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Research Article

Lymphocyte Subpopulations in the Peripheral Blood, Mesenteric Lymphoid Nodes and Colon in Experimental Chronic Ulcerative Colitis

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Abstract

In experimental chronic ulcerative colitis induced by dextran sulfate sodium salt the morphological changes in mesenteric lymph nodes and the composition of lymphocyte subpopulations measured by flow cytometry were studied. In chronic ulcerative colitis the reactive changes in the mesenteric lymph nodes are characterized by follicular hyperplasia and sinus reaction. In chronic ulcerative colitis the amount of CD4+CD25+FOXP3+ regulatory T cells was increased in the peripheral blood, colon and mesenteric lymph nodes. In the colon and mesenteric lymph nodes the amount of CD19+ B cells was increased which is typical for adaptive immunity and chronic inflammatory process. In the colon the amount of CD3+CD4+ T-helper was increased and in the mesenteric lymph nodes the amount of CD3+CD8+ cytotoxic T-lymphocytes was also increased.

ABBREVIATIONS

UC: Ulcerative Colitis; Tregs: Regulatory T Lymphocytes; iTreg: induced Regulatory T Lymphocytes; nTreg: natural Regulatory T Cells; DC: Dendritic Cells; DSS: Dextran Sodium Sulphate; Th: T helper; APC: Antigen Presenting Cells

INTRODUCTION

Inflammatory bowel diseases like Crohn's disease, ulcerative colitis (UC) and unclassified colitis are widespread in developed countries [1]. UC is an inflammatory disease of the colon with an unknown etiology and relapsing course, it affects the mucosal layer of rectum and other sections of the colon. Clinical symptoms include diarrhea, abdominal pain, gastrointestinal bleeding and weight loss. In case of a severe course, it could lead to abscess formation, which would require a surgical intervention [2]. In recent years the prevalence of ulcerative colitis has increased 15 times, 20% of patients with a history of long-term chronic inflammation develop colorectal cancer [3].

UC is a multifactorial disease. The pathogenesis of UC involves genetic factors, immune response, environmental factors and dysbiosis of commensal microbes and microbial products [4]. Chronic inflammation of colorectal mucosa is characterized by a predominant neutrophillic infiltration with crypt abscesses and ulceration of the epithelium, reepithelialization and cell turnover in the colonic mucosa, which leads to increased risk of errors in the cell cycle repair. Oxidative stress also has been linked to chronic inflammation and genomic instability that promotes progression of UC to cancer [5].

According to scientific data $CD4^+CD25^+FOXP3^+$ regulatory T lymphocytes (Tregs) display cytokine-independent suppressing properties and play a major part in the pathogenesis of UC [4,6,7].

The majority of Tregs differentiation in thymocyte development occurs late. Although the highest concentrations of thymic Tregs are detected in the medulla, Treg lineage commitment also occurs in the cortex. In human Treg cell development, medulla-resident cells and cortex-resident cells, including reticular epithelial cells and dendritic cells (DCs), collaborate to promote Foxp3 expression. Foxp3 expression confers selective survival of thymocytes that react to self-antigens. In addition to signals derived from the TCR complex, optimal Treg generation requires IL-2 receptor signal transduction. Once CD25 is upregulated in a TCR-dependent manner, antigen-independent activation of the IL-2 receptor completes Treg cell differentiation. Differences in the nature of peptides presented

by cortical and medullary APCs are predicted to generate distinct Treg repertoires, underscoring the importance of the site of Treg cell lineage commitment [8].

