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Title: Naive and memory CD4⁺ T-cells in the cerebrospinal fluid of children with aseptic meningitis following measles-mumps-rubella vaccination and enteroviral meningitis

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ABSTRACT

Background: We investigated the distribution of memory (CD45RO⁺) and naive (CD45RA⁺CD62L⁺) CD4⁺ T-cells as well as CD8⁺ T-cells and total T-cells in the CSF of children with aseptic meningitis following measles-mumps-rubella (MMW) vaccination and those with enteroviral meningitis.

Methods: Flow cytometric analysis of CSF cells was performed in 12 children with MMR vaccine-associated meningitis and 11 children with enteroviral meningitis.

Results: Percentages of total T-cells, CD4⁺ and CD8⁺ T-cells and monocytes in CSF of patients from the two groups were not significantly different. The majority of CD4⁺ T-cells in the CSF of both patient groups were of memory phenotype. Percentages of CSF naive CD4⁺ T-cells were increased in children with aseptic meningitis following MMR vaccination.

Conclusions: Further studies focused on the more detailed immunophenotyping of CSF cells are needed to fully establish the usefulness of flow cytometry in the diagnostic workup of inflammatory CNS diseases in children.

INTRODUCTION

Memory T-cells enter cerebrospinal fluid (CSF) directly from the systemic circulation, monitor the subarachnoid space (SAS) and return to secondary lymphoid organs in the absence of antigen (11, 12). Alternatively, in persons with inflammatory diseases of the central nervous system (CNS), memory T-cells participate in the local immune response.

The routine diagnostic approach to the analysis of CSF cells is usually limited to the cytomorphological description of cells (23). However, *ex vivo* immunophenotyping of CSF cells by flow cytometry provided a valuable insight into the local immune response in a variety of inflammatory and non-inflammatory CNS diseases. The phenotype of CSF cells has been extensively studied by flow cytometry in a variety of models including multiple sclerosis (MS), CNS malignant diseases, tick-borne encephalitis (TBE), neuroborreliosis, CNS malignant diseases and healthy adults (2,8,10,14,15,16,17,18,24,25). However, literature data on flow cytometric analysis of CSF cells in children are scarce.

Tabata et al described an increase in the percentage of CD4⁺ T-cells in the acute phase and of CD8⁺ T-cells in the recovery phase of aseptic meningitis in children (21). More recently, Häusler et al analysed the distribution of CD4⁺ and CD8⁺ T-cells, B-cells, NK cells, HLA-DR⁺ T-cells and memory T-cells (CD45RO⁺CD3⁺) cells in the CSF of children with and without primary inflammatory CNS diseases as well as in children with CNS diseases of uncertain aetiology (7). According to their study, the analysis of CSF lymphocyte subpopulations did not allow a distinction between patients with and without primary inflammatory processes. However, they recommended flow cytometry for the evaluation of CSF lymphocytes in children with chronic or unusual inflammatory CNS processes as well as in patients with very small amount of CSF available for analysis.

Studies in adults have shown the predominance of antigen-experienced memory CD4⁺ T-cells in the CSF of patients with a variety of inflammatory neurological diseases (2,3,6,13). Our hypothesis was that memory CD45RO⁺CD4⁺ T-cells also represent the majority of total CD4⁺ T-cells in the CSF of children with inflammatory CNS diseases. Increased percentages of naive CD4⁺ T-cells in the CSF of patients with different etiologies might represent a distinct immunopathogenic finding. Therefore, we compared the distribution of memory (CD45RO⁺) and naive (CD45RA⁺CD62L⁺) CD4⁺ T-cells, CD8⁺ T-cells and total T-cells in the CSF of children with aseptic meningitis following measles-mumps-rubella (MMW) vaccination and those with enteroviral meningitis. To the best of our knowledge, the distribution of lymphocyte subpopulations in the CSF of children with MMR vaccine associated aseptic meningitis and enteroviral meningitis has not been previously investigated.

