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THE STATE OF NEUTROPHILIC GRANULOCYTE INDICES IN PATIENTS WITH ACUTE INFECTED NECROTIZING PANCREATITIS DEPENDING ON THE TYPE OF PATHOGEN

The aim of the work *was to study the state of polymorphonuclear leukocytes (PMN) in patients with acute infected necrotizing pancreatitis (AINP) depending on the type of pathogen.* ***Methods.*** *In patients with AINP in the preoperative period and in patients of the control group, the metabolic activity of blood neutrophils in the SP and ST test with nitro-blue tetrazolium (NBT), as well as the PR of metabolic activity were determined in the blood. The percentage of active PMN in the phagocytosis reaction (a percentage of neutrophils involved in phagocytosis) and the number of absorbed Candida albicans particles on average by one PMN - PhI were studied.* ***Results.*** *Pseudomonas aeruginosa, Escherichia coli, Enterococcus faecalis, Klebsiella pneumoniae, Acinetobacter baumannii,and Staphylococcus epidermidis were the most common bacterial pathogens identified in AINP patients. The patients with G(–) microorganisms had a significantly increased number of formazan-positive blood PMN in the ST NBT test, as well as PhN of blood PMN compared to patients with G(+) microorganisms. The metabolic activity and phagocytic index of blood PMN in patients with infected APN did not differ depending on the monoculture or association with isolated microorganisms. However, patients with a monoculture of m/o had a significantly increased PMN blood phagocytic index compared to patients with microbial associations.* ***Conclusions.*** *In patients with acute necrotizing pancreatitis, there are disturbances in the functional activity of the PMN. The main directions of the disorders are a significant activation of the processes of blood PMN metabolism. A significantly increased PhN of blood PMN was found in patients with G(–) microorganisms compared to patients with G(+) microorganisms. It was found that patients with m/o monoculture had a significantly increased PhN of blood PMN compared to patients with microbial associations.*

Keywords: *acute infective necrotizing pancreatitis, absorptive activity, metabolic activity, Gram-negative microorganisms, Gram-positive microorganisms, polymorphonuclear leukocytes (PMN).*

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Acute pancreatitis (AP) is one of the most common inflammatory diseases of the digestive system, with a tendency to a constant increase in the proportion of this disease among patients with acute abdominal surgical pathology. The etiopathogenetic mechanisms of acute infected necrotic pancreatitis (AINP) can vary, and they invariably trigger an immune response that largely determines the severity and course of the disease. In general, AP is associated with a mortality rate of 1 to 5% due to either an excessive pro-inflammatory response or a strong compensatory inhibition of antibacterial defense mechanisms that lead to a severe necrotic form of infected pancreatitis (Glaubitz et al., 2023; Iannuzzi et al., 2022).

Recent data from microbiome studies elucidate the role of the microbiome in pancreatic diseases. Infection is a significant cause of mortality in patients with severe acute pancreatitis (SAP). However, the bacterial range is always evolving (Fan et al., 2020). Based on current understanding, bacteria translocate to the enecrotic accumulations from the small intestine. These findings align with further translational experiments demonstrating the migration of fungi and bacteria into the pancreas through the upper gastrointestinal tract (Fritz et al., 2010; Aykut et al., 2019; Pushalkar et al., 2018). Culture-based analyses revealed that *Enterococcus* spp., coagulase-negative *Staphylococcus,* and *Candida* spp. are commonly found in walled-off necrosis samples obtained by endoscopic puncture and aspiration (Sahar et al., 2018; Mowbray et al., 2018). Retrospective analysis concluded that the bacterial spectrum characteristics of 398 AP patients shows 48,4 % gram-negative bacterial strains, 41,2% gram-positive bacterial strains, and 10,4% fungal strains (Fan et al., 2020). A multihospital clinical study showed that the gut population of *Enterococcus* was higher and positively correlated with the serum levels of IL-6 in SAP patients, suggesting that the increase in Enterococci contributes to the severity of this disease (Tan et al., 2015).

