INTERNAL DISEASES

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Features of lymphocyte differentiation in ANCA-associated and virus-associated glomerulonephritis in children

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Glomerulonephritis is a heterogeneous group of immuno-inflammatory diseases. The purpose of the study: to analyze the subpopulation composition of lymphocytes in children with various types of glomerulonephritis. 50 children aged 1 to 16 years were examined: 1 group — 17 children aged 1 to 16 years with ANCA-associated glomerulonephritis; 2 group — 33 patients aged 2 to 16 years with virus-associated glomerulonephritis. Diagnostics of the subpopulation composition of lymphocytes was performed by flow cytometry using non-flushing technology using a three-color combination of monoclonal antibodies. It was found that the development of ANCA-associated glomerulonephritis is accompanied by signs of immunodeficiency against the background of a relative increase in the content of T-lymphocyte subpopulations. Differentiation of lymphocytes in children with virus-associated glomerulonephritis is ambiguous: the process goes either along the path of activation of antiviral protection, or along the path of development of insufficient antiviral activity of immunocytes. In children with virus-associated glomerulonephritis, a decrease in the level of B-lymphocytes was found, correlating with hypoimmunoglobulinemia A, which is an additional factor in reducing resistance on the mucous membranes. The study of lymphocyte differentiation in the development of glomerulonephritis is important for identifying the main immunological mechanisms involved in the pathogenesis of these diseases.

Keywords: flow cytometry, lymphocyte subpopulations, lymphocyte phenotype, glomerulonephritis, children.

Background

Glomerulonephritis is a heterogeneous group of immuno-inflammatory diseases characterized by diffuse exudative-proliferative damage of renal glomerular apparatus. The pathogenesis of glomerulonephritis is based on an imbalance in the mechanisms of regulation of immunocytes' functioning and renal glomerular cells [1–3].

Anti-neutrophil cytoplasmic antibody (ANCA) — associated glomerulonephritis (ANCA-GN) develops on the background of systemic vasculitis, in which autoantibodies to neutrophil cytoplasm antigens are serologically diagnosed [3; 4]. Renal damage in children with ANCA-associated vasculitis, which proceeds as glomerulonephritis, is one of the most frequent and dominant manifestations of systemic disease, characterized by a severe course with a high risk of acute kidney damage and chronic kidney disease with outcome in terminal renal failure. In development of ANCA-GN in children the role of infectious inflammation as well as B-lymphocytes producing autoantibodies has been confirmed. ANCA are specific IgG autoantibodies to neutrophil cytoplasm antigens and monocyte lysosomes. The features of the ANCA spectrum were revealed for various forms of ANCA-associated vasculitis: the appearance of antibodies to neutrophil myeloperoxidase (MPO-ANCA) is characteristic of microscopic polyangiitis, antibodies to proteinase-3 (PR3-ANCA) are characteristic of Wegener's granulomatosis. ANCA antibodies after interaction with the neutrophil receptor apparatus activate neutrophils. Activated neutrophils act on the walls of the capillaries of kidney glomeruli with the development of fibrinoid necrosis and inflammation [4–6].

In virus-associated glomerulonephritis (VA-GN) the etiological and/or pathogenetic role of viral infection has been established [7; 8], in particular, herpes virus infection types 1/2, 4, 5. The presence of herpes virus infection is associated with prognostically unfavorable variants of the course of glomerulonephritis resistant to immunosuppressive therapy. The development of VA-GN is accompanied by changes in the immune system [9]. The effectiveness of antiviral protection is determined by the characteristics of parameters of cellular immunity, quantitative and qualitative characteristics of cells with cytotoxic potential, interferonogenesis [9; 10]. Violation of the lymphocyte differentiation leading to a decrease in adaptive immunity is a predisposing factor for persistence and reactivation of latent viral infection [7; 8; 11]. Herpes virus infection inhibits the proliferation of T-lymphocytes, inhibits the activity of NK cells, reduces the cytotoxic potential of T-killers, disrupts the functioning of the monocyte-macrophage system, reduces the expression of MHC molecules thereby disrupting the presentation of viral antigens by macrophages [12]. Dysfunction of cellular component of immunity leads to a violation of the elimination of virus-containing immune complexes and their deposition in kidney glomerul [7].

