, $P = 0.031$) and γδ T-cells (−44%, $P = 0.023$). There was a trend toward fewer CD3+ cells ($P = 0.063$), CD45RA+ T-cells ($P = 0.054$), and CD45RO+ T-cells ($P = 0.088$).

There were no significant differences between the POM and OM/no tumor groups. As in the POM patients, there was a significant reduction in the relative size of the CD4+ T-cell subset (−17%, $P = 0.013$) and the CD4/CD8 ratio (−32%, $P = 0.0035$) compared with controls. The percentage of CD8+ T-cells was significantly higher (1.3-fold, $P = 0.012$).

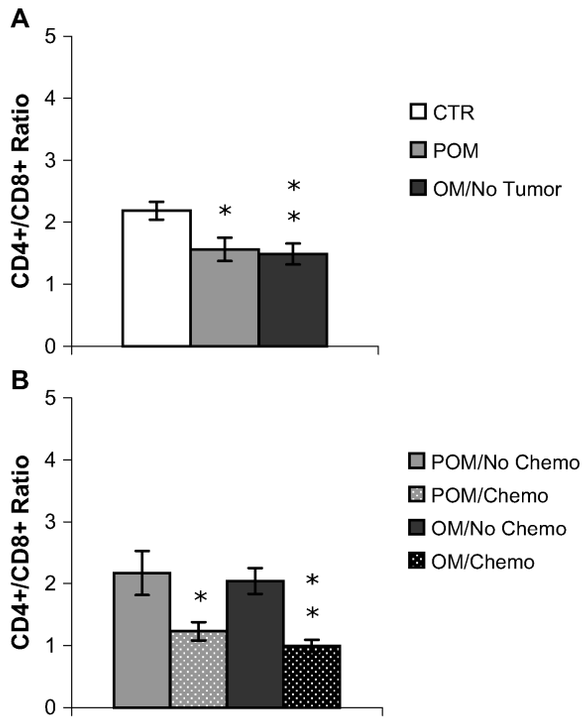

FIGURE 2. CD4/CD8 T-cell ratio. Ratios were computed from percentages of CD3+CD4+ and CD3+CD8+ T-cells. Data are means ± SEM. A, In the POM and OM groups, the ratio was significantly lower compared with controls (CTR). B, In POM or OM patients with prior tumor chemotherapy, the ratio was lower. Statistically significant by *t* test: *$0.01 \le P < 0.05$, **$0.001 \le P < 0.01$.

Relation to Clinical Variables

In the POM group, three principal clinical variables were evaluated: age (infant, toddler, preschool, school-age), duration (acute, intermediate, chronic), and severity (mild, moderate, severe). A significant relation to POM duration (Fig. 4) was found for the CD4/CD8 ratio ($F_{3,30} = 3.33$, $P = 0.033$, ANOVA) and the percentage of CD3+CD8+ cells ($F_{3,30} = 3.43$, $P = 0.029$), whereas for the percentage of CD3+CD4+ cells, it was only a trend ($F_{3,30} = 2.75$, $P = 0.059$). Similar relations were found for the OM/no tumor group. For the relative size of lymphocyte subsets in the POM patients, there was only a trend between the percentage of CD3+DR+ cells and severity ($F_{2,30} = 5.2$, $P = 0.099$, ANOVA). There was a trend toward a reciprocal relation between severity and the percentage of CD4+ T-cells ($F_{3,30} = 2.42$, $P = 0.085$) and the CD4/CD8 ratio ($F_{3,30} = 2.46$, $P = 0.082$).

Effect of Chemotherapy

Prior tumor chemotherapy (see Fig. 1) was associated with a significant increase in the relative size of the CD3+CD8+ subset (+34%, $P = 0.0032$) and in HLA-DR+ T-cells (2.4-fold, $P = 0.017$). However, these changes were not reflected in the absolute size of the lymphocyte subsets (see Fig. 3). The CD4/CD8 ratio was lower in the POM patients who had received chemotherapy, whether based on the relative (−43%, $P = 0.011$) or absolute (−63%, $P = 0.0093$) size of lymphocyte subsets.

