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L.M. Skivka[1], O.G. Fedorchuk[2], O.M. Pyaskovska[2], N.M. Khranovska[3], V.V. Pozur[1]

[1] T.G. Shevchenko Kyiv National University, Glushkov ave., 2, Kyiv, 03022, Ukraine, e-mail: realmed@i.com.ua;
[2] R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of NASU, Vasylkivska str., 45, Kyiv, 03022, Ukraine;
[3] National Cancer İnstitute Ministry of Public Health of Ukraine, Lomonosov str., 33/44, Kyiv, 03022, Ukraine

THE EFFECT OF *STAPHYLOCOCCUS AUREUS* WOOD 46 PEPTIDOGLYCAN ON CYTOTOXIC ACTIVITY OF MURINE MONONUCLEAR SPLENOCYTES

The effect of S. aureus Wood 46 *peptidoglycan (PG) on cytotoxic activity of mononuclear splenocytes in mice has been investigated. It was shown that the target-cells death indices upon addition of PG at the concentration of 500 μg/ml after 4h incubation were 1.5 times higher than corresponding indices in the control, but at lower concentrations PG did not influence cytotoxic activity of murine mononuclear splenocytes. 16 h incubation effector and target cells with PG resulted in a dose dependent increase of splenic mononuclear cells cytotoxicity by 1.5, 1.8, 2.0 and 2.5 times for 10, 25, 100 and 500 μg/ml of murein correspondingly.*

K e y w o r d s: peptidoglycan, cytotoxic activity, Lewis lung carcinoma.

Peptidoglycan (PG) is the major unique and essential component of the cell wall of virtually all of Gram-positive and Gram-negative bacteria [2]. PG and its muropeptide derivatives are considered as potential virulence factors [7]. PGs from different bacteria have distinguishing features but all of them are not present in eukaryotes and therefore they are excellent targets for innate immune system [1, 3, 6].

Two families of receptors — the members of proinflammatory interleukin-1 receptor family — Toll-like receptors (TLR) (in particular, TLR-2) and cytosolic proteins containing a nucleotide-binding oligomerization domain (NOD1 and NOD2) — play the key role in PG detection [3, 6]. Following ligand recognition TLR and NODs initiate intracellular signal transduction that results in the expression of genes involved in inflammation [9, 15]. PG detecting germ-line encoded receptors are expressed in the variety of cells and tissues including mucosal epithelial cells, monocytes, macrophages, T- and B-lymphocytes [11, 18]. Recently TLRs were found in NK cells [10]. These receptors allow NK cells to recognise directly bacterial structures besides their indirect activation with accessory cells through cytokine production. NK cells are one of the key players in tumor immunity, being ultimately responsible for destruction of malignant cells [8]. NK cytotoxic ability can be enhanced in vitro and in vivo by

cytokines, such as interleukin (IL)-2, IL-12, IL-15 and interferon alpha/beta (IFN-alpha/beta) [17]. Recently it was shown that some bacterial immunomodulators, such as CpG-containing DNA, can directly activate NK cell response to tumor cells [13]. The aim of our work was to investigate the effect of *S. aureus Wood 46* peptidoglycan on cytotoxic activity of mononuclear splenocytes in mice.

Materials and Methods

PG was isolated from the cell walls of *S. aureus* Wood 46 as described previously [12]. For the experiments the PG was dispersed by sonication (25 Hz, 60 s) on ice.

Splenic mononuclear cells (SMC) were isolated by centrifugation splenocytes in ficoll-verograffin gradient (ρ=1,077).

Lewis lung carcinoma (LLC), rat lymphosarcoma (LSR) and Yac-1 cells were obtained from the National Bank of Cell Lines and Tumor Strains (IEPOR, Kyiv, Ukraine). The single cell suspension was prepared as described previously [14] by mechanical disaggregation of tumor tissues. LLC cells were expanded in vitro in RPMI 1640 medium (Sigma, USA) supplemented with 2 mM L-glutamine, 10% FBS and 40 μg/ml gentamycin and were incubated at 37 °C in incubator with humidified atmosphere containing 5% CO_2.

NK-cell cytotoxic activity was determined by flow cytometry method. The target cell ($2x10^5$ cells/ml) and effector cells (murine momonuclear cells) ($4x10^6$cells/ml) were mixed in U-bottom wells of a 96-well microtiter plate at the E : T cell ratio of 20 : 1 in triplicate and incubated in 5% CO_2 atmosphere for 4 or 16 h. The target cells were incubated alone to estimate the rate of spontaneous death. Later incubation cells were collected into cytometric tubes and stained with propidium iodide (Sigma, USA) (2,5 μg/ml). After that the cells were analyzed using a FACSCalibur (Becton Dickinson) and CellOuest program. The cytotoxicity indices were calculated according to the formula: k = % dead cells in experimental probes − % dead cells in spontaneous probes [16].

Statistical analysis was performed using Student's t-test. *P* values < 0.05 were considered significant.

