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Immunological Monitoring of Osteogenesis Disorder

*Olga Viktorovna Berdyugina and
Kirill Alexandrovich Berdyugin*

Abstract

The study of immune responses of peripheral blood in bone regeneration for bone formation predicting complications is relevant. Studies were carried out on patients with the facial skeleton injury before and after stable osteosynthesis of the mandible in a fixing device for external fixation. Of the 136 patients, 17 people's bone tissue regeneration has been slow. Laboratory tests were carried out before and after the operation. The studies included the study of cellular immunity, humoral immunity, phagocytosis, acute phase proteins and cytokine status. The obtained data were processed using variation statistics methods and the modified theorem of T. Bayes. The study revealed that alternating stages of regeneration of bone tissue (inflammation, proliferation of osteoblasts, collagenogenesis and ossification) are accompanied by changes in the immunological status. A comparative study of the dynamics of immunological parameters at normal and slow osteogenesis had made it possible to establish criteria for delayed consolidation of bone tissue. Prognostic criteria before the operations include increasing the concentration of IgM and decreasing the concentration of C-reactive protein, in the early postoperative period-increasing the number of leukocytes, the concentration of tumor necrosis factor, IgM, as well as reducing the number of CD45⁺CD3⁺-cells, the complement activity and the amount of lactoferrin.

Keywords: monitoring of osteogenesis, prognostic criteria, nitro blue tetrazolium test, tumor necrosis factor, IgM, C-reactive protein

1. Introduction

Regulation of bone formation when damaged is regulated by a complex set of factors, including mechanical conditions for the formation of high-grade regeneration, vascular reactions, the influence of the neuroendocrine system, effects of metabolites and growth factors [1]. A reflection of the processes becomes dynamic of blood parameters, among which the most important are the changes in the immune status.

It is known that the immune system and bone tissue have broad functional and structural connections. The heterogeneity of cell population included in the immune system and the variety of regulators produced determine its ambiguous participation in the regulation of bone tissue regeneration.

Both macrophages and osteoclasts develop from the same progenitor cell of the monocytic line [2]. Both of these cells have the ability to destroy tissues, which leads

to the activation of repair processes carried out by cells of mesenchymal origin, fibroblasts or osteoblasts. From stromal cells expressing the most important regulators of osteoclastogenesis and being a natural microenvironment for normal hematopoiesis, osteoblasts develop [3].

Quantitative and qualitative changes in immunocompetent cells are due to lymphocyte-macrophage regulation of osteogenesis and are reflected regeneration of the bone-favorable or delayed.

An important role in the regulation of bone tissue regeneration belongs to immune cells-lymphocytes. In an animal experiment, it was shown that thymectomy leads to a significant delay in osteogenesis after a bone fracture [4]. Different lymphocyte populations influence the regulation of bone tissue regeneration ambiguously: when analyzing the cellular composition of the regeneration zone in difficultly fused fractures, a significant decrease in the number of T cells, the absence of mast cells and the predominance of fibroblasts and mononuclear cells was revealed [5]. There are reports that with severe mechanical polytrauma, deep disturbances of the cellular immunity are observed in the form of inhibition of the expression of CD2⁻DR⁺ receptors and a decrease in the absolute number of cells with CD4⁺ and CD8⁺ phenotypes [6]. It was also shown that the activity of NK cells is significantly reduced among the patients in the fracture zone, as well as in peripheral blood [7]. A number of authors have found that in animals with a deficiency of mature T lymphocytes, physiological metabolism in bone tissue is not disturbed [8]. However, under conditions when there was a need to increase bone metabolism (in trauma and after surgery), deficiency of T-lymphocyte function led to the formation of demineralized bone matrix and loss of density of existing bone tissue [9]. Clinical studies had also confirmed the role of T lymphocytes in the regulation of bone density. It was revealed that both CD4⁺ and CD8⁺ cells can produce factors that affect osteoclastogenesis [10]. There are a number of studies that show the inhibitory role of T lymphocytes in relation to the formation of osteoclasts and resorptive changes in the bone [11]. It is indicated that activated T lymphocytes can reduce osteoclastogenesis by producing INF- γ . In vitro, the removal of CD8⁺ T lymphocytes from a cell culture containing both osteoclast precursors and bone marrow cells significantly increases the formation of mature osteoclasts [12]. Some in vivo data indicate the involvement of the prostaglandin mechanism in this process [13]. In various pathological conditions, the nature of the influence of T lymphocytes on osteoclastogenesis is determined by both a set of locally secreted cytokines and the degree of differentiation of T lymphocytes [14]. Bone resorption caused by some cytokines secreted by T lymphocytes is often accompanied by further increased bone tissue formation, which leads to an increase in bone metabolism [15]. The direct molecular mechanisms of influence, as well as the conditions that determine the stimulating or inhibitory role of T lymphocytes in the regulation of bone tissue regeneration, are under study.

B lymphocytes are also involved in the regulation of bone tissue regeneration. In the bone marrow, the earliest precursors of B lymphocytes are in close contact with the endosteal surface of the bone, and the most mature cells of this series are located in the center of the bone marrow. Such a spatial organization of B lymphopoiesis in the bone marrow suggests that the cells of the osteoblastic germ located in the endosteum, as well as stromal cells located in the connective tissue stroma of the bone marrow, produce factors that influence the early precursors of B lymphocytes. It has been suggested that molecules like vascular cell adhesion molecules (VCAMs), expressed on the surface of stromal cells, mediate the binding of lymphocyte precursors, predominantly B lymphocytes, since the latter form an essential part of the entire lymphoid component of the bone marrow. It was also revealed that this molecule plays an important role in the phenomenon of homing of

lymphocytes into the bone marrow [16]. These cells and bone tissue molecules, as it turned out, ultimately realize their effect on B lymphocytes through the transcription factor Pax5 (B-cell specific activator). In the absence of this factor, maturation of B lymphocytes does not occur. In this case, immature pre-B cells can differentiate into other cells of bone marrow origin, for example, into macrophages, osteoclasts and other cells [17]. The lack of mature lymphocytes in bone tissue is reliably associated with an increased volume of trabecular bone tissue [18]. It is interesting to note that under physiological conditions [19], as well as under the conditions of ontogenesis [20], the lack of mature B lymphocytes practically does not affect the cellular balance and metabolism in bone tissue. However, under pathological conditions or external stimuli, the absence of mature B lymphocytes does not allow bone tissue to adequately respond to changing conditions [21].