Peripheral CD4 T Cells can gain the Foxp3 Expression. Naive CD4 T cells differentiate into specialized effector cells under the guidance of cytokines during T cell activation. TGF-b serves as the instructional cytokine that activates Smad transcription factors. Licensed Smad family members translocate to the nucleus to bind to DNA elements in the Foxp3 regulatory loci and enhance the Foxp3 transcription [9]. TGF-b induced Treg (iTreg) cells share many characteristics with natural regulatory T cells (nTreg) including in vitro anergy, and comparable CTLA-4 and CD45RB expression levels. Additionally, iTregs produce lower levels of IFN-g and IL-4, confirming previous reports that TGF-b attenuates Th1 and Th2 differentiation, respectively. However, the suppressive quality and the stability of these iTreg cells remain controversial. In some studies, iTregs were as suppressive as thymically derived nTregs in inhibiting the CD4 cell proliferation in vitro and in vivo.[7] Regulatory T-lymphocytes suppress the proliferation of $CD4^+CD25^-$ T-effector lymphocytes, thus inhibiting the development of inflammation and autoimmune reaction [10].

The quantitative evaluation of T-regulatory lymphocytes in mucosal biopsy of the colon of ulcerative colitis patients is proposed as one of diagnostic criteria by a number of authors [11,12].

Currently the investigation of inflammation in the colon is conducted on various experimental models of ulcerative colitis, one of the most frequently used is dextran sodium sulphate salt - DSS induced colitis [13]. Yet current scientific literature lacks any study, which evaluates changes in lymphocyte subpopulations in mesenteric lymph nodes and the colon wall in long-lasting chronic colitis.

Thus, our study of morphological changes in the colon in conjunction with the systemic and local alterations of lymphocyte subpopulations in experimental chronic ulcerative colitis is of current interest.

MATERIALS AND METHODS

Experimental animals

C57BL/6 male mice, 6-8 weeks old, weighting 18-20 g were purchased from the animal breeding facility «Stolbovaya» of the Federal State budget institution of science "Scientific Center for Biomedical Technology of the Federal Medical and Biological Agency" and maintained at the animal research facility at Research Institute of Human Morphology

Induction of DSS Colitis

Colitis was induced by an oral administration of 1% solution of DSS (wt/vol, mol wt 40,000, BioChemica, Germany) in drinking water ad libitum for 4 days at first and then on 10 – 12 and 21 – 25 day followed by drinking of tap water. The mice were checked each day for morbidity and their weight was recorded.

Sample Collection

Upon the sacrifice (64 day), the colon and mesenteric lymphoid nodes were dissected. The total blood was collected by venipuncture in EDTA-containing tubes (1.8 mg EDTA/1mL of blood) (Greiner Bio-One, Austria).

Histology

At autopsy, a two cm section of the rectum and mesenteric lymphoid nodes were fixed in the Bouin solution (composed of saturated picric acid, formaldehyde, glacial acetic acid). Samples were immersed in the fixative overnight and then transferred to 70°C ethanol. Tissues were processed by the Tissue-Tek VIP5Jr (Sakura, USA), embedded in paraffin by the Tissue-Tek TEC (Sakura, USA), sectioned at 5 μm and stained with Hematoxylin and eosin (H&E).

Isolation of intestinal lymphocytes

The mesenteric lymphoid nodes were homogenized in RPMI 1640 media by Potter – Elvehjem. Lymphocytes of the lamina propria were isolated from colon specimens using modifications of previously described techniques (Bowcutt R et al., 2015). The dissected mucosa was briefly incubated in RPMI 1640 media (ThermoFisher, USA) containing 2.5% heat-inactivated fetal bovine serum and 1 mM dithiothreitol (Sigma-Aldrich) to remove mucus. The mucosa was then incubated twice in RPMI 1640 containing 1 mM EDTA (Sigma-Aldrich) for 20 minutes at 37°C. Tissues were collected and incubated in isolation media containing RPMI 1640 supplement with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 U/mL streptomycin, 0.21 U/mg collagenase type D (Roche, Switzerland), 0.98 U/mg dispase II (Roche, Switzerland), 1 U/μL DNase I (ThermoFisher, USA) and subjected to shaking for 20 minutes at 37°C. Cells were washed with RPMI media. Cells were filtered through a 40 μm filter and counted.