The study of lymphocyte differentiation in the development of various types of glomerulonephritis is important for identifying the main immunological mechanisms involved in the pathogenesis of these diseases.

The aim of study: to analyze the subpopulation composition of lymphocytes in ANCA-associated and virus-associated glomerulonephritis in children.

Materials and methods

50 children aged 1 to 16 years were examined. Patients are divided into 2 groups in accordance with the type of GN:

Group 1 — 17 children aged 1 to 16 years with ANCA-GN. ANCA-GN is confirmed by a positive level of autoantibodies to MPO (2 children) or PR3 (15 children).

Group 2 — 33 patients aged 2 to 16 years with VA-GN. All children in group 2 revealed clinical and laboratory (immunoserodiagnosis) signs of active mono- or combined herpes virus infection (HSV — type 1/2, VEB — type 4, CMV-5 type).

Phenotypic characteristics of adaptive and innate immunity cells as well as their subpopulation composition was carried out by flow cytometry (NovoCyte, Acea, USA) by non-washing technology using a three-color combination of monoclonal antibodies conjugated with FITC/PE/PC5 fluorescent labels: IgG1(mouse)/IgG1(mouse)/CD45, CD3/CD19/CD45, CD3/CD4/CD45, CD3/CD8/CD45, CD3/ CD(16+56)/ CD45, CD3/HLA-DR/CD45, CD3/CD25/CD45 (Beckman Coulter,USA). Cell populations were isolated using heterogeneous gating.

Statistical processing of the research results was carried out using standard software packages for applied statistical analysis (Microsoft Office Excel 2010, Statistica for Windows v. 6.1). Methods of descriptive statistics included estimating the arithmetic mean (M), mean errors (m), and standard deviation (σ) for features that have a normal distribution, including indicators in the range of reduced and/or increased values. To assess the interdependence of the values, the methods of correlation analysis were used. Correlation strength was estimated according to the following classification: strong bond — ±0,7–1; average — ±0,3–0,699; weak — ±0–0,299.

Results and discussion

The cytometric analysis of the blood lymphocyte subpopulation composition in patients with ANCA-associated and virus-associated glomerulonephritis was carried out taking into account age norms.

The criteria for reducing antiviral protection were [7–9]:

1) a decrease in the absolute number of CD3+ CD8+ (T-cytotoxic lymphocytes)
2) reduction of CD3-CD8+ (activated NK-cells)
3) reduction of CD3-CD(16+56)+ (NK-cells)

The criteria for increasing antiviral protection were:

1) increase in the absolute number of CD3+ CD8+ (T-cytotoxic lymphocytes)
2) increased CD3-CD8+ (activated NK-cells)
3) increased CD3-CD(16+56)+ (NK-cells)

Transient secondary immunodeficiency was determined by:

1) a decrease in the absolute number of lymphocytes
2) a decrease in CD3+ lymphocytes (T-lymphocytes)
3) a decrease in CD3+ CD4+ lymphocytes (T-helpers)
4) a decrease in the immunoregulatory index (CD3+CD4+ / CD3+ CD8+)

The criteria for activation of the cellular component of immune system were:

1) increase in the absolute number of lymphocytes;
2) increased CD3+ (T-lymphocytes);
3) increase CD3+ CD4+ (T-helpers);
4) increased CD3+ CD8+ (T-cytotoxic lymphocytes);
5) increase in CD3+ HLADR+ and CD3+CD25+ (activated T-lymphocytes).

The average level of the studied subpopulations of lymphocytes in children with ANCA-GN and VA-GN is presented in Table 1.

Table 1. **Quantitative composition of lymphocyte subpopulations in children with different types of glomerulonephritis**