An important role in the regulation of bone tissue regeneration belongs to neutrophils. The impact of a damaging factor on the tissues leads to the development of acute inflammation, the main effectors of which are these cells. The outcome of the process largely depends on the reactivity of neutrophils [22]. Neutrophils, while not playing a key role in the repair phase, at the same time have an effect on collagenolysis and remodeling of the extracellular matrix of bone tissue. They produce factors that activate fibroblasts and metalloproteinases (collagenases, gelatinases and stromelysin), which can catabolize all the main products of extracellular matrix and play an important role in remodeling of bone matrix [23]. When studying the state of non-specific protection among the victims of severe mechanical polytrauma, it was found that regardless of the nature of the traumatic agent, the degree and severity of the damage, in almost all cases, certain changes in the immune system were observed. For example, in the first week after an injury, the polymorphonuclear neutrophil system was functionally inferior [6]. Currently, researchers are inclined to believe that the complex interactions between individual cells of the immune system during the entire post-traumatic period have not been sufficiently studied. However, it is widely known that bone damage, like any other traumatic effect, leads to an increase in the migratory ability of polymorphonuclear neutrophils [24]. At the same time, the mechanism, according to which a sharp decrease in this migratory ability is noted several times throughout the post-traumatic period, remains unclear [25]. A study of the role of polymorphonuclear leukocytes by other scientists showed that a decrease in the number of cells led to a significant decrease in the mechanical strength of the regeneration. This, according to the authors, was associated with the outflow of neutrophils into the focus of inflammation (hematoma revascularization), which was accompanied by the release of a large number of inflammatory mediators (serotonin, bradykinins and histamine). This process violated the revascularization of the forming bone callus and led to a decrease in its mechanical strength [26].

When bone tissue is damaged, reactive changes are noted not only in the cellular but also in the humoral component of the immune system. It was found that activated lymphocytes responded with a significant reduction in proliferation when interacting with the main component of the extracellular matrix-collagen both *in vitro* and *in vivo* [27]. It is also interesting to note that bone tissue injury leads to the production of autoantibodies to oxidized low-density lipoproteins, and the concentration of these autoantibodies is quite strongly related to the intensity of post-traumatic osteogenesis [28]. In the framework of the study of immunoregulation of osteogenesis, a violation of the synthesis of immunoglobulins by lymphocytes with damage to bone tissue was established [29]. Some authors also obtained data on a decrease in the synthesis of immunoglobulins class G in severe mechanical polytrauma [6].

Lactoferrin is an important regulator of osteocyte activity that increases bone formation *in vivo* [30]. Usually, lactoferrin is secreted under the influence of stimuli caused by inflammation, as it is contained in neutrophil secretory granules [31]. Lactoferrin affects the synthesis of chemokines, plays an important immunomodulatory function to reduce the high concentration of osteolytic cytokines such as TNF- α and IL-1 α [32] and stabilizes binding complexes [33]; therefore, its direct effects on the activity and development of osteocytes are apparently supplemented by these intermediary effects [30]. Reliable data have now been obtained, and it shows that lactoferrin stimulates osteoblastic growth and acts as a powerful factor in the survival of osteoblasts, preventing cell apoptosis. In addition, lactoferrin enhances the function of differentiated osteoblasts [34]. The effect of lactoferrin on the development of osteoclasts was evaluated in mouse's bone marrow cultures; as a result, it was found to exceed the response to such strong growth factors as insulin, amylin, IL-18, adrenomedullin, C-terminal telopeptides and calcitonin [35]. After local injection of lactoferrin, active bone growth was established, as well as the fact that it is a powerful factor in osteoblast growth, which can reduce bone resorption [30]. Lactoferrin acts on preosteoclasts and a large number of mature cells of this origin; however, it has no effect on bone resorption by isolated mature osteoclasts [30]. There is also a report on the effects of lactoferrin leading to bone resorption, which demonstrates that lactoferrin reduces bone function: a mixed rabbit bone cell culture is resorbed in a manner independent of RANK (receptor activator of NF- κ B)/RANKL (receptor activator of NF- κ B ligand)/OPG (osteoprotegerin) system [36]. Obviously, the identification of the mechanisms by which lactoferrin acts on bone cells is important due to the revealed possibilities of the demonstrated effects, and therefore this direction is being actively studied. The putative lactoferrin receptor is known to have been identified [37]. There is evidence that lactoferrin acts through a receptor-bound protein related to the low-density lipoprotein family (LRP) [38]. Identification of the LRP1 receptor as a functional lactoferrin receptor in osteoblasts explains the interaction mechanism and makes it possible to regulate the physiological or pharmacological effects on the bone, as well as the introduction of vitamins and other necessary substances into the bone tissue [39]. The same applies to the revealed LRP5 and LRP6 receptors, structurally associated with LRP1, which are necessary regulators of osteoblast functions [40]. Thus, lactoferrin, on the one hand, provides bone growth and, on the other hand, can perform the therapeutic function of a local agent to restore bone integrity after damage.

