Abstract. Background. In patients with newly diagnosed type 2 diabetes mellitus (T2DM), a small but statistically significant decrease in absolute number of natural killer (NK) cells (CD56+) in the peripheral blood (PB) was found, especially pronounced with concomitant obesity. In connection with the above, one of the main aims of the present report was to further study the role of NK cells in patients with newly diagnosed T2DM, including those with obesity. For this purpose, a combination of flow cytometry methods with scanning and transmission electron microscopy was used to determine the number and function of CD56+ cells in the PB of patients with T2DM. Materials and methods. We examined 144 patients of both sexes with newly diagnosed T2DM aged 40–70 years who had no other acute or chronic diseases and had not previously taken any hypoglycemic drugs. The control group consisted of 50 healthy individuals with normoglycemia aged 40–65 years who had not COVID-19 disease. Results. Our studies have found that in untreated patients with newly diagnosed T2DM compared to healthy controls, there is a very small statistically significant decrease in the absolute number of CD56+ cells in the PB. When combining flow cytometry (FACS method), transmission and scanning electron microscopy in patients with T2DM, a significant change in the ultrastructure of CD56+ cells was revealed, indicating a decrease in their function (an increase in the number of cells with a smoother surface of the cell membrane, a sharp decrease in the number of azurophilic granules in the cytoplasm considered the main producer of cytokines and perforins, the appearance of parallel tubular structures, biomarkers of decreased cell function, a disruption of the Golgi apparatus structure, granular endoplasmic reticulum, etc.). The data obtained indicate that at the initial stage of T2DM development in humans, there is a pronounced weakening in the function of NK cells and, consequently, the natural immune defense of the body and explain the increased susceptibility of diabetics to various viruses and infections, including COVID-19, and also more severe clinical course and increased mortality. Conclusions. The conducted studies show that in patients with T2DM who are at the initial stage of T2DM, there is a sharp disturbance in the submicroscopic organization of CD56+ cells, the most important cellular element of natural immunity, which suggests a weakening of the function of natural immune defense. It is the main cause of increased sensitivity of diabetic patients to infection with various pathogens, including the SARS-CoV-2, as well as more severe clinical course and increased mortality rate. Keywords: type 2 diabetes mellitus; obesity; viral diseases; COVID-19; NK cells expressing CD56 antigen; cytokines
Such cells are called large granular lymphocytes (LGL) [2].

The content of LGL in the PB of a healthy person according to the data of T. Timonen et al., one of the pioneers in the field of studying NK cells, ranges from 2 to 6 % and should not exceed 15 % in relation to the total number of leukocytes in the PB [3].

According to our data [2] when determining LGL in PB smears stained by Pappenheim from more than 100 primary donors, LGL amounted to 3.95 ± 0.30 % in relation to all leukocytes or 0.270 ± 0.020 × 10^9/L cells in relation to the absolute number of lymphocytes.

Until recently, it was believed that since LGL is a cytopathological homolog of NK cells, it was sufficient to determine the amount of LGL in PB to identify the function of natural immunity in humans. However, after the discovery of monoclonal antibodies against functionally different subpopulations of lymphocytes and the creation of methods for their precision counting, as well as obtaining enriched fractions of immunologically homogeneous cells using flow cytometers, it became possible to more accurately identify NK cells. It was established that human NK cells express specific antigens CD16 and especially CD56 on their cell membrane, i.e. are CD3–CD16+ and especially CD3–CD56+ cells. Determination of CD56+ cells has become the most accurate method for assessing the state of the NK system in the clinic. It was later found that there are two types of CD56+ cells: CD56+ bright cells and CD56+ dim cells. CD56+ bright cells make up 90 % of all CD56+ cells and have the highest cytotoxicity [4, 5].

Based on our previous publications [2] among residents of Ukraine, the content of CD56+ cells in PB relative to all leukocytes was 15.0 ± 1.9 %, and their absolute number relative to all lymphocytes was 0.312 ± 0.082 × 10^9/L cells.

It should also be taken into account that the level of CD56+ cells in a person can also be significantly influenced by age, gender and the degree of excess body weight.

At the same time, existing publications devoted to the study of the role of NK cells in patients with T2DM are few and contradictory, which is largely due to the fact that different authors used different methods for this purpose to evaluate it (determining the number, cytotoxicity, patients with T2DM at different stages of disease development and unequal compensatory therapy).