MATERIALS AND METHODS

Study design and patients:

This prospective, cross-sectional study was conducted at the University Hospital for Infectious Diseases "Dr. Fran Mihaljević", Zagreb, Croatia. We enrolled 12 children (4 females, median age 14,8 months, range from 12-46 months) with aseptic meningitis following MMR vaccination (MMR vaccine, Institute of Immunology, Inc., Zagreb, Croatia) caused by L-Zagreb mumps vaccine strain and 11 children (4 females, median age 20 months, range from 9-41 months) with enteroviral meningitis. The diagnosis of aseptic meningitis or enteroviral meningitis was based on clinical signs and symptoms, accompanied with CSF cytological and biochemical findings. The etiology of aseptic meningitis was determined after virus isolation from the CSF. Enteroviral meningitis was confirmed by cultivation and neutralization assays.

The patients underwent spinal tap at the Pediatric Infectious Diseases Department. CSF samples used for flow cytometry were collected as a part of routine diagnostic procedure. In patients with aseptic meningitis following MMR vaccination, lumbar puncture was performed after a median of 22 days after vaccination (range from 15-31 days).

Additionally, we enrolled 14 adults (7 females, median age 50, range from 24 to 77 years) with acute viral encephalitis.

Informed consent was obtained from all parents and adult patients. The study was approved by the local Ethics committee.

Samples:

Biochemical (protein concentration), cytological and flow cytometric tests were done on all CSF samples. CSF samples for flow cytometry were taken from the last aliquot

collected (reduction in the amount of contaminating erythrocytes), kept at room temperature and immediately processed.

Flow cytometry:

CSF cells were collected by centrifugation on 600g, for 5 min., at room temperature (Rotofix 32, Hettich Zentrifugen, Tuttlingen, Germany). The supernatant was removed and the pellet was resuspended in 200 µl of Izoton II Azide-free balanced electrolyte solution (Beckman Coulter, Inc., Fullerton, CA, USA).

Identification of lymphocytes and monocytes as well as the determination of lymphocyte purity in the gate was done by CD45 versus CD14 backgating strategy (CD45-FITC (clone T29/33) and CD14-RPE (clone TüK4) (Figure 1a and 1b, representative plots). Our results showed excellent lymphocyte purity (less than 1% of contamination within the gate) for all samples. The remaining part of the CSF was used for the analysis of lymphocyte subpopulation and was not divided into more than 3 aliquotes (in order to ensure reproducible results).

Percentages of CD4⁺ and CD8⁺ T-cells, truly naive CD45RA⁺CD62L⁺ and memory CD45RO⁺ CD4⁺ T-cells as well as monocytes in the CSF were determined by three-color flow cytometry by using a panel of monoclonal antibodies specific for CD14 (clone TüK4), CD3 (clone UCHT1), CD4 (clone MT310), CD8 (clone DK25) CD45RA (clone 4KB5), CD45RO (clone UCHL1) and CD62L (clone FMC46) conjugated with FITC, RPE or RPE-Cy5 (DakoCytomation A/S, Glostrup, Denmark). We used the following combinations of monoclonal antibodies: tube 1 (CD4-FITC/CD45-RO-RPE/CD3-RPE-Cy5), tube 2 (CD62L-FITC/CD45RA-RPE/CD4-RPE-Cy5) and tube 3 (CD3-FITC/CD14-RPE/CD8-RPE-Cy5) (representative plots Figures 1-4).

Non-specific binding to Fc receptors was blocked with 0.2 mg/ml of mouse IgG1 (clone MCG1, IQ Products, Groningen, Netherlands).

CSF cells were stained with antibody-fluorochrome conjugates by incubating for 15 minutes at room temperature, in the dark. Erythrocyte lysis was performed by using a non-wash method on Multi-Q-Prep System with ImunoPrep Reagent System (Beckman Coulter, Inc., USA).

CSF samples were analyzed on Epics XL-MCL and FC500 flow cytometers (Beckman Coulter, USA). We acquired 5000 events in the lymphocyte gates. Samples without 5000 events in the lymphocyte gates were excluded from the further analysis.