Flow Cytometry

Surface marker analysis was performed using freshly isolated cells. In the peripheral blood and isolated cells, $CD3^+CD4^+$ T-helper cells, $CD3^+CD8^+$ cytotoxic T lymphocytes, $CD3^-CD19^+$ B lymphocytes were determined by flow cytometry (Cytomics FC 500, Beckman Coulter, USA) using the CD3 FITC, CD19 PE, CD 8 PE-Cy7 and CD 4 PE-Cy5 antibodies (eBioscience, USA). For the detection of FOXP3, the cells were fixed and permeabilized using the FOXP3/Transcription Factor Staining Buffer Set, according to the manufacturer's protocol (eBioscience, USA). The antibodies used were CD4 FITC, CD25 PE, Foxp3 PE-Cy5 (eBioscience, USA). The cells were counted by the hematology analyzer Celltac alpha MEK-6400 (Nihon Kohden, Japan).

Statistics

The data are presented as median (lower quartile; upper quartile). Statistical significance between selected groups was evaluated using Mann-Whitney U test. A probability of $P<0.05$ was considered as significant.

RESULTS

In the control group of male C57Bl/6 mice there was no morphological change in the colon wall. In chronic ulcerative colitis there were multiple small epithelium covered ulcers in the colon mucosa and pronounced features of remodeling

– crypts were decreased in number, deformed, had enlarged lumens, there also were several crypt-abscesses. There were focal fibrosis of the mucosa and its inflammatory infiltration with lymphocytes, macrophages and plasmocytes, the infiltration was the most pronounced in the basal portion of the lamina propria mucosae (Figure 1a).

In the control group the mesenteric lymph nodes were morphologically normal. In chronic colitis there were reactive changes characterized by follicular hyperplasia and sinus reaction. Lymphoblasts prevailed in the wide bright centers of the hyperplastic lymph nodes, there was a small number of macrophages and a high amount of dying cells. In the severely enlarged medullary cords and sinus lumens there were aggregation of macrophages with an eosinophilic cytoplasm (Figure 1b,c).

During chronic colitis in the peripheral blood there was an increase in the absolute number of CD4+CD25+FOXP3+ regulatory T-lymphocytes; in the mesenteric lymph nodes there was an increase in the absolute number of CD3+CD8+ cytotoxic T-lymphocytes; CD19+ B-lymphocytes, and CD4+CD25+FOXP3+ T-regulatory T-Lymphocytes; in the colon wall there was an increase in the number of CD3+CD4+ T-helpers, CD19+ B-lymphocytes, CD4+CD25+FOXP3+ regulatory T-lymphocytes (Table 1).

