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Research of subpopulation CD3$^+$CD4$^-$CD8$^-$ double-negative T lymphocytes in kidney transplant recipients

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Introduction. *CD3$^+$CD4$^-$CD8$^-$ cells represent one of the subpopulations of T-regulatory lymphocytes. According to literature reports, an increase in the content of graft-infiltrating CD3$^+$CD4$^-$CD8$^-$ T cells was detected in the heart xenograft tissues of an experimental model with a long-term graft survival. The efficacy of CD3$^+$CD4$^-$CD8$^-$ infusion to induce skin graft tolerance was described. Some studies have shown that a decrease in CD3$^+$CD4$^-$CD8$^-$ content in peripheral blood of patients undergoing to hematopoietic stem cell transplantation was associated with the development of a graft-versus-host reaction.*

Objectives. *To study changes in the values of CD3$^+$CD4$^-$CD8$^-$ double-negative T lymphocytes in peripheral blood in kidney transplant recipients.*

Material and methods. *The study included 165 recipients who underwent kidney transplantation. The creatinine and urea concentrations in blood were determined before surgery, on day 7, and day 360 after transplantation. The content of* $CD3^{+}CD4^{-}CD8^{-}$ *lymphocytes was studied before surgery, on the 3rd, 7th, 30th, 90th, 180th and 360th days after surgery. Early graft function was assessed on day 7 after transplantation. The function was defined as a primary graft function at creatinine levels below 300 μmol/L. The delayed graft function was defined as creatinine values equal to or greater than 300 μmol/L and the need for dialysis in the first week after surgery. The satisfactory graft function after a year was characterized by the blood creatinine level below 150 μmol/L, absent episodes of graft rejection, and no need for dialysis in the first year of follow-up. There were 4 groups of recipients formed. The first group included patients with the primary graft function and satisfactory late graft function. The second group included patients with the primary graft function and late graft dysfunction. The third group included patients with the delayed graft function and satisfactory graft function. The fourth group included patients with the delayed graft function and late graft dysfunction.*

Results and discussion. *In the first and second groups, there were no significant differences in the blood level of* $CD3^{+}CD4^{-}CD8^{-}$ *during the year. After a year, a significant* $CD3^{+}CD4^{-}CD8^{-}$ *decrease was noted in the group with late graft dysfunction. A similar tendency was revealed in the third and fourth groups. In the fourth group (with the delayed graft function and late graft dysfunction), the level of* $CD3^{+}CD4^{-}CD8^{-}$ *was significantly lower only after a year of observation compared with the levels in the third group. A negative correlation was noted between the* $CD3^{+}CD4^{-}CD8^{-}$ *values and the creatinine and urea levels. Thus, high*

$CD3^+CD4^-CD8^-$ values in kidney transplant recipients after a year were associated with a satisfactory graft function.

Conclusions. *1. A stable 1-year satisfactory kidney graft function is characterized by an increase in the blood level of $CD3^+CD4^-CD8^-$ T lymphocytes. 2. A kidney graft dysfunction in the late post-transplant period is characterized by a decrease in the blood level of $CD3^+CD4^-CD8^-$ T lymphocytes.*

Keywords: $CD3^+CD4^-CD8^-$, T lymphocytes, renal graft dysfunction, kidney transplantation

DGF, delayed graft function

DN T lymphocytes, double negative $CD3^+CD4^-CD8^-$ T lymphocytes

GVHR, graft versus host reaction

KTR1, kidney transplant recipient group 1

KTR2, kidney transplant recipient group 2

KTR3, kidney transplant recipient group 3

KTR4, kidney transplant recipient group 4

LGD, late graft dysfunction

PGF, primary graft function

SLGF, satisfactory late graft function

Introduction

Regulatory T lymphocytes play an important role in various diseases [1, 2]. Double negative $CD3^+CD4^-CD8^-$ T lymphocytes (DN T-lymphocytes) represent one of the subpopulations of T-regulatory lymphocytes. This subpopulation accounts for 1-3% of peripheral T cells [3]. DN T lymphocytes can be involved in systemic inflammation and

tissue damage in autoimmune / inflammatory conditions, including systemic lupus erythematosus, Sjogren's syndrome, and psoriasis [4]. DN T lymphocytes have the ability to suppress antigen-specific auto-, allo- or xenoreactive $CD8^+$ T-lymphocytes [3, 5, 6], $CD4^+$ T lymphocytes [6–9] or B-lymphocytes [10, 11]