Integrins for extracellular matrix proteins also take part in the regulation of bone tissue regeneration. These receptors are a substrate for adhesion, migration and differentiation of fibroblasts and osteogenic cells [41]. Receptors provide a link between the cytoskeleton and the extracellular matrix, transmitting information about stretching and compression of bone tissue through, the cell membrane [42] and activate certain signaling pathways, affecting gene expression [43]. It was found that during the recirculation process, when the cells of the immune system migrate through tissues and interact with one of the main components of the extracellular matrix (collagen), and integrins act as receptors, the activation signal is combined with an antigen-recognizing receptor and is able to change the direction of action of immunocompetent cells [44]. It is known that the metabolism of bone tissue during damage is provided by numerous cytokines-IL-1 (interleukin), IL-3, IL-4, IL-6, IL-11, TNF- α (tumor necrosis factor), TNF- β , colony stimulating factors, leukemia inhibitory factor, INF- γ (interferon), TGF- β (transforming growth factor) [45, 46]. There is no doubt that the study of blood immune responses in patients with damage to the bone will assess their relationship with the passage of bone formation and find the results obtained practical application. Thus, current is the study of blood immunological reactions during the regeneration of bone tissue to predict complications osteogenesis.

2. Material and methods

The study included 136 patients with lesions of the facial skeleton before and after stable osteosynthesis of the mandible in a fixing device for external fixation. Limitation of the injury was on average 12.0 ± 3.0 days. Indications for the use of external fixation devices in damaged bone were complicated by primary and secondary shifts: mandibular fractures, in 92 persons (67.6%); disjoint fractures, in 20 persons (14.7%); fused fractures, in 21 persons (15.4%); and gunshot defects, in 3 people (2.2%). A study was authorized by the Ethics Committee, guided by the order of operation, standard operating procedures and international instruments, which are based on "Declaration of Helsinki of the World Medical Association" and its subsequent editions, UN documents and of the Council of Europe documents relating to the rights of the patient, Consolidated Guideline for Good Clinical Practice and the National Russian Federation Standard "Good Clinical Practice" GOST R 52379-2005 from 01.04.2006. Informed consent to participate in a research project in accordance with the "Statement of Ethical Control" had been obtained from all surveyed participants in this test.

The diagnosis is based on clinical and instrumental learning techniques using X-ray data, orthopantomography and CT. The volume of surgical intervention determines the nature and localization of lesions. External or intraoral access was used. The second type of access was a priority, since its implementation in the postoperative period was excluded orostoma development. The inner edge of the subsystems was fixed at 1 cm from the skin surface to avoid damaging the soft tissue. An important moment during the use of external fixation device was to restore a fractured bone axis creating contact bone fragments across the fracture surface and compression at the junction of the bone fragments. Some patients have been diagnosed with osteomyelitis. When the osteomyelitis was detected, osteosynthesis was done by opening and draining the purulent chamber. Surgical treatment of purulent focus was conducted by conventional rules.

Permanent rigid fixation of bone fragments and permanent functional damage to the load of the lower jaw were created to organize the normal course of the recovery process and a successful fight against purulent infection. These conditions of bone tissue restoration were achieved by the periodic-every 5–7 days-tension of weakened rods and displacement of rings of external retainer relative to each other. When dressing wounds and fistulas, great importance had been attached to providing constant drainage of purulent separable. Medical dressings are replaced in a timely and high-quality manner, taking into account the nature and stage of wound healing, microbial composition of purulent discharge.

An apparatus for external fixation after treatment was taken off when the following clinical signs of coalescence bone appeared: disappearance of soft tissue swelling, lack of mobility at the junction of the bone fragments during the clinical trial on the motility and on the basis of radiographic criteria: identifying fuzziness of contour ends of the fragments and improving optical densities in the gap region of bone damage. Terms of use of metal structures averaged 15.0 ± 3.0 days. Long-term results of treatment patients were followed up to 4 years.

Of the total patients (136 people) based on retrospective analysis of data, a group with slow regeneration of bone tissue was isolated, because of osteomyelitis presence. It amounted to 17 people or 12.5% of all patients. Consolidation of bone tissue in this group was observed after an average 43.0 ± 1.0 postoperative day. The control group consisted of patients whose postoperative period was uneventful. Their consolidation of bone tissue occurs on average through 29.0 ± 2.0 days after the imposition of an external fixation device. It occurred somewhat earlier than in the treatment with the other ways. Usually, the terms of consolidation of mandibular fractures make 34.5–39.7 days.

The main clinical indicators are as follows: the ratio in the group of patients by gender and by age, the amount of intraoperative blood loss, presence of concomitant pathology of the cardiovascular, respiratory, urinary systems and the gastrointestinal tract, and the presence of allergies and operations, which were previously carried out using steel structures in both groups significantly did not differ. Laboratory studies were performed preoperatively and after 3 and 10 days and 1 and 3 months after its execution. Blood was obtained in the morning fasting from subclavian vein using the phlebotomy method and from the cubital vein or veins on the back of the hand, to receive physiotherapy.

To assess the lymphocyte population composition, whole blood with K₃EDTA anticoagulant (ethylenediaminetetraacetic acid) was used at a concentration of 1.6 mg/ml (tube S-Monovette® 2.7 ml, Sarstedt, Germany), for determining the phagocytic activity of the cells-the blood with the anticoagulant heparin Li (lithium heparin) at a concentration of 16 IU/ml (tube S-Monovette® 7.5 ml, Sarstedt, Germany). Serum was used for obtaining tubes S-Monovette® 7.5 ml (Sarstedt, Germany) with polystyrene beads as activators of coagulation.