In patients with POM who had received chemotherapy, there was a negative correlation between prior tumor chemotherapy and the percentage of CD3+CD8+ cells ($r = -0.51$, $P = 0.035$) and a positive correlation with the CD4/CD8 ratio ($r = 0.61$, $P = 0.0088$). A positive correlation was found between prior tumor chemotherapy and the number of CD4+ cells ($r = 0.49$, $P = 0.066$) and NK+ cells ($r = 0.58$, $P = 0.023$). Due to the relatively small number of patients, multivariate analysis to look at the influence of chemotherapy was not possible.

There were no significant differences between the POM and OM/no tumor groups. In the OM group, chemotherapy was associated with a larger size of the CD8+ T-cell subset (1.6-fold, $P = 0.00082$) and a lower CD4/CD8 ratio (−51%, $P = 0.011$). There was a trend toward a reduced percentage of CD4+ T-cells ($P = 0.048$) and an increased percentage of CD3+ NK cells ($P = 0.052$). The absolute number of CD4+ T-cells was lower ($P = 0.018$).

Given the lack of significant differences between the POM and OM groups, we combined the groups to increase sample size for another analysis to resolve whether statistical

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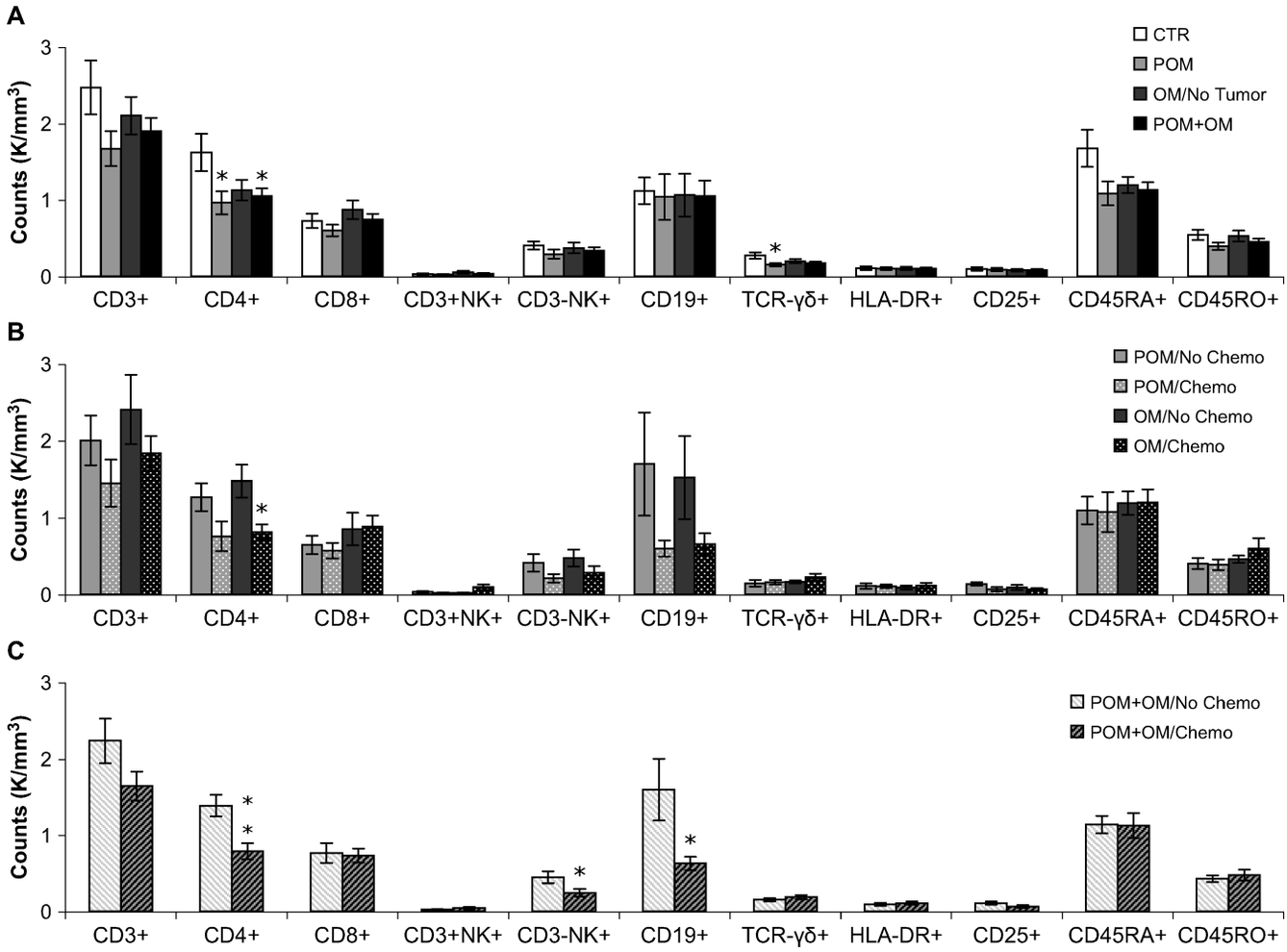