A number of authors found an increase in the count of DN T lymphocytes in heart allo- and xenotransplantation, where the course of post-transplant period proceeded without acute rejection episodes [12]. An adaptive transfer of Ag-activated DN T lymphocytes, according to some studies, can increase the graft survival of allo- or xenografts of the skin and heart [3, 8, 13]. In contrast to allogeneic $CD4^+$ or $CD8^+$ T cells, the infusion of allogeneic DN T lymphocytes induces the skin allograft tolerance without causing a graft versus host disease (GVHR = a graft versus host reaction) [14]. Early studies showed that the rate and total number of DN T lymphocytes were significantly reduced in patients with hematopoietic stem cell transplantation who developed GVHR, and the CD8/DN T lymphocyte ratio directly correlated with the GVHR severity [15]. It was also found that DN T lymphocytes were involved in anti-tumor immunity [16]. Given the above, it becomes clear that the role the DN T lymphocytes play in various diseases is important and poorly understood.

The mechanisms by which $CD4^+CD25^+$ Tregs mediate immune suppression have been extensively studied [17], but the mechanisms by which DN T cells mediate antigen-specific suppression remain poorly understood. Previous studies have shown that DN T lymphocytes can suppress immune responses through direct cytolysis of activated antigen-specific T-effector cells [3, 7, 8, 18, 19]. Also, one of the mechanisms of suppressing the immune response is the suppression by DN T

lymphocytes of the expression of costimulatory CD80 and CD86 molecules on the allogeneic dendritic cells induced by lipopolysaccharide [1]. In addition, DN T lymphocytes through a Fas-FasL interaction can cause the death of antigen-specific dendritic cells. [1].

This article presents the results of a study of the DN T lymphocyte dynamics in various courses of the post-transplant period in patients after kidney transplantation.

The study objective was to investigate the changes in the counts of $CD3^{+}CD4^{-}CD8^{-}$ double negative T lymphocytes in peripheral blood in kidney transplant recipients.

Material and methods

The study was conducted at the base of the Gomel State Institution *The Republican Research Center for Radiation Medicine and Human Ecology* (Gomel RRC RM&HE).

The study included 165 kidney allograft recipients with an end-stage chronic renal disease who underwent kidney allotransplantation in the Transplantation, Endocrine and Reconstructive Surgery Department of the State Research Center of Gomel RRC RM&HE. The post-transplant follow-up period was 12 months. The clinical study was conducted in conformity with the Helsinki Declaration of 1975 and approved by the Ethics Committee of the RRC RM&HE State Institution (Proceedings No. 5 dated 02.12.2013).

All patients had the serum creatinine and urea concentrations measured in dynamics: at preoperative stage, on day 7, and day 360 after transplantation. The content of $CD3^{+}CD4^{-}CD8^{-}$ lymphocytes was studied before surgery, on days 3, 7, 30, 90, 180, and 360 after surgery. Early renal graft function was evaluated by studying the blood creatinine level

on day 7 after surgery. The function was defined as a primary graft function (PGF) at creatinine levels below 300 μmol/L; the condition was classified as a delayed graft function (DGF) at values equal to or greater than 300 μmol/L, as well as when there was the need for dialysis in the first week after transplantation [20]. A satisfactory renal graft function after a year was characterized by the blood creatinine level below 150 μmol/L, absent episodes of graft rejection, and no need for dialysis during the first year of follow-up [21].

The main inclusion criteria in the study group were the following:

1. Primary renal transplantation.
2. Induction therapy with monoclonal anti-CD25 antibodies.
3. Three-component immunosuppressive therapy during the first 12 months of follow-up.