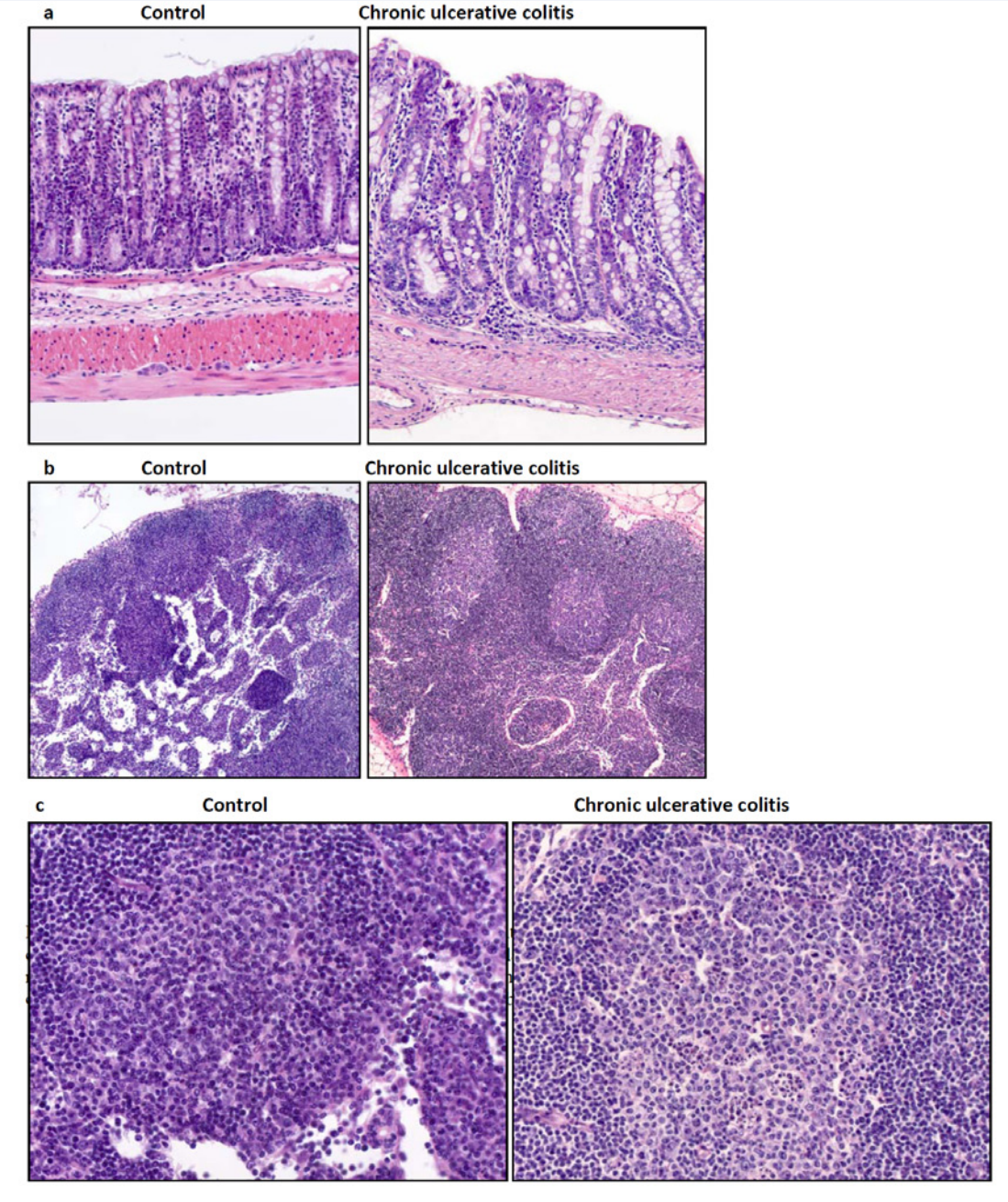

Figure 1

Table 1: Subpopulation of lymphocytes in the control and experimental group of male C57Bl/6 mice Me (0.25; 0.75).

Parameters		Peripheral blood	Mesenteric lymph nodes	Colon
		million/ml	million/ml	thousand/ml
White blood cells	Control	9.4 (8.8; 9.6)	27.25 (22.80; 27.5)	1100.0 (400.0; 1100)
	DSS	9.2 (7.9; 11.7)	31.6 (22.9; 50.7)	1150.0 (400.0; 1800.0)
Lymphocytes	Control	7.50 (7.06; 8.1)	19.44 (15.61; 20.48)	300.0 (160.0; 500.0)
	DSS	7.2 (6.26; 9.4)	26.37 (13.11; 42.17)	500.0 (166.6; 600.0)
T-helper cells CD3+CD4+	Control	1.17 (1.13; 1.23)	5.33 (4.52; 5.86)	11.4 (2.5; 14.7)
	DSS	0.8 (0.56; 1.19)	6.56 (3.69; 11.2)	40.5 (20.3; 66.0)*
Cytotoxic T lymphocytes CD3+CD8+	Control	1.01 (0.91; 1.17)	3.96 (3.38; 4.12)	6.0 (1.6; 9.6)
	DSS	0.82 (0.66; 0.98)	6.88 (4.79; 12.02)*	1.3 (0.5; 3.6)
B lymphocytes CD19+	Control	4.49 (3.04; 5.35)	5.69 (5.63; 5.79)	2.8 (0.9; 7.0)
	DSS	3.49 (2.56; 4.47)	12.6 (8.45; 17.01)*	40.8 (33.5; 121.5)*
Regulatory T lymphocytes CD4+CD25+Foxp3+	Control	thousand/ml		
		33.56 (25.66; 46.81)	267.46 (146.88; 316.94)	1.65 (0.51; 2.69)
	DSS	60.23 (44.2; 121.6)*	1100.8 (625.29; 1525.78)*	15.9 (3.1; 30.0)*