Lymphocyte subpopulations	ANCA-associated glomerulonephritis (n = 17)		Virus-associated glomerulonephritis (n = 33)	
	Relative quantity (M±σ)	Absolute quantity (M ± σ)	Relative quantity (M ± σ)	Absolute quantity (M ± σ)
B- lymphocytes CD19+	13,2 ± 4,1	369,2 ± 133,1	14,2 ± 4,9	572,8 ± 320,8
T- lymphocytes CD3+	76,9 ± 5,4	2121,1 ± 247,8	73,8 ± 9,6	2523,4 ± 792,3
T-helpers CD3+CD4+	40,0 ± 7,3	1096,8 ± 225,4	38,8 ± 10,4	1316,5 ± 284,0
T-cytotoxic limphocytes CD3+CD8+	30,7 ± 3,7	847,1 ± 141,5	28,8 ± 4,5	1036,7 ± 284,0
Activated NK cells CD8+NK	3,1 ± 0,9	88,0 ± 35,3	3,6 ± 2,8	142,2 ± 123,2
NK- lymphocytes CD3-CD16+56+	7,9 ± 2,5	221,9 ± 90,9	11,3 ± 8,6	477,1 ± 436,8
NKT- lymphocytes CD3+CD16+56+	2,0 ± 1,4	51,2 ± 31,4	2,8 ± 1,6	71,0 ± 32,0
Activated T-lymphocytes CD3+HLA-DR+	1,3 ± 0,7	39,0 ± 20,2	2,25 ± 2,1	43,7 ± 27,9
Activated T-lymphocytes CD3+CD25+	2,8 ± 1,9	71,4 ± 44,6	5,6 ± 3,7	207,2 ± 147,6
γδ-T-lymphocytes	6,1 ± 1,9	173,0 ± 67,8	6,1 ± 2,9	168,4 ± 168,0
Immunoregulatory index CD3+CD4+/CD3+CD8+	1,4 ± 0,4		1,4 ± 0,4	

Taking into account the age norm, in children of group 1 in 47,0 % of cases relative T-lymphocytosis is observed due to an increase of NKT-lymphocytes (35,3 %), T-lymphocytes with early activation marker CD25 (23,5 %), cytotoxic T-lymphocytes CD3+CD8+

(23,5 %), T-helpers CD3+CD4+ (11,8 %). The ratio of CD3+CD4+/CD3+CD8+ (immunoregulatory index) decreased on average to 0,95 ± 0,28 in 47.0 % of patients. Thus, the development of ANCA-GN is accompanied by signs of an immunodeficiency state on the background of relative T-lymphocytosis.

NKT lymphocytes are an element of innate immunity, they have features of both T- and NK-lymphocytes, the classic phenotype is CD3+CD56+ and/or CD16+. The activation of NKT-cells is accompanied by a cytokine "burst" — the rapid secretion of a large amount of cytokines IL-2, IL-5, IL-6, IL-10, IL-17, IL-31, TNF-alpha and GM-CSF which contribute to the progression of autoimmune process.

A decrease in the absolute (on average up to 10,7 ± 2,2 cells / μL) and relative (on average up to 0,87 ± 0,32 %) number of T-lymphocytes with the marker of late activation HLA-DR is detected in 29.4 %. The proliferation of activated T-lymphocytes, which trigger a cascade of reactions leading to the formation and maintenance of the inflammatory process, plays an important role in the development of autoimmune inflammation. An increase in the levels of cytokines produced by activated T-lymphocytes is a predictor of the progression of the autoimmune process.

In B-cell component absolute inhibition of marker CD19 expression (on average up to 140,5 ± 9,5 cells / μL) is observed in 35,3 % of children in group 1, relative (up to 6,0 ± 1,7 %) — in 23,5 %.

The absolute and relative quantity of NK-cells CD3-CD(16+56)+ (on average, up to 44,2 ± 3,7 cells/μl and 3,55 ± 1 %, respectively) which are an early source of gamma-interferon at the site of the immune response is reduced in 23,5 % of the examined. At the same time 17.6 % showed a decrease in the relative quantity of activated natural killers CD8+NK to the level of 0,97 ± 0,21 %.

According to the literature, there is a correlation between the homeostatic proliferation of lymphocytes induced by lymphocytopenia and the development of an autoimmune disease [13; 14]. The pathogenesis of autoimmune inflammation is based not only on the mechanisms of realization of the activity of autoaggressive cells of T- and B-cell origin, but also mechanisms that disrupt the normal functioning of immunocompetent suppressor cells suppressing their suppressive activity which leads to the emergence and formation of clones of autoaggressive cells of the immune system [14].