Neutrophils are an important component of the innate immune system, mediating both defense against pathogens and inflammation through numerous processes, including phagocytosis, release of granular enzymes, oxidative burst, and neutrophil extracellular trap formation (Lehman & Segal, 2020).

Increased and sustained activation of neutrophils is a major determinant of pancreatic inflammation and injury (Wan et al., 2021).

Their huge number, constant production, high cytotoxicity, and ability to create extracellular traps underlie their ability to effectively protect the body from the negative effects of microbial pathogens. However, neutrophils are much more than immune sentinels, as evidenced by the wide range of their functions, found in the context of tissue homeostasis, regeneration, or chronic pathology.

Over more than a century, neutrophils (microphages) have been recognized as the fastest and most aggressive immune cells to respond to infection. They are a large army of non-proliferative cells that are constantly searching for microbial pathogens, adhere to a strict circadian regime, live only a few hours, and after contact with microorganisms must be quickly eliminated so as not to cause concomitant damage to the macroorganism. Paradoxically, the mechanisms that they use to protect the macroorganism and to further influence other aspects of body function remain largely unexplored to this day.

The aim of the work is to investigate the state of polymorphonuclear leukocytes (PMN) counts in patients with AINP depending on the type of pathogen.

Materials and Methods. The study is based on the analysis of the results of the examination of 24 patients with AIPN (purulent-necrotic forms and pancreatic abscess) in the preoperative period, who were treated at the National Scientific Center of Surgery and Transplantation named after A.A. Shalimov of the National Academy of Medical Sciences of Ukraine, and 21 healthy donors.

According to the Declaration of Helsinki, all patients were informed and gave their consent for the use of their data (examination results) in the study.

There were 22 men and 2 women (aged 29 to 65 years) among the patients in the main group, which structurally corresponds to a higher prevalence of purulent complications in male patients. The control group consisted of 21 healthy volunteer donors, including 11 men and 10 women (aged 26 to 53 years).

Bacteriological studies of blood and abscess contents were performed to determine the etiology of purulent-septic complications of AINP.

The phagocytosis study was performed according to the method described by K.F. Chernushenko (1985). We evaluated the absorption activity of PMN: the percentage of active PMN in the phagocytosis reaction — phagocytic index (PhI) and the number of absorbed Candida albicans particles on average by one PMN — phagocytic number (PhN).

The NBT test reaction was performed according to Park et al. (1968) and Bengt Bjorksten (1974). The metabolic activity of neutrophils in the spontaneous (SP) and stimulated (ST) tests with nitroblue tetrazolium (NBT), as well as the reserve (R) index of metabolic activity, as the difference in active PMN in the ST NBT and SP NBT tests, was determined.

The reactions were performed using venous heparinized blood cells. One drop (0.02 mL) of blood plasma pre-settled for erythrocyte sedimentation and 0.02 mL of medium 199 was added on a grease-free glass slide. Then, the NBT solution was added to the glass slide and gently mixed, and further incubated at 37 ˚C for 30 min in a humid chamber. For the determination of Yeast-stimulated recovery, in addition to the above ingredients, 0.02 mL suspension of nonviable *C. albicans* at a concentration of 1 million m.b./mL obtained by heating was added to a water bath for 1 hour.

The preparations were air-dried by placing the glass in the upright position. The bulk of the liquid flowed down on the glass. The glass slides are fixed with Nikiforov's mixture and counterstained with 0.1% neutral red stain for 3 min. The slides were air-dried, and the percentage of granulocytes with intracellular deposits of formazan, NBTpositive cells, were counted by light microscopy.

100 PMNs were counted for examining spontaneous NBT-reducing activity and stimulated (ST) NBT-reducing activity. The phagocytic activity of neutrophils was evaluated by two indicators: PhI and PhN.