FIGURE 3. The absolute size of PBMC subsets. Data are means ± SEM. A, In POM patients, the number of CD3+CD4+ cells and γδ+ T-cells was significantly lower than controls (CTR), but the differences did not reach significance in the OM group. There was a trend toward lower CD45RA+ and CD45RO+ T-cells. B, Prior tumor chemotherapy in POM patients was not associated with significant changes. C, When the POM and OM groups were combined, the chemotherapy group showed significant decreases in CD4+ T-cells, CD3− NK cells, and CD19+ B-cells. Asterisk signifies statistical significance by *t* tests: *$0.01 \leq P < 0.05$, **$0.001 \leq P < 0.01$.

trends were real effects of chemotherapy. In the chemotherapy group, the relative subset size of CD3+ cells ($P = 0.037$), CD8+ T-cells (1.6-fold, $P = 0.000016$), γδ+ T-cells (1.5-fold, $P = 0.045$), and HLA-DR+ T-cells (2.2-fold, $P = 0.0029$) was significantly larger. Both the relative (−25%, $P = 0.044$) and absolute (−61%, $P = 0.034$) size of the CD19+ B-cell pool was smaller. Also, the absolute size of the pool of CD4+ T-cells (−43%, $P = 0.0016$) and CD3− NK cells (−47%, $P = 0.029$) was smaller. The 46% reduction in the CD4/CD8 ratio was highly significant ($P = 0.00015$).

Tumor chemotherapy did not significantly reduce neurologic severity in the POM patients (see Table 1). In the OM group, there was a trend toward a reduction in total score ($P = 0.057$). When the POM group was combined with the OM group, there was no significant effect of chemotherapy.

Effect of Other Therapies

When chemotherapy-treated patients were excluded, the number of children with POM who had been treated with immunotherapy was too small for analysis by type of treatment (e.g., ACTH, IVIG, or steroids). Because there were no significant differences between the POM and OM groups, we combined them and compared all immunotherapy-treated children (n = 9) to controls. There were no statistically significant differences in PBMC subsets. The sample size was insufficient to reliably compare monotherapy and combination therapy.