According to modern concepts, T2DM in humans is a chronic low-gradient inflammation characterized by the presence of hyperglycemia and insulin resistance (IR) [6–8]. At the same time, the number of T2DM patients in the world as a result of the current diabetic pandemic, of which 90 % are T2DM patients, continues to grow and has now reached 537 million [9].

According to H. Wang et al., in most T2DM patients, on the contrary, there was a significant decrease in the relative and absolute content of CD56+ cells, which was accompanied by a weakening of their cytotoxic activity [5]. A decrease in the content of NK cells (CD56+ cells) in the PB of patients with T2DM was also observed by other authors [12, 13].

As is known, NK cells, like neutrophils and monocytes/macrophages, are a population of effector cells of natural immunity in humans before the appearance of “memory” cells. However, it has recently been shown that NK cells can also participate in adaptive immunity, leading to the formation of “memory” cells [14].

The mechanism of cytotoxic effect of NK cells on target cells is very complicated.

According to the data of a number of researchers summarized in the review [15], it consists of two main factors: 1) cytotoxic action of perforins and some cytokines secreted by azurophilic granules that directly and quickly kill infected or malignant target cells; 2) the activation of death cell receptors located on target cells via binding of the death ligands, e.g. Fas ligand and TRAIL ligand. In addition, NK cells are able to induce target cell lysis via antibody-dependent, cell mediated cytotoxicity with the participation of the FcyRIII receptor.

An important factor in understanding the role of NK cells in patients with T2DM is that the most of such patients are characterized by obesity [16], which, which, just like T2DM, is classified as chronic low-gradient inflammation [14]. Moreover, the curve of the annual increase in the number of patients with T2DM runs parallel to the curve of the increase in the number of obese people around the world [17], which has now reached 2 billion people, i.e. every eleventh inhabitant of our planet suffers from obesity [18]. An increase in BMI by 5 kg/m^2 increases the mortality rate of patients with T2DM by 30 % [19].

Since it is now firmly established that T2DM in humans is a chronic low-grade inflammation characterized by hyperglycemia and insulin resistance [6, 8], then obesity should now be defined as a chronic low-grade inflammation characterized by metabolic disorders and overweight, since according to the figurative statement of G.S. Hotamisligil, inflammation and metabolism are two sides of the same coin [20].

Despite the huge number of publications concerning the nature of obesity and its role in numerous pathologies in humans, there are very few controversial works devoted to the study of the content and function of CD56+ cells in obesity [8].

At the same time, according to some authors [21–23] obesity in normoglycemic people is associated with a decrease in the number of NK cells in the PB, while other authors [15, 24, 25] did not find a significant change in content and cytotoxic activity of EK-cells compared to healthy individuals of normal weight.

There is especially insufficient information about the role of NK cells in T2DM patients with comorbid obesity. Yet, according to genetic studies [26, 27] in T2DM patients with concomitant obesity, a decrease in the expression activity of NK cell receptors KIR-2D34, KIR2DL3, NKG2D, NKG2A and changes in other receptors were found compared to T2DM patients without excess body weight.

In connection with the above, one of the main goals of the present report was to further study the role of NK cells in patients with newly diagnosed T2DM, including those com-
plicated by obesity. For this purpose, a combination of flow cytometry methods with scanning and transmission electron microscopy was used to determine the number and function of CD56+ cells in the PB of patients with T2DM.

Materials and methods

We examined 144 patients of both sexes with newly diagnosed T2DM aged 40–70 years, who had no other acute or chronic diseases, and who had not previously taken any hypoglycemic drugs. The control group consisted of 50 healthy individuals with normoglycemia aged 40–65 years and who had not COVID-19 disease.

During the research, the authors adhered to the principles of bioethics: the Basic Provisions of Human Rights and Biomedicine. Declaration of Helsinki of the World Medical Association on Ethical Principles for International Human Research. All subjects independently and voluntarily signed an informed agreement to participate in research.

BMI was calculated by dividing body weight in kilograms by the square of height in meters. According to the international classification, a BMI value between 18.5 and 24.9 kg/m² is considered normal, and from 25.9 to 29.9 kg/m² is characteristic of overweight. BMI of 30.0–34.9 kg/m² is considered obese. At higher BMI values (> 35.0 kg/m²), a diagnosis of severe obesity is made, and at BMI of 40.0 kg/m², a diagnosis of morbid obesity is made [28].