To evaluate the immune status, a standard set of laboratory tests has been used complete with modern diagnostic methods [47]. The number of leukocytes was determined using hematology analyzer Cell-Dyn 1700 (Abbot, USA), reagents and equipment from the firm Abbott. Differentiation of population composition of leukocytes was carried out in the Romanovsky-Giemsa stained smears. Stab neutrophil and segmented neutrophil cell ratio was calculated [48]. Lymphocyte subpopulations were determined by flow cytometry on the device Coulter®Epics®XL (Beckman Coulter, USA), using a monoclonal antibody produced by Beckman Coulter. Lysis of erythrocytes was performed by using the sample preparation station Coulter®Q-Prep (Beckman Coulter, USA) and reagents Immunoprep, manufactured by Beckman Coulter. Quality control was performed using gauge particles Flow Check. In vitro activation of T lymphocytes with phytohemagglutinin was performed and evaluated in the reaction of inhibition of leukocyte migration [49]. The metabolic activity of neutrophils was evaluated in the reduction reaction of nitro blue tetrazolium peroxide radicals under the action of cells and evaluated by light microscopy [50]. Spontaneous and latex-stimulated activity was determined, their ratio was calculated (stimulation index). The ability of the neutrophils to kill was determined using cytochemical cell study data. Myeloperoxidase activity was determined by Grantham-Knoll [51], and the result was expressed by the average cytochemical coefficient of Kaplow [52]. The level of the lysosomal cationic cytoplasmic proteins was determined by reaction with bromophenol blue, and the results are also expressed as the average cytochemical factor [53]. The content of serum immunoglobulin classes A, M and G was determined by enzyme immunoassay. Lysozyme activity was evaluated by gel-diffusion test for the ability to lyse culture *M. lysodeikticus* [54], and the functional state of the complement system was adjusted to 50% by assessment of hemolysis of sheep erythrocytes [49]. Contents lactoferrin and cytokines (IL-1 α , IL-8, TNF- α , IL-10 and IL-1ra) were determined using the method of two-site “sandwich” ELISA-variant using test systems from firms “Protein contour” (St. Petersburg, Russia), “Cytokine” (St. Petersburg, Russia), “Vector-Best” (Novosibirsk, Russia) and BioSource International (USA) on immunoassay equipment Stat Fax (Awareness Technology Inc., USA). The concentration of C-reactive protein was determined by turbidimetric and ceruloplasmin - at Ravin [55].

The selection of antibiotic therapy in osteomyelitis was performed based on bacteriological examination of wound and aspirate purulent contents of the cavity. The evaluation included a microscopic examination of a Gram stain, inoculation of culture media production by bioMérieux (France). Identification of microorganisms and determination of their sensitivity to antibiotics were carried out by the

analyzers Vitek 2-compact and ATB-Expression (bioMérieux, France). Bacteriological examination most frequently detected *Staphylococcus aureus* (92–95% of cases), among others were *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Acinetobacter baumannii*, *Enterococcus faecalis* and *Proteus vulgaris* (the proportion of each of them in the total number of cases was not more than 1%).

Statistical data processing was carried out using the “Microsoft Office Excel 2007” program and “Statistica for Windows v.6.1”. It included an assessment of the data on the normality of the distribution in the test groups and was based on data on the mathematical expectation of 0 and the corresponding standard deviation equal to 1. Kolmogorov–Smirnov test, Lilliefors, Shapiro–Wilk and χ^2 were used to test the hypothesis that the sample has a Gaussian (normal) distribution. To compare the variance of the studied samples, F-Fisher criterion was used. Statistical hypothesis is considered confirmed with a significance level of $p < 0.05$. Modified theory of T. Bayes was used to identify the prediction criteria [56]. Calculation of diagnostic sensitivity, diagnostic specificity and diagnostic information content of immunological tests was performed using the following Eqs. (1)–(3) [57].

$$\text{Diagnostic sensitivity} = \left(\frac{TP}{TP + FN} \right) \times 100 \quad (1)$$

$$\text{Diagnostic specificity} = \left(\frac{TN}{TN + FP} \right) \times 100 \quad (2)$$

$$\text{Diagnostic informative laboratory test} = \left(\frac{TP}{TP + FP} \right) \times 100 \quad (3)$$

where TP is the true-positive results: the number of patients with complications who were correctly classified using this criterion; FP is the false-positive results: the number of patients without complications, which have been erroneously attributed to the number of patients with complications as a result of this test; TN is the true-negative results: the number of patients without the complications that were correctly classified with the help of this test; and FN is the false-negative results: the number of patients with complications who were misclassified using this test.

3. Results and discussion

As a model for studying the dynamics of immunological parameters in normal and complicated osteogenesis, the study used laboratory parameters in patients with lesions of the facial skeleton. Injury of facial skeleton, especially with slow consolidation of bone tissue, leads to disturbances of body functions and generating esthetic defects. Therefore, the creation of forecasting system complications of osteogenesis in the recovery of fractures of the lower jaw becomes important. To address the issue of participation of immunological reactions in the restoration of bone tissue, first consider the dynamics of the main laboratory parameters during normal consolidation lesions of the mandible. Before operation values, immunological parameters were compared to known literature data [58, 59].

3.1 Features of immune responses of peripheral blood in normal bone consolidation

Before surgery, along with a slight increase in the relative number of CD45⁺CD3⁺-, CD45⁺CD19⁺-cells revealed increased production of IgA and IgM (Table 1). There was also a decrease in the activity of oxygen-dependent (nitro blue