Statistical processing of the immunological results of the study was performed using the parameters of variation statistics — relative values (p), mean values (x), mean errors of the mean and relative values (mr and mx), and estimation of the reliability of differences (P) ("Microsoft Excel" and "STATISTICA 6" programs by "Stat SOFT" for working with data in "Windows"). To compare the quantitative indicators of independent groups, in the case of normal distribution, the Student's test was used.

Results. The distribution of patients depending on the isolated pathogens is presented in Diagram 1.

In the etiopathogenesis of AINP, the following pathogens were the leading ones: *Pseudomonas aeruginosa, Escherichia coli, Enterococcus faecalis, Klebsiella pneumoniae, Acinetobacter baumannii,* and *Staphylococcus epidermidis.*

The main Gram-negative G(–) bacteria in AIPN patients were *P. aeruginosa* (24%), *E. coli* (13.33%), *K. pneumoniae* (10.67%), *A. baumannii* (13.33%), and *Citrobacter freundii* (6.67%). These five G(–) bacteria accounted for 68% of all bacterial pathogens identified in patients with AIPN. The main Gram-positive G(+) bacteria in patients with AIPN were *E. faecalis* (10.67%), *S. epidermidis* (9.33%), *E. faecium* (4%), and *Staphylococcus aureus* (2.67%). The main fungi in patients with AINP were *Candida* spp (5.33%). Among the G(–) bacteria, *Pseudomonas fluprescesns, Erwinia* spp, and *Hafnia alvei* are easily detected in patients with AIPN.

The results indicated that there were 11 patients with G(-) culture results, 5 patients with G(+) culture results, and 8 patients with both G(–) and G(+) microorganisms among the AINP patients.