From the group of patients with newly diagnosed T2DM and comorbid obesity, a subgroup of individuals with very high BMI values (more than 35.5 kg/m²) was identified and the same indicators of the content of NK cells in the PB were also determined.

When diagnosing prediabetes and T2DM, the EASD and ADA recommendations were used [9].

The content of CD56+ cells in PB was determined by flow cytometry method using a FACStar plus laser cytofluorimeter from Becton Dickinson and panels of monoclonal antibodies against the CD56 membrane antigen from Becton Dickinson and Dakopats (Denmark). For this purpose, mononuclear cells from heparinized PB were isolated by differential centrifugation into a Ficoll-Hypaque density gradient followed by further incubation in plastic dishes (cups) in a CO₂ incubator to purify monocytes. After this, the isolated cell fractions were treated with monoclonal antibodies to the CD56 antigen labeled with fluorescein isothiocyanate (FITZ).

The ultrastructure of CD56+ cells in the PB was studied using transmission and scanning electron microscopy. The object of the study was NK cells of theuffy coat isolated by differential centrifugation and a Ficoll-Hypaque density gradient from heparinized venous PB directly taken from the vein, as well as enriched concentrates of the number of CD56+ cells obtained using flow cytometry. The studied samples were fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer with the addition of 2% glucosan (glucose), postfixed with 1% osmium tetroxide in the same buffer, passed through alcohols of decreasing concentration, anhydrous acetone and enclosed in Araldite from Fluka Chemical Co, Ltd. (Switzerland). Ultrathin sections were prepared with an LKB-8800 microtome and examined under a JEM-100C electron microscope (Japan).

For scanning electron microscopy, fixed samples of a homogeneous cell suspension of CD56+ cells were applied to aluminum disks coated with poly-L-lysine and dried at a critical point in a carbon stream and filed with high-carat gold (high quality gold) on an IFC-1100 filer. The preparations were examined under a JEM-100C electron microscope with an ASID-4C scanning attachment [29].

Statistical processing of the obtained data was carried out by the method of variation statistics with a standard statistical calculation package using the LibreOffice Calc program.

Results

Our studies have established that in untreated patients with newly diagnosed T2DM, compared with healthy controls, there is a very small statistically significant decrease in the absolute number of CD56+ cells in the PB (Fig. 1).

In a flow cytometric study of patients with newly diagnosed T2DM, as can be seen from Table 1, a small but statistically significant decrease in absolute number (10^9/l) of CD56+ cells (by 9.25%) was found compared with those in normoglycemic individuals, which is consistent with the data of some other authors [5, 12, 13]. At the same time, a particularly pronounced decrease in the relative (by 18.3%) and absolute (by 16.0%) number of CD56+ cells in the PB compared to the norm was observed in patients with T2DM with severe obesity (BMI > 35.5 kg/m²), which suggests that this is largely due to excess body weight.

At the same time, in a recently created model of T2DM in mice with normal weight, a significant decrease in the cytoxic activity of NK cells was found [30], which also confirms a certain role of this disease in the development of hypofunction of NK cells.

This suggests that the decrease in the number of NK cells in patients with the initial form of T2DM is a consequence of a combination of two inflammatory processes, i.e. characteristic for the pathogenesis of T2DM and concomitant obesity.

The most demonstrative results were discovered by us when studying the ultrastructure of CD56+ cells in the PB of patients with T2DM using scanning and transmission electron microscopy. Moreover, it should be noted that submicroscopic changes in CD56+ cells of patients with T2DM and normoglycemic individuals isolated both from heparinized native PB and in an enriched suspension of CD56+ cells obtained using flow cytometry (FACS method) were identical.

![Figure 1. Large cytoplasmic lymphocytes. PB containing azurophilic granules in the cytoplasm (LGL) (A) and not containing granules in the cytoplasm. Pappenheim staining (B). Light optical microscopy, 9,000×](image)
When scanning electron microscopy, which gives a three-dimensional image of the object of lymphocytes isolated from the PB of a healthy person using differential centrifugation in a Ficoll-Hypaque density gradient, a significant diversity in the architectonics of the surface of their cell membrane was discovered [28]. In healthy people of the control group, it is possible to identify at least 6 different types and transitional types of CD56+ cell surface. The majority (about 45–55%) of CD56+ cells in a normoglycemic person are characterized by the presence of a significant number of small, densely located microvilli on the surface (Fig. 2A). The number of CD56+ cells (about 20–30%) have a smooth surface with a small number of elongated villi (Fig. 2B). Patients with T2DM are characterized by a sharp decrease in the number of CD56+ cells (up to 15–20%) with a large number of microvilli (Fig. 2A), and increase in the number of CD56+ cells (up to 30–50%) with a smooth surface and a small number of microvilli (Fig. 2B). The type of CD56+ cell with ridge-like projections (outgrowths) is quite rare in T2DM patients with severe obesity.