Laboratory parameters	Normal values	Before surgery	3 days	10 days	1 month	3 months
Total number of white blood cells, 10 ⁹ /l	4.0–9.0	6.85±0.33	6.68±0.45	7.30±0.51	7.35±0.55	7.30±0.48
Total number of neutrophils, 10 ⁹ /l	2.00–5.80	4.34±0.26	4.40±0.27	4.62±0.43	4.70±0.30	4.88±0.35
Ratio stab neutrophil/segmented neutrophils	0.02–0.06	0.03±0.01	0.02±0.01	0.02±0.01	0.03±0.01	0.03±0.01
Total number of monocytes, 10 ⁹ /l	0.09–0.60	0.54±0.05	0.45±0.05	0.69±0.05*	0.50±0.05	0.52±0.05
Total number of lymphocytes, 10 ⁹ /l	1.2–3.0	1.89±0.09	1.56±0.27	2.08±0.22	2.15±0.09*	2.10±0.09
CD45 ⁺ CD3 ⁺ -cells, %	46.0–56.0	60.00±2.00	63.33±1.67	51.29±6.36	62.40±7.17	62.33±0.88
CD45 ⁺ CD19 ⁺ -cells, %	4.0–8.0	9.50±1.50	9.67±1.17	9.71±0.64	9.20±1.98	7.33±0.33
Leukocyte migration inhibition test with phytohemagglutinin, %	0–30	8.03±0.24	23.02±1.25*	13.50±1.08*	5.06±1.03	22.09±1.11*
Nitro blue tetrazolium test spontaneous, %	10–20	34.05±9.10	23.67±4.77	31.50±6.36	32.40±9.05	24.00±9.64
Nitro blue tetrazolium test stimulated, %	50–100	31.00±4.00	35.56±5.59	37.13±5.94	52.20±4.46*	49.67±4.17*
Nitro blue tetrazolium test, stimulation index	>1.3	1.00±0.20	1.83±0.27*	1.52±0.26	1.73±0.14*	2.81±0.56*
Myeloperoxidase activity of neutrophils, average coefficient cytochemical	1.9–2.8	1.91±0.25	0.70±0.12*	2.28±0.33	2.10±0.08	0.69±0.09*
Activity cationic proteins neutrophilic granulocytes, average coefficient cytochemical	1.6–1.9	1.35±0.12	1.22±0.11	1.27±0.02	1.36±0.12	1.22±0.05
Lactoferrin, ng/ml	700–1500	825.10±12.60	501.09±12.65*	1250.90±18.34*	836.21±25.16	500.03±12.89*
IgA, g/l	1.38–2.50	2.89±0.23	2.69±0.31	2.89±0.45	3.18±0.18	2.03±0.29
IgM, g/l	0.92–2.10	2.27±0.03	1.41±0.23*	1.62±0.24*	1.60±0.22*	2.10±0.63
IgG, g/l	8.5–15.8	12.35±2.05	11.73±0.58	12.56±0.77	12.30±0.57	11.97±2.56
Lysozyme, mkg/ml	28.6–31.0	16.23±1.17	20.80±1.56*	12.25±2.37	8.02±1.88*	14.90±2.04
Total hemolytic complement activity, arbitrary units/ml	40.0–42.0	50.28±2.06	36.35±5.00*	45.30±2.28	48.06±2.26	36.71±13.38
IL-1α, pg/ml	0–50	22.06±2.04	21.03±1.54	625.18±12.36*	20.14±1.98	19.25±1.15
IL-1ra, pg/ml	0–500	35.24±0.22	40.08±1.45*	609.65±12.54*	81.07±2.59*	39.66±3.08
TNF-α, pg/ml	0–50	11.17±6.23	25.03±6.17	141.25±33.75*	39.50±2.98*	24.37±7.02

Laboratory parameters	Normal values	Before surgery	3 days	10 days	1 month	3 months
IL-8, pg/ml	0–50	42.88±3.64	76.03±3.60*	415.12±11.98*	70.47±2.65*	39.74±2.99
IL-10, pg/ml	<1.0	2.31±0.08	0.07±0.02*	0.02±0.01*	0.09±0.03*	0.92±0.04*
Ceruloplasmin, g/l	0.24–0.42	0.53±0.02	0.40±0.05*	0.55±0.10	0.29±0.04*	0.33±0.07*
C-reactive protein, mg/l	0–8	11.50±5.50	7.50±2.95	30.84±13.40	1.40±0.25*	0.61±0.02*

* *p* < 0.05 compared with preoperative levels.

Table 1.
 Immunological parameters of peripheral blood in the regeneration of bone tissue.

tetrazolium test) and oxygen-independent (neutrophil activity cationic proteins) killing mechanisms, lowering the concentration of serum lysozyme.

Major changes were detected due to the inflammatory reaction induced by damage to the bone tissue in the mandible fracture, which was evaluated on the basis of a complex of acute-phase proteins. Prior to surgery, in particular, it was revealed that there was an increase in the most sensitive C-reactive protein with increasing concentration of ceruloplasmin and complement activity, indicating the presence of acute inflammation (**Table 1**).

On day 10 after surgery, the number of monocytes increased by 27.8% ($p < 0.05$) compared to preoperative level, and at 1 month after surgery, the number of lymphocytes increased by 13.8% ($p < 0.05$); the composition of population lymphocytes did not change (**Table 1**). It is known that a recognized mitogen for assessing the activity of T cells is phytohemagglutinin. According to the stress tests with phytohemagglutinin, a gain in the functional activity of the cells during the early postoperative period was identified, that is, for 3–10 days after surgery (**Table 1**). In the same period, there was a decrease in the concentration of IgM by 37.9% ($p < 0.05$) with the subsequent restoration of its level in the peripheral blood (**Table 1**). Postoperatively, increased activity of “early” killing mechanisms was also observed: an increase in lactoferrin levels by 51.6% ($p < 0.05$) and the rise of total production of reactive oxygen species (nitro blue tetrazolium test) on 83.0% ($p < 0.05$). From the humoral immunity, a significant decrease in complement activity was revealed to be 27.7% ($p < 0.05$), followed by rapid recovery at 10 days. These changes appear to have been associated with the participation of complement in the elimination of foreign substance in the development of inflammatory response.

Due to the fact that not only cellular and humoral reactions played an important role in the regulation of immunological reactions, but also the effect of a number of low molecular weight peptides (cytokines) was noted, the dynamics of some of them was studied. In particular, it was found that in the postoperative period there was an increase in the concentration of IL-1 α ($p < 0.05$), an activator of the initial stages of the immune response (**Table 1**). This is likely to determine the development and course of a large number of immunological reactions in the reduction of bone tissue. Simultaneously, the increasing concentrations of receptor antagonist IL-1–IL-1ra were revealed, which limited the development of systemic inflammatory response (**Table 1**).

Concentrations of IL-1 α and IL-1ra were increased to respectively 28.4 times and 17.3 times to the 10th day of observation compared to preoperative values. A similar trend was also characteristic of TNF- α ($p < 0.05$), similar to having IL-1 phlogogenic properties. On day 10, there was a significant increase in the level of another factor inflammation-IL-8-whose concentration in the blood was increased 9.7 times as compared to preoperative values (**Table 1**). However, during the activation process, the decreased concentrations of IL-10 were revealed, which is a factor that inhibits the synthesis of most of the cytokines. Its concentration in the serum was reduced more than 100 times ($p < 0.05$). The inflammatory response in normal bone regeneration had little activity and duration.