Results and discussion

To research the effect of PG on mice NK cells cytotoxic activity against syngeneic tumor cells we have used Lewis lung carcinoma cells as the targets. Previously we have performed comparative investigation of cytotoxic activity of murine mononuclear splenocytes with LLC and traditional for murine system target cells (Yac-1 and LSR). The target-cells lysis indices in the probes with these three types of target cells were quite comparable (Table).

Table

Cytotoxic activity of mononuclear splenocytes of mice

Target cells	Yac-1	LSR	LLC
Cytotoxicity indexes	16,9±0.4	15.0±0.4	15.5±0.6

These results allowed us to considere LLC as adequate target cells for murine NK cells cytotoxic activity testing and to use them in further experiments.

Incubation of E: T mixture with PG at the concentration of 10, 25 and 100 μg/ml for 4 h has no effect on cytotoxic activity of murine mononuclear splenocytes (Fig. 1). However upon addition of PG at the concentration of 500 μg/ml for 4 h, the target cells lysis indices were 1.5 times higher than corresponding indices in control. Our experiments are performed on total suspension of SMC containing a certain portion of dendritic cells (DC). It is well documented that DC can activate NK cells [5]. But in murine system only early-stimulated mature DC can activate NK cells. Besides DC require period > 4 h to acquire NK cell stimulatory capacity after exposure to different stimuli (in our case — with PG) and to perform the activation procedure [4]. Therefore we suppose that after 4 h incubation with PG augmentation of the target cells death mediated by direct activation of NK cells lytic potential.

16 h incubation of effector and target cells with PG resulted in a dose dependent increase of splenic mononuclear cells cytotoxicity by 1.5, 1.8, 2.0 and 2.5 times for 10, 25, 100 and 500 μg/ml of murein respectively (Fig. 1).

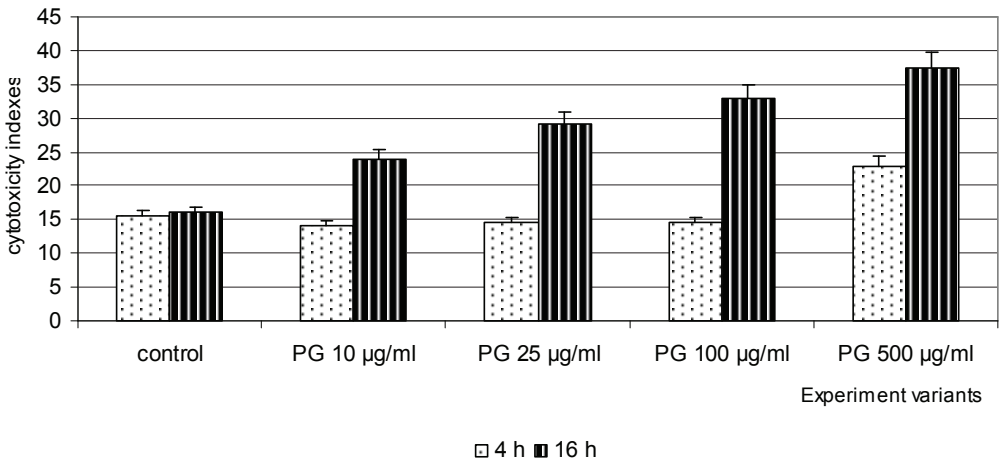

Fig. 1. Effect of PG from *S. aureus* Wood 46 on cytotoxic ativity of SMC of mice after 4 h and 16 h incubation

Probably after a long term incubation with PG augmentation of cytotoxic activity of murine SMC is the result of additional activation of NK cells by DC presented in suspension and matured after contact with murein. Since elevation of SMC cytotoxic activity in 4 h tests occurred only upon addition of PG at the maximal concentration, it is suggested that the higher concentration of murein is required for stimulation of lytic activity of NK cells than that for maturation and activation of DC.

It is necessary to point that addition of PG in the probes with target cells without effector cells resulted in dose dependent augmentation of LLC cells death irrespective of incubation period and had no influence on LSR cells (Fig.2). Incubation of LLC with PG for 4 h was leading to the increase of cell death without distinct dose dependence. Maximal cell death stimulation was observed upon addition of PG at the concentration of 500 μg/ml. More prolonged incubation of LLC with PG also resulted in slight augmentation of cell death (significant only upon addition of PG at the concentration of 500 μg/ml). PG has no effect on viability of LSR cells. It is known that TLRs can induce apoptosis in eucariotic cells. Our results suggest that PG has selective apoptotic effect on tumor cells.

As stated above TLR-2, NOD1 and NOD2 are involved in PG recognition. Though a number of authors has proved the presence of TLRs in broad spectrum of tumor cells, data concerning TLRs expression in LSR are absent. Probably it is precisely this fact that explains different reaction of LLC and LSR on PG.