There were 100 men (60.6%), and 65 women (39.4%). Age ranged from 19 to 71 years old, the mean age was 45.95 ± 0.94 years [95% CI 44.9; 47.81]. Before transplantation, 81.21% of the patients were on programmed hemodialysis and 18.79% were on peritoneal dialysis. The median blood creatinine level before kidney transplantation was 705.0 [579.0; 920.0] μmol/L, and that of urea was 17.0 [13.9; 20.2] mmol/L. The mean cold ischemia time was 12.14±0.32 hours [95% CI 11.50; 12.77]. A negative result of direct cross-match test was observed in 100% of cases.

The mean duration of stay on dialysis was 32.9 ± 2.45 months [95% CI 28.06; 37.76]. With respect to dialysis duration, the following patient distribution was noted: 23 patients (13.94%) for 5 years or more, 103 (62.42%) patients for from 1 year to 5 years, and 39 (23.64%) patients for up to 1 year.

Considering the status of the primary and late graft functions, 165

recipients were allocated into four groups. Group 1 (KTR1) included the patients with the primary graft function and satisfactory graft function (n = 76), Group 2 (KTR2) included the patients with the primary graft function and late graft dysfunction (n = 17), Group 3 (KTR3) included those with the delayed graft function and satisfactory late graft function (n = 44), and Group 4 (KTR4) included the patients with the delayed graft function and late graft dysfunction (n = 28).

All patients received immunosuppressive therapy according to the clinical kidney transplantation protocols (Appendix 1 to Order No. 6 of the Republic of Belarus of the Ministry of Health dated 05.01.2010). The immunosuppressive therapy regimen included the induction therapy with monoclonal anti-CD25 antibodies, calcineurin inhibitors in combination with mycophenolate (87.35%) or azathioprine (12.65%), as well as corticosteroids. Monoclonal anti-CD25 antibodies were administered twice at a dose of 20 mg on days 0 and 4. Thus, 72.89% of patients received cyclosporine as a calcineurin inhibitor, and 27.11% received tacrolimus.

To determine the immunological characteristics of the kidney transplant recipients, the flow cytometry technique was applied using a FaCsCanto II flow cytometer (Becton Dickinson and Company, BD Biosciences, USA) complete with a sample preparation station applying monoclonal antibodies CD4PC7, CD8FITC, CD3PC5.5 (Beckman Coulter and BD, USA) using mono-, bi- and six-parametric analysis according to the manufacturer's instructions applying multiple advanced gating. The immunological examination of patients was performed before surgery and on days 3, 7, 30, 90, 180, and 360 after surgery.

The methodology for determining the relative and absolute counts of lymphocyte subpopulations

Blood was drawn from the ulnar vein into tubes with an anticoagulant (ethylenediaminetetraacetic acid). To determine the expression of DN T-lymphocyte surface markers by flow cytometry, the samples were prepared by using a non-washing technology in the panel for assessing the T-cell activation capacity. Monoclonal antibodies CD4PC7, CD8FITC, CD38PE, CD3PC5.5, Anti-HLADR APC (Beckman Coulter and BD, USA) were added to 100 μl of blood in amounts recommended by the manufacturer. The samples were incubated for 15 minutes in the dark at room temperature. OptiLyse B lysis solution was used for erythrocyte lysis. Samples were analyzed on a FACS CantoII flow cytometer (BD, USA). At least 10,000 events were accumulated. The T-lymphocyte population was defined as $CD3^+$ cells in the SSClow$CD45^+$bright gate, typical for lymphocytes. The double-negative population was assessed according to the histogram gated by $CD3^+$ T lymphocytes taking into account the expression of $CD4^+$ and $CD8^+$ receptors.

In the $CD3^+CD4^-CD8^-$ quadrant, DN T-lymphocytes were determined. It should be noted that the DN T-lymphocyte population was not subject to immunophenotypic differentiation into subpopulations and could include DN T lymphocytes with the expression of both TCRαβ and TCRγσ cells. To calculate the absolute content of DN T lymphocytes, the results of a complete blood count were used, the sample being taken from that very tube on the same day.