It is known that the regeneration of bone tissue composed of several sequentially successive stages: inflammation, proliferation of osteoblasts, collagenogenesis and ossification. In this case, additional studies were conducted 3 months after the operation, at the last stage when the external fixing device was dismantled, reparative regeneration is completed, the bone tissue has formed and the mandible functioned normally. By the time of full functional bone formation, immunological parameters were within normal limits.

In a number of patients with osteomyelitis (17 people) based on clinical and radiographic data, delayed regeneration of bone tissue of the lower jaw was revealed. It occurs 1.5 times later than normal consolidation ($p < 0.05$).

3.2 Features of peripheral blood response in slow consolidation of the mandible

In this group of patients, before surgery, differences in immunological parameters from data obtained on the bone consolidation without complicating osteomyelitis were revealed. The number of monocytes (**Table 2**) was lower, than in the group with the slow regeneration of bone tissue by 59.3% ($p < 0.05$). There was a significant attenuation of the bactericidal activity of mechanisms: the level of lactoferrin was reduced 3.5 times ($p < 0.05$) was only 33.6% compared with the normal values. The same can be mentioned for complement activity-30.2% of the lower limit of normal and below 4.2 times ($p < 0.05$) than in the group with normal bone regeneration. Increased concentration of 37.4% IgM ($p < 0.05$) in peripheral blood also exceeded 49.0% and the normal values (**Table 2**). There was increased functional activity of $CD45^+CD3^+$ -cells and it was more than 3.5 times ($p < 0.05$)-differences in the recorded response of leukocyte migration inhibition (**Table 2**). Serum lysozyme was reduced by 5.8 times. Interestingly, the concentration of the receptor antagonist of IL-1ra concentration was lower for IL-1 α (**Table 2**) that was not observed in patients with normal bone consolidation. IL-8 levels were reduced by 1.8-fold ($p < 0.05$) (**Table 2**) in comparison with patients with the other group.

The postoperative period was characterized by leukocytogenesis activation: leukocytosis was marked with the change in the ratio of group cells to the segmented cells. On day 3, a reduction in the number of T cells was shown (according to the dynamics of $CD45^+CD3^+$ -cells) 40.8% ($p < 0.05$), confirming previously published data [60]. Recovery of these cell populations occurred in 10 days (**Table 2**). Perhaps this change in the amount of T cells in the early postoperative period resulting, ultimately, in disruption of bone regeneration, because it is known that both by generating INF- γ , and through prostaglandin mechanism of these cells involved in suppressing bone destruction and the formation of osteoclasts. Decrease in the relative number of B cells (based on dynamics of $CD45^+CD19^+$ -cells) by 55.6% ($p < 0.05$) was observed later, on day 10 (**Table 2**).

Important changes were noted by the indicators characterizing the phagocytic activity of neutrophils. Amplification reactions were detected at 3 days after surgery when the spontaneous and stimulated superoxide radical production by neutrophils' superior control values was in the subgroup of 1.6- to 1.7-fold ($p < 0.05$). It can be assumed that the changes in neutrophil phagocyte system in the blood reflect the processes occurring in bone tissue. Of interest for the study was the dynamics of the content of lactoferrin at the stages of bone tissue consolidation (**Table 2**). As noted earlier, prior to the operation, its level was significantly reduced. Due to the fact that lactoferrin causes proliferation of osteoblasts and bone growth, it is not excluded that lowering the level of lactoferrin in the blood is also one of the reasons for slow regeneration of bone tissue. Later, after the operation, the lactoferrin content was always significantly lower in patients with delayed bone formation compared with patients who have bone fusion that took place at the usual time.

Slow bone formation was accompanied by significant increase (72.0%) in IgM levels (**Table 2**). The revealed changes were statistically significant differences between these patients with normal bone tissue regeneration. Inflammatory response was characterized by pronounced dynamics of slow-reacting acute-phase proteins. Specifically, on day 3, after surgery, ceruloplasmin concentration was higher by 1.9-fold ($p < 0.05$) than in patients with normal bone regeneration. The functional activity of T cells throughout the observation period was 2.0- to 3.3-fold higher ($p < 0.05$) than in patients with normal consolidation, while it exceeds the normal value of 1.5 times (**Table 1**). Active cationic proteins and myeloperoxidase neutrophilic granulocytes were higher than in patients with complication of osteogenesis, respectively, by 33.6% ($p < 0.05$) and 3.1-fold ($p < 0.05$). The