Cytological analysis of the CSF:

The number of cells per μl of CSF was determined by counting in a Fuchs-Rosenthal chamber. CSF sediments were prepared by using a Shandon Cytospin 3 (Life Sciences International Europe, Astmoor, Runcorn, Cheshire, UK) for 5 min. at 300g. One CSF sediment sample was stained with My-Grunwald-Giemsa stain for routine cytological analysis. Percentages of granulocytes, lymphocytes and monocytes were determined after analyzing 200 cells in the sediment.

Isolation and determination of viruses

One ml of CSF specimen was obtained from all patients for viral diagnostics. CSF samples from patients with aseptic meningitis after MMR vaccination were cultured on Vero cells. The presence of mumps virus was determined by hemadsorption with 0.5 % guinea pig erythrocyte suspension and a neutralization test with mumps polyclonal sera (Institute of Immunology, Inc., Zagreb, Croatia). RT-PCR for SH gene, detection in agarose gel, sequencing of SH genes (on ABI Prism 377 automatic DNA sequencer, Applied Biosystems,

Foster City, Ca, USA) and alignment with SH gene of L-Zagreb mumps vaccine strain was then done as previously described (9).

CSF samples from patients in whom enteroviral aseptic meningitis was suspected were cultivated on GMK (green monkey kidney) cell culture obtained from WHO Labnet, National Public health Institute (KTL- Kansanterveyslaitos Folkhälsoinstitutet), Helsinki, Finland. Enteroviruses were detected by observation of a cytopathic effect in the previously mentioned cell culture. Identification of enterovirus isolates was done by neutralisation assay (by using microtechnique). For neutralisation assay, we used pools of specific equine hyperimmune sera that have been prepared at the National Institute of Public Health and Environment (RIVM- Rijksinstituut voor Volksgezondheid en Milieuhygiene) Bilthoven, The Netherlands and were provided by WHO (Department of Immunization, Vaccines and Biologicals), Geneva. Each virus was tested in duplicate against trivalent pooled polio antiserum (PP pool), a coxsackievirus B1-6 pool (CP pool), and seven pools against coxsackievirus A9 and 20 echoviruses (pool A-G). Confirmation was done using neutralization test assay with type-specific antisera prepared at Central Public Health Laboratory, London (26).

Statistical analysis

Statistical analysis was performed by using SAS (version 8.2., SAS Institute, Cary, North Carolina, USA). The comparison between two independent groups was done by using Wilcoxon two-sample test ($p < 0.05$ was considered significant).

RESULTS

Cytological and biochemical analysis of the CSF

We compared the results of standard cytomorphological and biochemical analysis of the CSF from children with aseptic meningitis following MMR vaccination and enteroviral meningitis. Statistically significant differences in the total CSF WBC count, percentage of segmented granulocytes, percentage of total and atypical lymphocytes as well as reactive monocytes and protein concentration were determined (Table 1).

Median number of total CSF WBC count in children with aseptic meningitis following MMR vaccination (1018.1 cells/ μ L) was significantly higher compared with enteroviral meningitis group (median 299.5 cells/ μ L, $p < 0.0001$). CSF of children with enteroviral meningitis (median 14.0%) contained significantly higher percentages of segmented granulocytes compared with MMR vaccine-associated aseptic meningitis group (median 4.5%, $p < 0.0001$). Median number of CSF WBC in adult patients with acute viral encephalitis was 112.5 (16-1160 cells/ μ l).

Percentages of total (median 56.3%) and atypical (median 8.07%) lymphocytes in children with aseptic meningitis following MMR vaccine were significantly increased compared with enteroviral meningitis group (median 32.2% and 2.1%, respectively, $p < 0.0001$ for both comparisons). Similarly, median percentage of reactive monocytes in the MMR vaccine-associated aseptic meningitis group (11.0%) was significantly increased compared enteroviral meningitis group (9.0%, $p = 0.034$). Adult patients with acute viral encephalitis had a median of 1% of neutrophils (range 0-7%), 85.5% of lymphocytes (range 47-96%) and 1% of plasmacytes (range 0-5%).