Since, according to modern concepts [15, 31], in the mechanism of killing by NK cells leading to the death of target cells, the main role is played by the secretion of such cytotoxic compounds as perforin and granzyme B, as well as the destruction of numerous receptors on the cell membrane associated with apoptosis, then the obtained our data of a significant change in the ultrastructure of the surface of CD56+ cells confirms the data on a significant change in the cell membrane of NK cells involved in killing target cells.

We found especially pronounced results during transmission electron microscopy of CD56+ cells in PB of T2DM patients. The majority of CD56+ cells in patients at the initial stage of T2DM development, compared with those in normoglycemic individuals, had a more rounded surface with a reduced number of microvilli on the plasma membrane (Fig. 3B). The nucleus in such cells was located eccentrically, often with the presence of a ring-shaped nucleolus.

The cytoplasm of CD56+ cells in T2DM patients, in contrast to the norm, was very variegated. On equatorial sections, it contained a cell center of condensed-type mitochondria, enlarged by the Golgi complex and an increased number of cytolsomes, pubescent vesicles that is a sign of secretory activity of cells [32], and short tubules of granular endoplasmic reticulum.

One of the rather important features of the CD56+ cells ultrastructure in patients with T2DM, compared with those in normoglycemic individuals, was the more frequent appearance of multivesicular bodies and parallel tubular structures (PTS) (Fig. 3D). As is known, the appearance of an increased number of PTS in the cytoplasm is a sign of a decrease in the functional activity of NK cells [33, 34].

The most characteristic feature of changes in the ultrastructure of CD56+ cells in patients with T2DM, compared with those in healthy individuals was a sharp decrease in azurophilic (AZ) granules in their cytoplasm (Fig. 3A, B). In T2DM patients, vacuoles with residues of AZ granules were also more common. In this regard, it is important to emphasize that in recent years it has been discovered that AZ granules in NK cells are one of the producers of many vital cytokines and chemokines involved in the pathogenesis of IR and T2DM [28, 35]. There are several publications that show a significant association between increased levels of pro-inflammatory cytokines (IL-1β, IL-6, IL-17, TNFα) and CD56+ cells in the PB of patients with prediabetes and T2DM [5, 12, 26, 27, 36]. Particular importance is also attached to the increase in the interferon family, which is considered to be strong antiviral and antitumor agents [14, 37, 38]. AZ granules are also involved in the secretion of such transforming proteins as perforin and granzyme B involved in the killing of target cells [5, 15].

At the same time, particularly pronounced changes in the ultrastructure of CD56+ cells in PB were observed in T2DM patients with concomitant obesity.
Discussion

As is known, a huge number of review publications are devoted to such an important problem as the interdependence (correlation) between obesity and T2DM [39]. However, unfortunately, only a few publications are devoted to studying the role of NK cells in obesity, especially in patients with T2DM [5, 14, 20, 35, 40]. At the same time, there is evidence that obese people without diabetes, like patients with diabetes, experience similar but less pronounced changes in natural immunity. There are isolated publications that show that in normoglycemic obese individuals, especially women, there is a significant decrease in the number of NK cells and a weakening of their cytotoxicity in vitro [21, 24]. Removal of a large amount of adipose tissue in obese women (35.3 ± 4.5 kg/m²) with a low content of NK cells also led to normalization of the level of NK cells and an increase in their cytotoxicity [24, 41].

Our electron microscopic studies of CD56+ cells showed that patients with newly diagnosed T2DM with obesity have submicroscopic changes similar to patients with T2DM without obesity, but more pronounced. In addition, in patients with T2DM, especially with high BMI values (> 35.5 kg/m²), single CD56+ LGL with impaired nuclear ultrastructure in the form of the nuclear membrane detachment and release of chromatin into the cytoplasm were found in the studied samples (Fig. 3C).