Statistical processing of the results was performed using the Statistica 10.0 software package. Descriptive statistics of qualitative characteristics have been represented by absolute and relative

frequencies, and quantitative characteristics have been presented in the following format: the mean (confidence interval) − M [Confidence -95%; +95%] and the median (interquartile range) − Me (Q25; Q75). To compare the values, we used the method of numerical characteristics (Mann–Whitney U Test, Wilcoxon Matched Pairs Test) with an estimate of the distribution of variables. The analyzed correlation of variables was assessed using Spearman Rank Order Correlations. The results were considered statistically significant when the reached significance level was equal to or less than 0.05.

Results and discussion

The results of biochemical examination of patients in the studied groups are presented in Table 1.

Table 1. Biochemical parameters of patients in the studied groups, Me (Q_{25}; Q_{75})

Parameter	Day	Patient groups			
		KTR1	KTR2	KTR3	KTR4
Creatinine, *μmol/L*	0	649.5 (569.0; 927.5)	705.0 (596.0; 920.0)	745.0 (566; 864.0)	818.0 (615.0; 1005.0)
	7	148.0 (114.0; 196.5)	192.0 (146.0; 197.0)	500.0* (352.0; 638.0)	545.0* (426.0; 738.0)
	360	107.0 (96.0; 121.0)	204.0* (152.0; 277.0)	109.0* (97.0; 126.0)	201.0*,** (169.5; 260.0)
Urea, mmol/L	0	19.2 (16.8; 22.2)	16.8* 14.3; 18.6	15.2* (10.2; 17.3)	15.8* (11.5; 17.55)
	7	10.35 (7.8; 14.5)	10.3 (7.8; 16.5)	21.5* (17.4; 36.05)	23.45* (16.9; 36.7)
	360	7.2 (5.9; 10.6)	11.2* (10.3; 14.0)	7.65 (6.4; 15.9)	12.3* (6.7; 17.9)

Notes:
* $p < 0.05$ when compared to KTR1;
** $p < 0.05$ when compared to KTR3.

The comparative analysis of the studied parameters in the four groups revealed statistically significant differences between the groups at the selected time-points of the postoperative follow-up period. Before surgery, a statistically significant excess of the urea level was revealed only in the KTR1 group compared to that in the KTR2 group ($p_{0Mann–Whitney\ U\ Test}$= 0.020). On day 7 of the investigation, no statistically significant differences in the urea level between the KTRT1 and KTR2 groups were seen ($p_{7Mann–Whitney\ U\ Test}$= 0.758). On day 360, the urea level in the KTR2 group was statistically significantly higher than in the KTR1 group ($p_{360Mann–Whitney\ U\ Test}$= 0.0003). On day 7, creatinine was statistically significantly lower in the KTR1 group than in the KTR2 group, although, according to the renal graft function assessment criteria, the function was considered as a primary one ($p_{7Mann–Whitney\ U\ Test}$= 0.039). On day 360 of investigation, the creatinine level in the KTR2 group met the LGD criteria and was statistically significantly higher than in the KTR1 group ($p_{360Mann–Whitney\ U\ Test}$ = 0.001). On day 7 of investigation, the creatinine level in the KTR4 group was statistically significantly higher compared to the value in the KTR3 group ($p_{7Mann–Whitney\ U\ Test}$= 0.005). Comparison of the blood urea between the KTR3 and KTR4 groups did not reveal statistically significant differences either on day 7 or day 360 of the investigation ($p_{0Mann–Whitney\ U\ Test}$= 0.477 and $p_{360Mann–Whitney\ U\ Test}$= 0.167, respectively). After a year of follow-up, the creatinine level in the KTRT3 group decreased and met the criteria of a late SLGF, and in the KTRT4 group it was statistically significantly higher than in the KTR3 group ($p_{360Mann–Whitney\ U\ Test}$ = 0.007).

The results of measuring the DN T lymphocyte content in kidney transplant recipient groups with various course of the post-transplant period are presented in Table 2.