Concentration of total CSF proteins was also significantly increased in children with aseptic meningitis following MMR vaccination (median 0.40 mg/mL) compared with

enteroviral meningitis group (median 0.32 mg/mL, $p=0.013$). Median concentration of CSF proteins in adults with acute viral encephalitis was 960 mg/l (range 480-3624 mg/l).

Lymphocyte subsets in the CSF

Distribution of total T-cells, CD4⁺ and CD8⁺ T-cells, monocytes as well as naive and memory CD4⁺ T-cells in the CSF of children with aseptic meningitis following MMR vaccination and in children with enteroviral meningitis is presented in Table 2.

Median percentages of total T-cells (81.3%) and monocytes (8.2%) in children with aseptic meningitis following MMR vaccination were not significantly different compared with enteroviral meningitis group (median 76.3% and 9.9%, respectively).

Similarly, distribution of CD4⁺ (median 42.9 vs. 44.8%, respectively) and CD8⁺ T-cells (median 32.8 vs. 26.5%, respectively) in the CSF of children with MMR vaccine-associated meningitis and enteroviral meningitis group were not significantly different.

The majority of CD4⁺ T-cell subpopulation in the CSF from both patient groups was of memory phenotype (CD45RO⁺). Percentages of CD45RO-expressing CD4⁺ T-cells in the CSF of children with aseptic meningitis following MMR vaccination (median 85.1% of total CD4⁺ T-cells) and enteroviral meningitis (median 87.7% of total CD4⁺ T-cells) were also not significantly different.

Percentages of total CD4⁺ T-cells coexpressing CD45RA and CD62L (associated with naive phenotype) in the CSF of both patient groups were low. Median percentage of naive CD4⁺ T-cells in children with MMR vaccine-associated aseptic meningitis (median 12.8% of total CD4⁺ T-cells) was significantly higher compared with enteroviral meningitis group (median 5.5% of total CD4⁺ T-cells, $p<0.039$).

Percentages of T-cells and CD8⁺ T-cells in the CSF (median 81% and 19.4%, respectively) and blood (median 75.3% and 24.4%, respectively) of patients with acute viral encephalitis were not significantly different. Contrary to this finding, percentages of CD4⁺ T-cells in the CSF of adults with acute viral encephalitis (median 70.1%) was significantly higher compared with the blood (median 55.1%, $p < 0.001$).

DISCUSSION

This study compares the distribution of CSF lymphocyte subpopulations in children with aseptic meningitis following MMR vaccination and those with enteroviral meningitis. Percentages of total T-cells, CD4⁺ and CD8⁺ T-cells and monocytes in the two groups were not significantly different. The majority of CD4⁺ T-cells in the CSF of both patient groups were of memory phenotype. Percentages of CSF naive CD4⁺ T-cells were increased in children with aseptic meningitis following MMR vaccination.

T-cells in the CSF are responsible for the immune surveillance of the subarachnoid space and for the initiation of the local immune response. The number and composition of CSF cells evaluated by standard cytology is an important part of routine diagnostics in CNS diseases of various aetiologies.

Application of flow cytometry for the analysis of CSF cells provided a valuable insight into the local immunity. More importantly, several studies documented a correlation between the percentage of selected CSF lymphocyte subpopulations with other clinical or laboratory parameters suggesting a possible diagnostic usefulness of cytometry in this setting (15,18,20,21,24).

The major obstacle to the more frequent application of flow cytometry for the analysis of CSF cells has been the low number of cells and their fragility. However, recent studies have reported the optimization of flow cytometry for CSF analysis which will certainly contribute to the more frequent use of this application (13,19). For this study, we selected a non-wash method and a commercially-available automated flow cytometry sample preparation system. It is the opinion of the authors that the methodology described in our study enables a consistent and standardised sample preparation for CSF flow cytometry.