*=p<0.05
Abbreviations: DSS: Dextran sulfate sodium

DISCUSSION

The clinical manifestations of inflammatory bowel disease, diagnostics and treatment remain an actual problem for modern gastroenterology. The clinical manifestations of ulcerative colitis vary a lot and depend on the localization and extent of inflammation, activity of disease, presence or absence of complications [14]. UC is difficult to diagnose, because the pathological processes may be similar to Crohn's disease and unclassified colitis. At present time there are no pathognomic laboratory and immunological markers for this disease. The most informative method is endoscopy with diagnostic biopsy. The most specific morphological criteria of ulcerative colitis are erosions and ulcers, alterations of crypt histoarchitectonics, reparative changes of epithelium, lymphoid-plasmocytic inflammatory infiltration of mucosa and its basal compartment in particular [15]. According to research of inflammatory bowel diseases in pediatric patients by A. I. Andreev and colleagues (2010), there is an increase in regulatory T-lymphocytes (FOXP3+) in the lamina propria mucosa of the colon, which is interpreted by the authors as one of additional diagnostic criteria of ulcerative colitis. According to our data, in experimental chronic ulcerative colitis in adult male C57Bl/6 mice the absolute number of regulatory T-lymphocytes increases in the peripheral blood, mesenteric lymph nodes and colon mucosa. Previously we showed that in acute UC the amount of regulatory T-lymphocytes decreases in the peripheral blood, but increases in the mesenteric lymph nodes [16,17]. J. Maul and colleagues (2005) showed that the number of CD4+CD25 high FOXP3+ regulatory T-lymphocytes in the peripheral blood of patients with UC decreases during aggravation and increases during remission [18]. This information corresponds with our own results. Thus, the content of T-regulatory lymphocytes in the peripheral blood may be one of diagnostic criteria of aggravations or remissions in the clinical course of ulcerative colitis in humans. The change of regulatory T-lymphocytes content, at the same time as the increase of effector T-lymphocytes, reflects an imbalance in the Th1- and Th2 response. In acute ulcerative colitis the immune response is polarized mostly towards the Th1 type: in the peripheral blood there is a decrease in the content of regulatory T-lymphocytes, and among the cells of mesenteric lymph nodes there is a decrease in the number of T-helpers and an increase in number of cytotoxic T- and regulatory T-lymphocytes [16], whereas in chronic ulcerative colitis the immune response is polarized towards Th2 response [19].

Our data show an increase in number of B- and regulatory T-lymphocytes in the mesenteric lymph nodes, with a hyperplasia of B- and T- zones and an increase in number of macrophages and lymphocytes in sinuses, which is typical for a chronic inflammation. In the colon there is distinct lymphoid-plasmocytic infiltration of the lamina propria mucosa and submucosal layer, more pronounced in the distal part of the colon, there is an increase in number of T-helpers, B- and regulatory T-lymphocytes. In male C57Bl/6 mice the absolute number of B-lymphocytes in the peripheral blood does not change significantly, but increases in the mesenteric lymph nodes and in the wall of the colon. The number of regulatory T-lymphocytes increases in the peripheral blood, mesenteric lymph nodes and colon mucosa. L. Wang and colleagues (2005) showed reciprocal regulatory interactions between the levels of B- and T-lymphocytes on the model of dextran induced colitis. With the adoptive transfer of B-lymphocytes the severity of colitis decreases and the number of regulatory T-lymphocytes restores in B-cell deficient genetically modified mice [20,21].

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