Table 2. Absolute and relative count of $CD3^+CD4^-CD8^-$ double-negative T lymphocytes in kidney transplant recipients, Me (Q_{25}; Q_{75})

Day	Measure Unit	$CD3^+CD4^-CD8^-$ double-negative T lymphocytes			
		KTR1	KTR2	KTR3	KTR4
0	Rel. x %	3.8 (2.98; 4.5)	4.09 (3.4; 4.57)	4.93 (2.51; 5.42)	3.64 (2.51; 5.4)
	10^9 cells/L	0.015 (0.013; 0.028)	0.02 (0.014; 0.045)	0.017 (0.015; 0.023)	0.022 (0.013; 0.057)
3	Rel. x %	1.89 (1.54; 2.16)	1.71 (1.48; 2.22)	4.3* (3.27; 5.05)	3.75* (2.95; 4.5)
	10^9 cells/L	0.12 (0.007; 0.019)	0.011 (0.004; 0.023)	0.025* (0.016; 0.029)	0.019* (0.012; 0.043)
7	Rel. x %	1.93 (1.47; 2.43)	1.85 (1.22; 2.43)	4.6* (2.9; 6.13)	4.19* (3.06; 5.0)
	10^9 cells/L	0.03 (0.02; 0.048)	0.024 (0.017; 0.032)	0.061* (0.034; 0.105)	0.063* (0.041; 0.072)
30	Rel. x %	1.93 (1.8; 2.11)	2.08 (1.9; 2.28)	5.12* (1.4; 7.71)	5.77* (3.8; 6.74)
	10^9 cells/L	0.031 (0.02; 0.048)	0.033 (0.021; 0.04)	0.06 (0.012; 0.082)	0.078* (0.043; 0.115)
90	Rel. x %	5.56 (3.56; 6.92)	5.56 (2.92; 7.32)	6.12* (4.66; 7.89)	5.15 (4.05; 6.12)
	10^9 cells/L	0.116 (0.061; 0.16)	0.1 (0.047; 0.176)	0.124* (0.101; 0.169)	0.101 (0.069; 0.124)
180	Rel. x %	4.49 (3.28; 5.52)	3.59 (3.3; 3.77)	3.5 (2.66; 4.4)	3.71 (2.66; 4.4)
	10^9 cells/L	0.09 (0.052; 0.125)	0.082 (0.048; 0.084)	0.072 (0.043; 0.096)	0.09 (0.048; 0.102)
360	Rel. x %	4.58 (3.33; 5.8)	2.76* (2.22; 3.33)	3.94 (2.48; 7.44)	2.5*,** (1.6;3.43)
	10^9 cells/L	0.1 (0.071; 0.128)	0.04* (0.036; 0.071)	0.099 (0.044; 0.161)	0.049 (0.02; 0.099)

Notes:
* $p < 0.05$ when compared to KTR1;
** $p < 0.05$ when compared to KTR3.

When comparing two groups of patients (KTR1 and KTR2), no statistically significant differences in the level of the DN T lymphocyte

subpopulation were seen within a year ($p_{0 Mann–Whitney\ U\ Test}$= 0.402, $p_{3 Mann–Whitney\ U\ Test}$= 0.471, $p_{7 Mann–Whitney\ U\ Test}$= 0.721, $p_{30 Mann–Whitney\ U\ Test}$= 0.191, $p_{90 Mann–Whitney\ U\ Test}$= 0.85, $p_{180 Mann–Whitney\ U\ Test}$= 0.347). After a year of follow-up, a statistically significant decrease in this cell subpopulation was noted in the KTR2 group (late graft dysfunction) ($p_{360 Mann–Whitney\ U\ Test}$= 0.001).