The majority of studies on CSF flow cytometry are limited to adult patients. A relatively large number of studies is available dealing with the expression of identification, activation and maturation surface markers as well as chemokine receptors on CSF cells from adults inflammatory, non-inflammatory or malignant CNS diseases in adults as well as healthy adult controls (2,8,10,14,15,16,17,18,24,25). However, the application of flow cytometry in the analysis of pediatric CSF samples could be even more important due to the well established problems with the cytological analysis of these samples. The CSF volumes available from pediatric patients are usually very limited, cell numbers are often relatively small and cytogram interpretation of cellular morphological characteristics is sometimes complicated.

One of the most important issues in the diagnostic application of CSF flow cytometry in children is the lack of age-related normal values due to obvious ethical issues. Our opinion is that comparison of the lymphocyte subsets distribution in children with inflammatory diseases of different etiologies, similarly to the concept of our study, might be a more realistic approach.

Literature data on flow cytometric analysis of pediatric CSF samples are scarce. One early study described an association between the changes in the percentages of CD4⁺ and CD8⁺ T-cells and disease stage (acute versus recovery phase) in children with aseptic meningitis (21). A more recent study described the analysis of several lymphocyte subsets including CD4⁺ and CD8⁺ T-cells, B-cells, NK cells, HLA-DR⁺ T-cells and CD45RO⁺CD3⁺ cells in the CSF from children with and without primary inflammatory CNS diseases (7). The authors reported that abnormal distribution of the selected lymphocyte subpopulations (compared to previously established referral values for adults) was observed in a very small number of patients. Additionally, the observed changes in the CSF immunophenotype did not allow the distinction between patients with or without primary inflammatory CNS diseases

and were quite heterogeneous, particularly for HLA-DR antigen expression. Nevertheless, the authors concluded that flow cytometry should be used for CSF analysis when evaluating children with chronic or unusual inflammatory CNS processes. The analysis of basic lymphocyte subpopulations in the CSF of children (T-cells, monocytes, CD4⁺ T-cell, CD8⁺ T-cells) did not prove to be diagnostically useful. However, a more detailed immunophenotyping of CD4⁺ T-cells enabled us to find differences between the two patient groups. It is our opinion that more detailed immunophenotyping of CSF lymphocyte subpopulations will be helpful in evaluation of a possible diagnostic usefulness of CSF flow cytometry in children. Comparison of CSF lymphocyte distribution in pediatric and adult patients in our study revealed major differences in the distribution of CSF CD4⁺ T-cells and CD8⁺ T-cells. Our results emphasise the necessity for further comparative studies.

To the best of our knowledge, the analysis of CSF in children with aseptic meningitis following MMR vaccination as well as in children with enteroviral meningitis has been limited to routine cytological and biochemical tests (1,4,5,22). Therefore, we applied flow cytometry for the analysis of CSF lymphocyte subpopulations in these two groups of pediatric patients.

A number of studies in adults with inflammatory CNS diseases demonstrated a preferential recruitment of antigen-experienced memory CD4⁺ T-cells from the blood to the CSF (2,3,6,13). Our study has shown that memory CD4⁺ T-cells also dominate the CSF CD4⁺ T-cell subpopulation in children with aseptic meningitis following MMR vaccination and those with enteroviral meningitis. In our opinion, this result supports the hypothesis that activated memory CSF T-cells are the principal cellular subpopulation responsible for the local immune response in patients with inflammatory CNS diseases, irrespective of etiology or age.

Our study has also shown increased percentages of naive CD4⁺ T-cells in the CSF of children with MMR-associated aseptic meningitis versus those with enteroviral meningitis. The observed difference needs to be carefully interpreted due to the relatively small number of patients in this study and further studies are needed to estimate the possible diagnostic relevance of this result.

CD45-RA-positive CD4⁺ T-cells are usually associated with naive state. Combination of CD4⁺CD45RA⁺CD62L⁺ has a high specificity for naive cells found in the peripheral blood mononuclear cells. In our study on CSF T-cells we used CD45RA/CD62L coexpression as a criteria for naive cells but can not exclude the possibility that combinations of other surface markers might better represent naive T-cell subpopulation in the CNS.