In children of group 2, in 63,6 % signs of activation of antiviral protection were found: relative T-lymphocytosis due to an increase in the relative quantity of T-helpers CD3+CD4+ (up to 51,2 ± 2,2 % in 33.3 % of the examined), cytotoxic T-lymphocytes CD3+CD8+ (on average up to 35,7 ± 1,1 % in 24.2 %) providing adequate antiviral immunity due to the cytokine production as well as direct cytotoxic effects on the virus. In 27,3 % a decrease in the relative quantity of T-helpers was detected, on the background of a decrease in the immune ratio (CD3+CD4+/CD3+CD8+) up to level 1,1 ± 0,3 in 51,5 % of cases, which indicates the development of secondary transient immunodeficiency and supports long-term persistence of the virus. A decrease in the number of immune cells with cytotoxic potential is noted: the relative quantity of NK-cells up to 4,1 ± 0,3 % (51,5 % of children), activated CD8+NK-cells in average up to 1,2 ± 0,1 % (30,3 %), NKT-lymphocytes (12,1 %) decreases. It causes insufficiency of antiviral activity of cellular immunity and can contribute to the transition of acute infection into a chronic form.

In one third of patients in group 2 B-lymphocytopenia was detected (relative in 33,3 % of cases, absolute in 30,3 %), which correlates with hypoimmunoglobulinemia A:

r = –0,63 and r = –0,99, respectively. Hypoimmunoglobulinemia A is an additional factor in reducing mucosal resistance.

Patients of group 2 were found to have B-lymphocytopenia: relative (relative decrease in B-lymphocytes to 6,1 ± 0,8 %) in 33,3 % of cases, as well as absolute (absolute decrease in cells to 176,5 ± 6,5 cells/μl) in 30,3 % of cases. The relative and absolute quantity of B-lymphocytes correlates with hypoimmunoglobulinemia A (r = –0,63 and r = –0,99, respectively). Hypoimmunoglobulinemia A is an additional factor in reducing mucosal resistance.

Table 2 presents the features of lymphocyte differentiation in children of studied groups.

Table 2. **Cell dysfunction indicators in children with ANCA- associated glomerulonephritis and virus- associated glomerulonephritis**

The nature of immunological changes	ANCA-associated glomerulonephritis (n = 17)	Virus-associated glomerulonephritis (n=33)	Significance of differences, p
B-lymphocytopenia	4 (23,5 %)	11 (33,3 %)	> 0,05
T-lymphocytosis	8 (47,0 %)	21 (63,6 %)	> 0,05
Transient secondary immunodeficiency	8 (47,0 %)	17 (51,5 %)	> 0,05
NK-cells CD8+NK-cells	4 (23,5 %) 3 (17,6 %)	17 (51,5 %) 10 (30,3 %)	< 0,05 > 0,05
T-cytotoxic cells NKT-cells	4 (23,5 %) 6 (35,3 %)	8 (24,2 %) 0 (0,0 %)	> 0,05 < 0,01

An increase in the absolute quantity of γδ-T-lymphocytes in children of group 1 is observed in 77,8 % of cases, in children of group 2 — in 40 %. A high level of γδ-T-lymphocytes — factors of innate immunity — usually accompanies inflammatory processes on the mucous membranes, including the mucous membranes of the urinary tract.

About half of children in groups 1 and 2 have transient secondary immunodeficiency. A decrease in the number of cells with the cytotoxic potential of NK-cells is significantly more often observed in the group with VA-GN (p < 0,05). More than a third of children with ANCA-GN have an increased level of NKT cells (p < 0,01 compared with the VA-GN group).

Conclusions

1. The development of ANCA-GN is accompanied by signs of an immunodeficiency state (a decrease in the immune ratio, the level of B-lymphocytes, natural killers) on the background of a relative increase in the content of subpopulations of T-lymphocytes.

2. The differentiation of lymphocytes in children with VA-GN is ambiguous: the process goes either along the path of activating antiviral protection or along the path of developing deficiency of antiviral activity of immunocytes.

3. In children with VA-GN a decrease in the level of B-lymphocytes was revealed, which correlates with hypoimmunoglobulinemia A which is an additional factor in reducing mucosal resistance.

4. It is recommended to include a study of the subpopulation composition of lymphocytes in the standard of diagnostics of disorders of the immune status in children with different types of GN in order to assess violations of the parameters of the cellular component of immunity.

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