This study complements the previous our works, which showed that, along with neutrophils and monocytes, the content and function of another one of the most important cell elements of natural immunity as NK cells also changes in patients with T2DM.

At the same time, submicroscopic studies of CD56+ cells in patients with newly diagnosed untreated T2DM, especially with morbid obesity, indicating a pronounced decrease in their functional activity, can serve as one of the explanations for the majority of publications that describe a decrease in the cytotoxic activity of NK cells in vitro experiments in patients with diabetes [15, 21, 31, 42]. Moreover, a pronounced decrease in the cytotoxic activity of NK cells has been described even in patients with prediabetes and T2DM with a normal content of NK cells in the PB [43, 44].

It is also important to note that in very old people (including 100–120 years), the number of CD56 cells and NK activity are completely preserved or even exceed normal values. This is explained by natural selection — individuals with low NK cell activity die early from viral diseases and malignant neoplasms, while individuals with high natural immune defense become long-livers [37, 45].

The data obtained on the function of CD56 cells in humans can be used to develop new methods for the prevention of type 2 diabetes.

Figure 3. Ultrastructure of CD56+ cells (LGL) in the PB of patients with newly diagnosed T2DM: A — CD56+ cell in healthy individuals, 8,000×; B — in patients with newly diagnosed T2DM (BMI of 25.1 kg/m²), 8,000×; C — in a T2DM patient with obesity (BMI of 39.8 kg/m²); D — a fragment of CD56+ cell with the presence of longitudinal and transverse PTS and at the cell center (arrows), 50,000×

Conclusions

Thus, the conducted studies show that in patients with T2DM who are at the initial stage of T2DM, there is a sharp disturbance of the submicroscopic organization of CD56+
cells — the most important cellular element of natural immunity, which suggests that in such patients the weakening of the function of natural immune defense is the main reason for increased sensitivity in diabetic patients to infection with various pathogens, including the SARS-CoV-2, as well as their more severe clinical course and increased mortality rate.

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Резюме. Актуальность. У хворих із вперше виявленим цукровим діабетом 2-го типу (ЦД2) спостерігається невелике, але статистично виразне зниження абсолютного числа природних клітин-кілерів (CD56+), що підтверджується структурно-функціональними змінами природних клітин-кілерів у периферичній крові. Гіпоглікемічні препарати, в якості яких не хворіли на COVID-19, зменшують кількість CD56+ клітин у ПК, але статистично вірогідне зниження абсолютного числа природних клітин-кілерів CD56+ здійснюється лише у нелікованих пацієнтів із вперше діагностованим гострим та хронічним захворюванням і раніше не приймаючих гіпоглікемічних препаратів. Контрольна група становище природних клітин-кілерів у периферичній крові (ПК), підтвердженої проточною цитометрією (FACS методом) і супутніх трансмісійної та скануючої електронної мікроскопії у хворих на ЦД2 було виявлено значні зміни у ультраструктурі СD56+ клітин. Це вказує на зменшення їхньої функції, а саме збільшення кількості клітин з більш гладенькою поверхнею клітинної мембрани, різке зменшення кількості азурофільних гранул у цитоплазмі, які вважаються основним продуктом цитокінів і перфоринів, повну паралельно-трубчастих структур, біомаркерів зниження функції клітин, порушення структури апарату Гольджі, гранульарного ендоплазматичного ретикулума та ін. Отримані дані свідчать про зниження трофічної активності природного імунітету, що свідчить про ослаблення клітинної апаратури, зміни якої диктуються структурно-функціональними змінами природних клітин-кілерів у периферичній крові, що обумовлюється гіпоглікемічним впливом гіпоглікемічних препаратів. Висновки. Проведені дослідження показують, що у хворих на початковій стадії ЦД2 спостерігається різке зменшення субмікрової клітинної частини організму, різке зниження кількості природних клітин-кілерів у периферичній крові, що вказує на зниження трофічної активності природного імунітetu, що свідчить про ослаблення клітинної апаратури, зміни якої диктуються структурно-функціональними змінами природних клітин-кілерів у периферичній крові, що обумовлюється гіпоглікемічним впливом гіпоглікемічних препаратів. Ключові слова: цукровий діабет 2-го типу; ожиріння; вірусні захворювання; COVID-19; нк-клітини, що експресують CD56 антиген; цитокіні