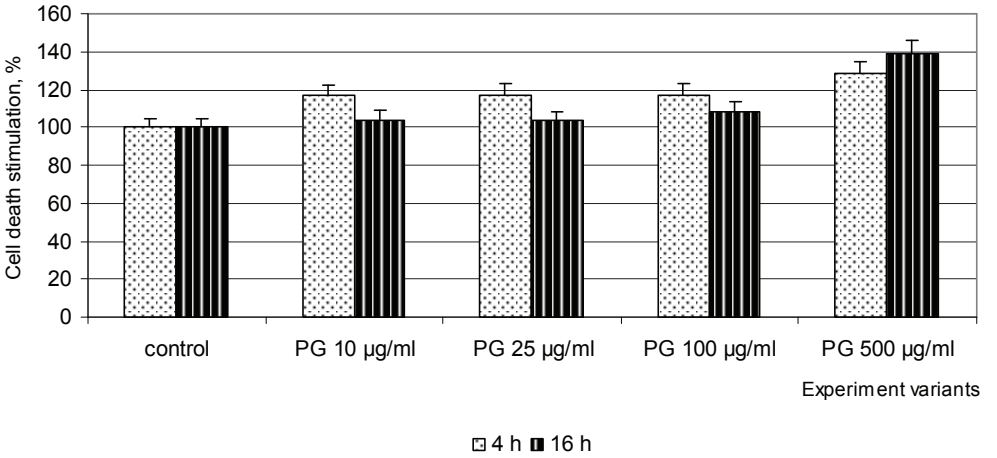

Fig. 2. Effect of PG from *S. aureus* Wood 46 on LLC-cells viability after 4 h and 16 h exposure

Thus we suppose that by the reason of different TLRs expression PG can have selective apoptotic effect on tumor cells. In that case PG may elevate target-cells lysis in E: T mixture by direct toxic action on the target cells and by activation of lytic potential of effector cells.

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Л.М. Скивка[1], А.Г. Федорчук[2], О.Н. Пясковская[2], Н.Н. Храновская[3], В.В. Позур[1]

[1] Киевский национальный университет имени Тараса Шевченко,
просп. Глушкова, 2, Киев, 03022, Украина, e-mail: realmed@i.com.ua;
[2] Институт экспериментальной патологии, онкологии и радиобиологии
имени Р.Е. Кавецкого НАНУ ул. Васильковская, 45, Киев, 03022, Украина;
[3] Национальный Институт Рака МЗ Украины
ул. Ломоносова, 33/44, Киев, 03022, Украина

ВЛИЯНИЕ ПЕПТИДОГЛИКАНА *STAPHYLOCOCCUS AUREUS* WOOD 46 НА ЦИТОТОКСИЧЕСКУЮ АКТИВНОСТЬ МОНОНУКЛЕАРНЫХ СПЛЕНОЦИТОВ МЫШЕЙ

Реферат

В настоящей работе исследовано влияние пептидогликана (ПГ) *S. aureus* Wood 46 на цитотоксическую активность мононуклеарных спленоцитов мышей. Показано, что добавление ПГ в концентрации 500 мкг/мл при 4-часовой инкубации в 1,5 раза усиливает гибель клеток-мишеней, тогда как ПГ в более низких концентрациях достоверно не влияет на цитотоксическую активность мононуклеарных спленоцитов мышей. 16-часовая инкубация с ПГ результируется дозозависимым усилением цитотоксичности мононуклеарных клеток в 1,5; 1,8; 2,0 и 2,5 раза при добавлении ПГ в концентрациях 10, 25, 100 и 500 мкг/мл соответственно.

К л ю ч е в ы е с л о в а: пептидогликан, цитотоксическая активность, карцинома легкого Льюис.

Л.М. Сківка[1], О.Г. Федорчук[2], О.М. Пясковська[2], Н.М. Храновська[3], В.В. Позур[1]

[1] Київський національний університет імені Тараса Шевченка,
просп. Глушкова, 2, Київ, 03022, Україна
e-mail: realmed@i.com.ua;
[2] Інститут експериментальної патології, онкології та радіобіології
імені Р.Є. Кавецького НАНУ, вул. Васильківська, 45, Київ, 03022, Україна;
[3] Національний Інститут Раку МОЗ України
вул. Ломоносова, 33/44, Київ, 03022, Україна

ВПЛИВ ПЕПТИДОГЛІКАНА *STAPHYLOCOCCUS AUREUS* WOOD 46 НА ЦИТОТОКСИЧНУ АКТИВНІСТЬ МОНОНУКЛЕАРНИХ СПЛЕНОЦИТІВ МИШЕЙ

Реферат

В роботі досліджено вплив пептидоглікана (ПГ) *S. aureus* Wood 46 на цитотоксичну активність мононуклеарних спленоцитів мишей. Показано, що додавання ПГ в концентрації 500 мкг/мл при 4-годинній інкубації в 1,5 рази посилює загибель клітин-мішеней, тоді як ПГ в нижчих концентраціях достовірно не впливає на цитотоксичну активність мононуклеарних спленоцитів мишей. 16-годинна інкубація з ПГ результується дозозалежним посиленням цитотоксичності мононуклеарних клітин у 1,5; 1,8; 2,0 і 2,5 рази при додаванні ПГ в концентраціях 10, 25, 100 і 500 мкг/мл відповідно.

К л ю ч о в і с л о в а: пептидоглікан, цитотоксична активність, карцинома легені Льюїс.