In conclusion, distribution of T-cells, CD4⁺ and CD8⁺ T-cells and memory CD4⁺ T-cells in the CSF of children with aseptic meningitis following MMR vaccination is similar to that observed in children with enteroviral meningitis. However, percentages of naive CD4⁺ T-cells are increased in the CSF of children with MMR vaccine-associated meningitis. Further studies focused on the more detailed immunophenotyping of CSF cells are needed to fully establish the usefulness of flow cytometry in the diagnostic workup of inflammatory CNS diseases in children.

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Table 1. Cytological and biochemical analysis of the cerebrospinal fluid from children with aseptic meningitis following measles-mumps-rubella (MMR) vaccination (n=12) and enteroviral meningitis (n=11)

Parameter	Aseptic meningitis after MMR vaccination	Enteroviral meningitis	P ^a Between group differences
Cell count (cells/ μ L)	1018.1 (466.5 – 1237.0)	299.5 (37.0-283.0)	< 0.0001
Segmented granulocytes (%)	4.54 (0-3.0)	41.0 (12.0-71.0)	< 0.0001
Lymphocytes (%)	56.3 (45.5-69.5)	32.2 (10.0-47.0)	< 0.0001
Atypical lymphocytes (%)	8.07 (0-6.0)	2.1 (0-2.0)	< 0.0001
Monocytes	13.6 (8.0-17.0)	13.2 (8.0-18.0)	not significant
Reactive monocytes (%)	11.0 (6.0-14.5)	9.0 (3.0-13.0)	0,0338
Proteins (mg/mL)	0.40 (0.24-0.48)	0.32 (0.24-0.36)	0,0130

^a between group differences, Willcoxon test, $p < 0.05$ was considered significant

-values expressed as median, values in parentheses are interquartile range (75% of the sample ranges)

Table 2. Lymphocyte subpopulations in the cerebrospinal fluid from children with aseptic meningitis following measles-mumps-rubella (MMR) vaccination (n=12) and enteroviral meningitis (n=11)

Lymphocyte subpopulation	Aseptic meningitis after MMR vaccination	Enteroviral meningitis	P ^a Between group differences
T-cells (%)	81.3 77.4-84.7	76.3 66.7-87.6	N.S.
CD4 ⁺ T-cells (%)	42.9 33.3-51.6	44.8 38.7-50.0	N.S.
CD8 ⁺ T-cells (%)	32.8 27.2-36.7	26.5 20.3-34.8	N.S.
Memory CD45RO ⁺ CD4 ⁺ T-cells (%)	85.1 81.1-92.2	87.7 85.0-95.0	N.S.
Naive CD45RA ⁺ CD62L ⁺ CD4 ⁺ T-cells (%)	12.8 5.8-20.3	5.6 3.2-7.3	0.039
CD4/CD8 ratio	1.4 1.0-7.8	1.8 1.4-2.1	N.S.
Monocytes (%)	8.2 4.4-11.5	9.9 3.6-8.8	N.S.

^a between group differences, Willcoxon test, p<0.05 was considered significant

-values expressed as median, values in parentheses are interquartile range (75% of the sample ranges)

Table 3. Lymphocyte subpopulations in the cerebrospinal fluid and blood of patients with acute viral encephalitis

Lymphocyte subpopulations (%)	Acute meningoencephalitis (n=14)		P ^a
	CSF	PB	
Median (IQR)			
T-cells	81.0 76.0-89.9	75.3 70.2-78.5	N.S.
CD4 ⁺ T-cells	70.1 65.6-73.0	55.1 50.1-60.2	P<0.001
CD8 ⁺ T-cells	19.4 15.2-23.5	24.4 19.4-25.6	N.S.

^a Comparison of lymphocyte subpopulations in the CSF vs. PB in patients with acute viral encephalitis

* Between group comparison (* p<0.05, Mann-Whitney test)

IQR, interquartile range, N.S., not significant