Laboratory parameters	Normal values	Before surgery	3 days	10 days	1 month	3 months
Total number of white blood cells, 10 ⁹ /l	4.0–9.0	7.08±0.50	9.08±0.66 ^{*,#}	8.43±0.47 [*]	6.13±0.38	7.50±0.51
Total number of neutrophils, 10 ⁹ /l	2.00–5.80	4.37±0.38	6.95±0.48 ^{*,#}	5.17±1.01	3.63±0.39 [#]	4.76±0.69
Ratio stab neutrophil/segmented neutrophils	0.02–0.06	0.04±0.01	0.08±0.01 ^{*,#}	0.06±0.01 [#]	0.04±0.01	0.02±0.01
Total number of monocytes, 10 ⁹ /l	0.09–0.60	0.32±0.07 [#]	0.52±0.15	0.30±0.11	0.59±0.10 [*]	0.58±0.18
Total number of lymphocytes, 10 ⁹ /l	1.2–3.0	2.09±0.20	1.40±0.22 [*]	2.63±0.55	1.70±0.10 [#]	1.94±0.22
CD45 ⁺ CD3 ⁺ -cells, %	46.0–56.0	63.77±3.24	38.50±3.51 ^{*,#}	46.47±3.54 [*]	63.61±2.98	47.74±3.64 ^{*,#}
CD45 ⁺ CD19 ⁺ -cells, %	4.0–8.0	7.03±0.89	9.06±2.01	4.31±0.59 ^{*,#}	5.88±0.47 [#]	12.71±0.36 ^{*,#}
Leukocyte migration inhibition test with phytohemagglutinin, %	0–30	28.45±5.46 [#]	45.45±5.07 ^{*,#}	44.73±6.98 [#]	42.07±5.32 [#]	91.09±12.34 ^{*,#}
Nitro blue tetrazolium test spontaneous, %	10–20	41.12±5.96	37.02±3.99 [#]	23.07±6.34 [*]	45.23±5.87	25.31±5.34 [*]
Nitro blue tetrazolium test stimulated, %	50–100	31.09±3.88	60.50±1.54 ^{*,#}	38.18±4.67	68.34±5.46 ^{*,#}	39.96±6.13
Nitro blue tetrazolium test, stimulation index	>1.3	0.75±0.21	1.64±0.24 [*]	1.65±0.13 [*]	1.51±0.22 [*]	1.50±0.14 ^{*,#}
Myeloperoxidase activity of neutrophils, average coefficient cytochemical	1.9–2.8	1.88±0.14	2.19±0.18 [#]	2.12±0.15	1.37±0.19 [#]	0.77±0.12 [*]
Activity cationic proteins neutrophilic granulocytes, average coefficient cytochemical	1.6–1.9	1.42±0.07	1.63±0.08 ^{*,#}	1.36±0.12	1.55±0.11	1.26±0.07
Lactoferrin, ng/ml	700–1500	235.14±22.63 [#]	451.09±17.35 ^{*,#}	956.88±16.44 ^{*,#}	566.22±23.26 ^{*,#}	355.84±14.87 ^{*,#}
IgA, g/l	1.38–2.50	2.48±0.41	2.59±0.19	2.46±0.15	2.06±0.21 [#]	2.68±0.31
IgM, g/l	0.92–2.10	3.12±0.38 [#]	1.05±0.19 [*]	2.80±0.25 [#]	0.78±0.13 ^{*,#}	2.86±0.34
IgG, g/l	8.5–15.8	15.30±2.36	11.26±2.20 [*]	14.13±1.87	6.31±0.69 ^{*,#}	14.51±1.45
Lysozyme, mkg/ml	28.6–31.0	2.78±0.49 [#]	3.87±0.56 [#]	14.21±2.33 [*]	3.87±0.49 [#]	13.41±2.17 [*]
Total hemolytic complement activity, arbitrary units/ml	40.0–42.0	12.07±1.18 [#]	44.93±2.51 [*]	13.46±1.49 [#]	44.03±2.66 [*]	44.07±2.37 [*]
IL-1α, pg/ml	0–50	30.24±3.08 [#]	234.07±21.46 ^{*,#}	604.26±18.29 [*]	36.41±2.65 [#]	18.33±2.14 [*]
IL-1ra, pg/ml	0–500	18.36±1.17 [#]	100.51±13.96 ^{*,#}	321.61±15.96 ^{*,#}	24.74±2.96 ^{*,#}	13.22±1.74 ^{*,#}

Laboratory parameters	Normal values	Before surgery	3 days	10 days	1 month	3 months
TNF- α , pg/ml	0–50	22.65 \pm 6.58	62.50 \pm 11.54 ^{*,#}	75.12 \pm 9.67 ^{*,#}	100.33 \pm 9.89 ^{*,#}	102.61 \pm 10.24 ^{*,#}
IL-8, pg/ml	0–50	24.15 \pm 4.78 [#]	90.32 \pm 3.66 ^{*,#}	180.74 \pm 14.85 ^{*,#}	55.41 \pm 6.74 ^{*,#}	41.65 \pm 8.97 [*]
IL-10, pg/ml	<1.0	2.19 \pm 0.07	0.08 \pm 0.02 [*]	0.04 \pm 0.01 [*]	0.04 \pm 0.01 [*]	0.85 \pm 0.06 [*]
Ceruloplasmin, g/l	0.24–0.42	0.50 \pm 0.02	0.75 \pm 0.16 [#]	0.66 \pm 0.08	0.51 \pm 0.12	0.99 \pm 0.09 ^{*,#}
C-reactive protein, mg/l	0–8	9.09 \pm 0.22	6.01 \pm 1.12 [*]	0.14 \pm 0.03 ^{*,#}	0.12 \pm 0.03 ^{*,#}	0.14 \pm 0.04 ^{*,#}

^{*} $p < 0.05$ compared with preoperative levels.

[#] $p < 0.05$ compared with uncomplicated osteogenesis.

Table 2.
 Immunological parameters of peripheral blood at slowing bone formation.

concentration of lysozyme in the same period was significantly reduced by 5.4 times (Table 2). The dynamics of cytokine concentrations also had differences: on day 3 after surgery significantly, namely more than 11-fold ($p < 0.05$), increased concentration of IL-1 α , one of the stimulants of osteoclasts (Table 2). However similar changes were observed in patients with normal bone tissue regeneration. Most likely, the ratio between the level of IL-1 α and IL-1ra after surgery (on day 10) played an important role in the violation of regeneration, when in patients with normal bone consolidation it was equal (Table 1), and at late bone consolidation, the level of IL-1ra was almost two times lower than the concentration of IL-1 α (Table 2 and Figure 1).

After one month, when the regeneration of bone tissue in the other group was completed, in patients with delayed consolidation, it was in the stage collagenogenesis. Features of the dynamics of immunological parameters are as follows: the number of neutrophils and lymphocytes in peripheral blood was reduced by 23.0% ($p < 0.05$) and 21.0% ($p < 0.05$) compared to those of patients with normal bone regeneration. Inhibition signs of humoral immunity were shown: IgA level was lower by 35.3% ($p < 0.05$), the concentration of IgM (Table 2), and IgG – 2.0 times ($p < 0.05$) was compared with the results in patients with the normal regeneration of the bone tissue. There was also a decrease in myeloperoxidase activity by 34.8% ($p < 0.05$) and lysozyme concentration by 2 times ($p < 0.05$) reduced (Table 2). One month after surgery, the receptor level antagonist of IL-1 was higher concentration of IL-1 α (Table 2) and TNF- α content was increased by 2.5-fold ($p < 0.05$), while the functional activity of the CD45⁺CD3⁺-cells (Table 2), as already mentioned, was increased by 8.3-fold ($p < 0.05$).