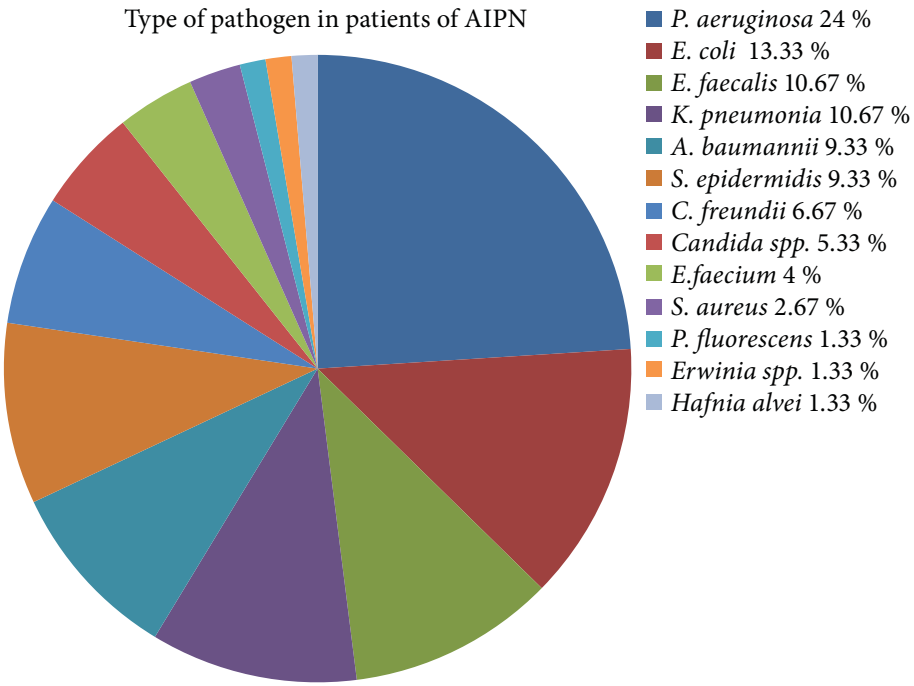

Fig. 1. The distribution of AIPN patients depending on the isolated pathogens

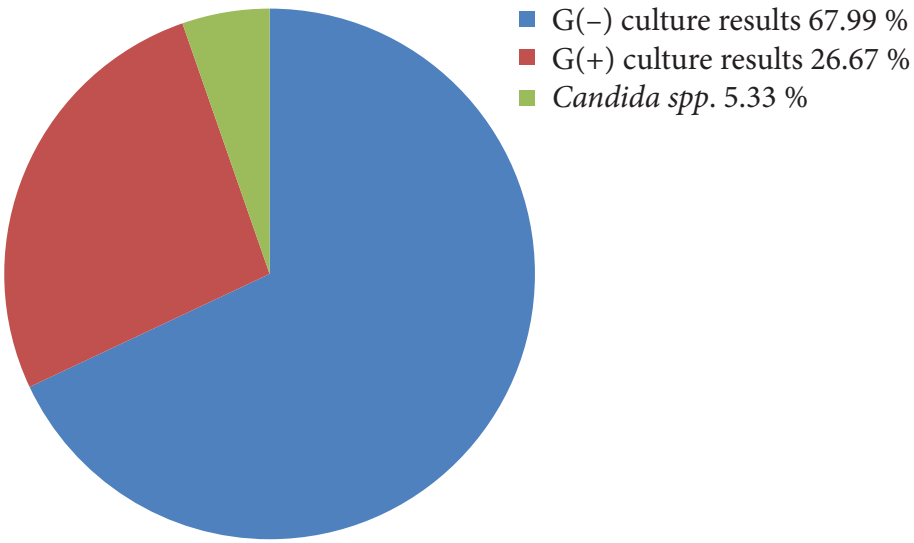

Fig. 2. The distribution of culture results in AIPN patients depending on the type of infectious agent

The distribution of culture results across the AIPN patients depending on G(–) and G(+) culture results or *Candida* spp are presented in Diagram 2.

Culture-based analyses revealed 67.99 % gram-negative bacterial strains, 26.67% gram-positive bacterial strains, and 5.33% fungal strains.

The results of the study of the metabolic and phagocytic activities of blood PMN in AINP patients depending on the type of pathogen, namely, belonging to G(–) and G(+) microorganisms and with a mixed form of infection, are presented in Table 1.

Significant changes in the metabolic activity of blood PMN were found in AINP compared with donors. AINP patients had a significantly increased metabolic activity of blood PMN in the SP NBT test and a significantly decreased PR of blood PMN. In AINP patients with gram-negative cultures of microorganisms, a significantly increased PhN of blood PMN was found compared to the control group. In AINP patients with gram-positive cultures of microorganisms, a significantly reduced PhN PMN of blood was observed compared to healthy subjects. Comparison of the study results for the metabolic and phagocytic activities of blood PMN in patients with G(–) and G(+) microorganisms, only G(–) microorganisms, and only G(+) microorganisms did not reveal any significant differences.

Table 1. **Comparison of the metabolic and phagocytic activities of blood neutrophilic granulocytes of the groups with different bacterial culture results in AINP patients**

Group	SP NBT, %	ST NBT, %	PR, %	PhI, %	PhN, conventional units.
Donors n=21	13.6 ± 2.3	63.7 ± 3.0	42.5 ± 4,8	70.7 ± 2.9	3.85 ± 0.22
Patients with G(-) microorganisms n=11	53.7 ± 5.7	78.5 ± 2.7*	23.8 ± 6.2	56.0 ± 3.7	4.62 ± 0.67*
Patients with G(+) microorganisms n=5	40.6 ± 5.2	67.8 ± 3.7	28.0 ± 7.7	67.3 ± 6.6	2.84 ± 0.25
Patients with G(-) and G(+) microorganisms n=8	44.4 ± 8.7	71.1 ± 6.8	26.7 ± 7.9	65.3 ± 6.8	3.81 ± 0.99

* — The difference between the number of formazan-positive blood PMN in the ST NBT test and PhN blood PMN is significant in AINP patients in the group with G(-) microorganisms compared with those in patients with G(+) microorganisms ($P<0.01$ — $P<0.05$).