When analyzing the dynamics of the $CD3^{+}CD4^{-}CD8^{-}$ T lymphocyte levels in KTR3 and KTR4 groups, a similar trend was revealed. In the KTR4 group (with delayed graft function and late graft dysfunction), this cell subpopulation was statistically significantly lower only at 1 year of follow-up compared to the value in the KTR3 group ($p_{0 Mann–Whitney\ U\ Test}$= 0.589, $p_{3 Mann – Whitney\ U\ Test}$= 0.163, $p_{7 Mann–Whitney\ U\ Test}$= 0.502, $p_{30 Mann–Whitney\ U\ Test}$= 0.407, $p_{90 Mann–Whitney\ U\ Test}$= 0.222, $p_{180 Mann–Whitney\ U\ Test}$= 0.993, $p_{360 Mann–Whitney\ U\ Test}$= 0.039). The dynamics and differences in the absolute values of this T-lymphocyte subpopulation in groups KTR1 and KTR2, KTR3 and KTR4 corresponded to those in the relative ones ($p_{0 Mann\text{-}WhitneyU\ Test}$= 0.397, $p_{3 Mann\text{-}Whitney\ U\ Test}$= 0.612, $p_{7 Mann\text{-}Whitney\ U\ Test}$= 0.2, $p_{30 Mann\text{-}Whitney\ U\ Test}$ = 0734, $p_{90 Mann\text{-}WhitneyU\ Test}$= 0.99, $p_{180 Mann\text{-}Whitney\ U\ Test}$= 0.567, $p_{360 Mann\text{-}Whitney\ U\ Test}$= 0.025).

Based on the data obtained, it can be noted that in the pre-transplant period there were no differences in the DN T-lymphocyte content between the studied groups. In the groups with PGF, the values of this lymphocyte subpopulation progressively decreased, possibly in response to the ongoing immunosuppressive therapy, and remained at a stable low level during the first month. In the groups with DGF, there was no statistically significant dynamics of DN T-lymphocytes during the first month. On day 90, the growth of this subpopulation was revealed in all groups; meantime, the increase was more significant in the PGF groups

than in the DGF groups.

Based on the data obtained, higher values of DN T lymphocytes in kidney transplant recipients on day 360 of follow-up were associated with a satisfactory late graft function, both in the KTR1 group with the stable function throughout the period, and in the KTR3 group, where initially (on day 7), a delayed graft function was noted.

Considering the obtained results, we performed a correlation analysis between the values of the $CD3^{+}CD4^{-}CD8^{-}$ T lymphocyte subpopulations and the biomarkers of the renal transplant function (Table 3).

Table 3. The level of correlation between the values of $CD3^{+}CD4^{-}CD8^{-}$ T lymphocyte counts and biomarkers of the renal graft function (Spearman Rank Order Correlations)

Parameter	Day	Measure Unit	Correlation Index, r (p)	
			Creatinine	Urea
DN T lymphocytes	0	Rel. x %	-0.11 (p=0.29)	-0.11 (p=0.29)
		10^{9} cells/L	$^{+}$0.14 (p=0.24)	-0.10 (p=0.38)
	360	Rel. x %	**-0.53 (p<0.001)**	**-0.38 (p<0.001)**
		10^{9} cells/L	**-0.33 (p<0.001)**	**-0.21 (p=0.028)**

As it follows from Table 3, on day 360, negative correlations appear between the values of $CD3^{+}CD4^{-}CD8^{-}$ T lymphocytes and the biomarkers of the renal transplant function. Thus, an increase in the level of DN T-lymphocyte subpopulation is associated with a good renal graft function. Measuring the level of $CD3^{+}CD4^{-}CD8^{-}$ T-lymphocytes can be used as an additional laboratory characteristic of the compensatory regulatory reaction of the immune system during kidney transplantation.

Conclusions

1. Patients with a stable, satisfactory peripheral renal graft function during the first year are characterized by increased blood levels of a $CD3^{+}CD4^{-}CD8^{-}$ T lymphocyte subpopulation.

2. An impaired renal graft function in the late post-transplant period is characterized by a decreased level of $CD3^{+}CD4^{-}CD8^{-}$ T lymphocytes.

3. Changes in the content of $CD3^{+}CD4^{-}CD8^{-}$ T lymphocyte subpopulations can be used as an additional laboratory sign of the compensatory regulatory response of the immune system during kidney transplantation.

4. The study of $CD3^{+}CD4^{-}CD8^{-}$ T lymphocyte subpopulations in patients after kidney transplantation makes it possible to predict variants of the post-transplant period course, which will allow developing potential therapeutic strategies for treating this patient population.

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