It is interesting that in circumstances where bone regeneration has been completed, 3 months after the operation, the factors of immune responses, causing the growth of bone, are reduced. Thus, the concentration of lactoferrin was reduced by 40.5% ($p < 0.05$) and the number of T cells by 23.4% ($p < 0.05$). During this same period, the factors of immune reactions associated with the destruction of the bone tissue continue to maintain high concentrations. In particular, TNF- α level was increased by 4.2-fold ($p < 0.05$) (Table 2). The functional state of T cells in the neutrophil migration inhibition of the reaction was increased by 4.1 times (Table 2) metabolic functional activity of neutrophils to normal.

Based on the study, criteria were developed by predicting delayed bone consolidation in the treatment of injuries of the lower jaw (Table 3).

These criteria allow for different stages of treatment (before surgery, for 3 or 10 hours after surgery) to predict the development of delayed consolidation of bone

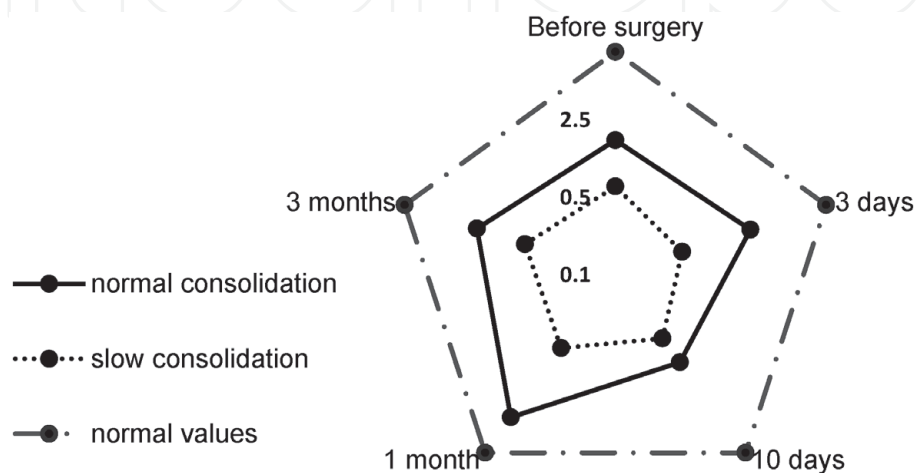


Figure 1. The ratio of the concentration of IL-1 α to IL-1ra, a logarithmic scale was used to represent the data.

Prognostic criteria	Morbidity osteogenesis	Average for osteogenesis	The diagnostic sensitivity of the test laboratory	The diagnostic specificity of the test laboratory	Diagnostic informative laboratory test
Before surgery					
IgM, g/l	↑ 2.4	0.9–2.3	85.4%	94.6%	87.3%
C-reactive protein, mg/l	↓ 9.3	9.4–17.0	79.2%	81.2%	83.2%
3 days					
Total number of white blood cells, 10 ⁹ /l	↑ 6.9	4.0–6.8	92.3%	80.1%	93.1%
CD45 ⁺ CD3 ⁺ -cells, %	↓ 45.0	46.0–70.0	90.4%	92.3%	91.7%
Nitro blue tetrazolium test spontaneous, %	↑ 33.0	10.0–32.0	91.4%	77.8%	96.3%
Lactoferrin, ng/ml	↓ 499.0	500.0–1500.0	81.3%	88.4%	86.2%
TNF-α, pg/ml	↑ 51.0	0–50.0	92.0%	90.9%	94.1%
10 days					
Total hemolytic complement activity, arbitrary units/ml	↓ 36.0	37.0–54.0	91.5%	93.0%	92.6%
IgM, g/l	↑ 2.7	0.9–2.6	87.7%	88.3%	89.5%

Table 3.
The criteria for predicting delayed consolidation of bone tissue in the treatment of lesions of the mandible.

tissue. For each of the criteria, diagnostic sensitivity, diagnostic specificity and informative test are calculated, that is the ability to predict the possible development of delayed consolidation of bone tissue.

To test the statistical hypotheses were recruited separate independent test samples of 49 people. It included patients with the same embodiments of bone damage and with the same kind of surgical treatment as the two major groups of the study. The criteria developed in this study showed that the coefficient of determination in this independent test sample was in the range of 84.2–97.5%. The joint use of two or more prognostic indicators allowed to increase the value of a diagnostic test for 3–4%. This fact gives more opportunities to clinicians in predicting delayed bone formation.

4. Conclusion

The study revealed that the stages of regeneration of bone tissue (inflammation, proliferation of osteoblasts, collagenogenesis and ossification) are accompanied by changes in the immunological status. When delayed consolidation of bone tissue revealed differences of response of individual parts of the immune system, a comparative study of the immunological parameter dynamics at normal and slow osteogenesis is possible to establish criteria for delayed consolidation of bone tissue. Prognostic criteria to the operations include increasing the concentration of IgM

and decreasing in concentration of C-reactive protein in the early postoperative period-increasing the number of leukocytes, the concentration of tumor necrosis factor, IgM, the production of superoxide anion in nitro blue tetrazolium test, as well as reducing the number of CD45⁺CD3⁺-cells, complement activity and the content of lactoferrin.

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Conflict of interest


The authors declare no conflict of interest.

Author details

Olga Viktorovna Berdyugina and Kirill Alexandrovich Berdyugin*
Institute of Immunology and Physiology, Ural Branch of Russian Academy of Sciences, Ekaterinburg, Russia

*Address all correspondence to: berolga73@rambler.ru

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