Table 2. **Comparison of the metabolic and phagocytic activity of blood neutrophilic granulocytes in AINP patients depending on isolated monoculture or association of microorganisms**

Group	SP NBT, %	ST NBT, %	PR, %	PhI, %	PhN, conventional units.
Donors n=21	13.6 ± 2.3	63.7 ± 3.0	42.5 ± 4.8	70.7 ± 2.9	3.85 ± 0.22
Patients with one isolated microorganism n=8	49.6 ± 4.4	75.2 ± 2.5	25.1 ± 4.8	61.1 ± 3.7	4.40 ± 0.77
Patients with association of microorganisms n=16	44.4 ± 8.7	71.1 ± 6.8	26.7 ± 7.9	65.0 ± 6.3	2.33 ± 0.33*

* — The difference between the PhN of blood PMN in AINP patients is significant in the group with monoculture compared to those in patients with m/o association ($P<0.05$).

Next, we compared the data on the patients with monoculture and with microbial associations of pancreatic infection. Monoculture was obtained in 33.3% of patients, and microbial associations in 66.7%. Comparison of the results of the study of the metabolic and phagocytic activities of blood PMN in AINP patients depending on the presence of one isolated pathogen (monoculture) and several detected pathogens (associations) are presented in Table 2.

The metabolic and phagocytic activity of blood neutrophil granulocytes in AINP patients with isolated monoculture and association of microorganisms were significantly different from similar indicators of healthy individuals. The metabolic activity and phagocytic index of blood PMN in AINP patients were not significantly different between the two groups — with monoculture or association of isolated m/o. However, patients with a monoculture of m/o had a significantly increased PMN blood phagocytic index compared with patients with microbial associations.

Discussion. It is known that the pathogenicity factors of many pathogens disrupt the evolutionarily formed mechanisms of regulation of the macroorganism's immune defense (Pakbin, Brück & Rossen, 2021; Wang et al., 2021). We found pathogens in AINP patients , among which Gram-negative bacteria were the most prevalent. The pathogens identified by us in AINP patients are consistent with the classical concepts of the etiopathogenesis of acute pancreatitis (Chengsi Zhao et al., 2023). We compared the metabolic and phagocytic activity of blood PMN in AINP patients depending on whether the pathogen belongs to the Gram-positive or Gram-negative flora. Patients with isolated G(–) microorganisms had a significantly increased number of formazan-positive blood neutrophils in the ST NBT test and a significantly increased PhN of blood neutrophils compared to patients with G(+) microorganisms. A stimulated NBT test characterizes the potential ability of the PMN to respond with a «respiratory explosion» to adequate irritation. The phagocytic number reflects the intensity of phagocytosis. Thus, G(–) microorganisms do not block the production of oxygen-dependent bactericidal factors and the absorption activity of PMN compared to G(+) microorganisms, which is explained by different molecules and structures that make up the cell wall of microorganisms and different antigenicity and functions of biological membranes.

Gram-negative bacteria and lipopolysaccharide (LPS) are likely to be more potent triggers of the innate immune response. Many pathogens have evolved means to prevent phagocytosis or to resist its effects on phagocytic cells. (Thi, Wibowo, & Bernd Rehm, 2020). Some pathogens try to do that by producing substances that extracellularly intoxicate phagocytes. *S. aureus* can secrete various membrane- damaging toxins that can cause cell lysis and death (Eileen Uribe-Querol & Carlos Rosales, 2017).

In our studies, it was found that patients with microorganisms monoculture had a significantly increased PhN of blood PMN compared to patients with microorganisms association. This indicates that the bacteria in associations are able to avoid phagocytizing blood PMN. They are highly interactive and possess an extraordinary repertoire of intercellular communication and social behavior, including quorum sensing (Azimi S, Klementiev, & Whiteley M, Diggle, 2020). Another explanation for the differences in PhN between the study groups may be the fact that the phagocytic response is dose-dependent with an increase in the number of bacteria (Skjeflo et al., 2019). The studies by Giuditta Fiorella Schiavano et al. (2016) revealed an unexpected level of specificity in the elimination of bacteria in phagosomes and showed that completely different mechanisms are required to destroy different types of bacteria.