DISCUSSION

This study identified abnormalities of lymphocyte distribution in peripheral blood from children with POM. Both the relative and absolute size of the CD4+ T-cell subset was significantly lower, as was the CD4/CD8 ratio. Because there were no significant differences between the POM and OM without tumor groups, these abnormalities are attributable to OM rather than neuroblastoma. We are aware of no previous studies and only two case reports in which blood lymphocytes were immunophenotyped in OM. A low CD4/CD8 ratio of 0.7

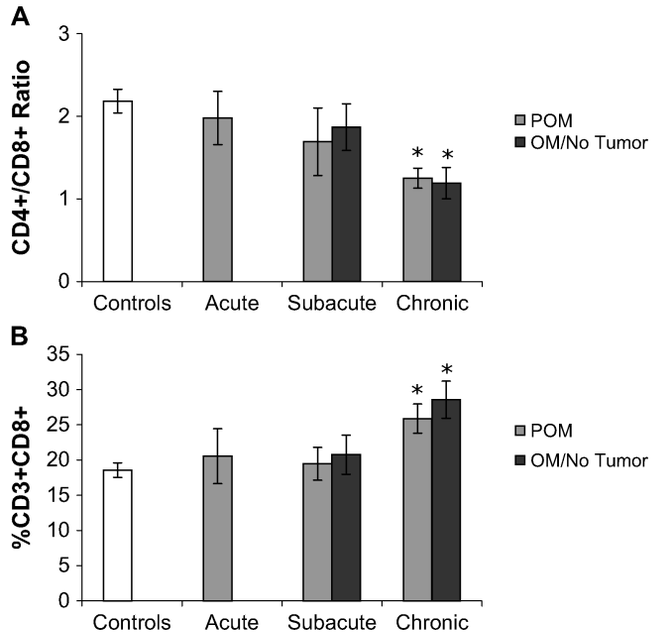

FIGURE 4. Effect of duration of disease on the (A) CD4/CD8 ratio and (B) percentage of CD3+CD8+ cells. Duration was defined as acute (≤0.3 years), subacute (>0.3 years but ≤1 year), or chronic (>1 year). In both (A) and (B), the chronic group was significantly different than controls, $P < 0.05$, Duncan's multiple range test.

(normal 1.5–2) was found in a 14-month-old with neuroblastoma, but an elevated CD4/CD8 ratio of 3.48 (normal 0.6–2.9) was reported in another 14-month-old boy.[14,15]

The failure of tumor chemotherapy (principally cyclophosphamide) to restore the CD4+ T-cell subset was noteworthy, as was the increased percentage of CD8+ T-cells. Perhaps cyclophosphamide is not the ideal chemotherapeutic agent for POM. However, ours was a descriptive observation, and prospective longitudinal studies, such as those in progress at our center, are needed. Chemotherapy did reduce circulating B-cells, which may be relevant to proposed B-cell mechanisms in POM.[6] Also, the chemotherapy was directed at the tumor using high-dose neuroblastoma protocols over a relatively short period, which may not be optimal in chronic autoimmune disorders such as POM.

Reduction in CD4+ T-cells in peripheral blood has been reported in other neurologic disorders, such as epilepsy,[16] a subset of autism,[17] and active multiple sclerosis.[18] The significance of this abnormality is unclear, but CD4+ T-cells play an important role in the pathogenesis of chronic inflammatory autoimmune diseases.[19] Although we entertained the possibility that low CD4+ cells may be a factor in illness-induced relapse in OM, a major clinical problem, the degree of CD4+ T-cell reduction is not likely to pose a risk factor for infection based on data from acquired immune deficiency.

Reduced CD4+ T-cells and a reduced CD4/CD8 ratio were also found in the CSF in POM patients.[8] However, other CSF abnormalities, such as expansion of B-cells and γδ T-cells, were not present in the circulating lymphocyte pool. Peripheral blood γδ T-cells, an "unconventional" T-cell,[20] may be lowered in POM due to their increased presence in CSF, but no such relation was found for B-cells. This is unfortunate, because CSF B-cell and γδ T-cell percentages correlated with neurologic severity. We conclude that immunophenotyping studies of the PBMC do not replace those of CSF lymphocytes. Longitudinal studies during various immunotherapies will be necessary to determine whether PBMC phenotypic abnormalities have utility as treatment markers.

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