Conclusions. *P. aeruginosa, E. coli, E. faecalis, K. pneumoniae, A. baumannii,*and *S.epidermidis*

were the most active bacterial pathogens identified in patients with AINP.

It was found that AINP patients with G(–) microorganisms had a significantly increased number of formazan-positive blood PMN in the ST NBT test, as well as a significantly increased PhN of blood PMN compared to patients with G(+) microorganisms. Our study first reported that patients with isolated microorganisms monoculture have a significantly increased blood PMN PhN compared to patients with microbial associations.

Conflicts of Interest. The authors declare that there is no conflict of interest.

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СТАН ПОКАЗНИКІВ НЕЙТРОФІЛЬНИХ ГРАНУЛОЦИТІВ У ХВОРИХ НА ГОСТРИЙ ІНФІКОВАНИЙ НЕКРОТИЗУЮЧИЙ ПАНКРЕАТИТ ЗАЛЕЖНО ВІД ТИПУ ЗБУДНИКА

Мета роботи — вивчити стан поліморфноядерних лейкоцитів (ПМЯЛ) у хворих на гострий інфікований некротичний панкреатит (ГІНП) залежно від типу збудника. **Методи.** У пацієнтів з ГІНП у доопераційному періоді та у пацієнтів контрольної групи визначали метаболічну активність нейтрофілів крові в спонтанному (Сп) та стимульованому (Ст) тестах із нітросинім тетразолієм (НСТ), а також показник резерву (ПР) метаболічної активності в крові. Вивчали відсоток активних ПМЯЛ у реакції фагоцитозу —фагоцитарний індекс (ФІ — відсоток нейтрофілів, залучених до фагоцитозу) та кількість поглинених часточок *Candida albicans* у середньому одним ПМЯЛ — фагоцитарне число — ФЧ. **Результати.** *Pseudomonas aeruginosa, Escherichia coli, Enterococcus faecalis, Klebsiella pneumoniae, Acinetobacter baumannii* та *Staphylococcus epidermidis* були найпоширенішими бактеріальними збудниками, виявленими в пацієнтів з ГІНП. У пацієнтів , які мали Г(–) мікроорганізми, було виявлено значно більшу кількість формазан-позитивних ПМЯЛ крові в Ст-НСТ-тесті, а також (ФЧ) ПМЯЛ крові порівняно з пацієнтами з Г(+) мікроорганізми. Метаболічна активність та фагоцитарний індекс ПМЯЛ крові в пацієнтів з ГІНП не відрізнялися залежно від монокультури або асоціації виділених мікроорганізми. Однак пацієнти з монокультурою м/о мали значно збільшений ФІ ПМЯЛ крові порівняно з пацієнтами з мікробними асоціаціями. **Висновки.** У пацієнтів з ГІНП спостерігаються порушення функціональної активності ПМЯЛ. Основними напрямами порушень є значна активація процесів метаболізму ПМЯЛ крові. У пацієнтів з Г(–) мікроорганізми було виявлено значно збільшений ФЧ ПМЯЛ крові порівняно з пацієнтами з Г(+) мікроорганізми. Встановлено, що у пацієнтів з монокультурою мікроорганізми був значно більший PhN ПМЯЛ крові порівняно з пацієнтами з мікробними асоціаціями.

Ключові слова: *гострий інфікований некротичний панкреатит (ГІНП), поглинальна активність, метаболічна активність, грам-негативні Г(–) мікроорганізми, грам-позитивні Г(+) мікроорганізми, поліморфноядерні лейкоцити (ПМЯЛ).*