ЕКСПЕРИМЕНТАЛЬНІ РОБОТИ

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THE ROLE OF α7 NICOTINIC ACETYLCHOLINE RECEPTORS IN B LYMPHOCYTE ACTIVATION

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The involvement of nicotinic acetylcholine receptor (nAChR) α7 subtype in B lymphocyte activation has been investigated. B lymphocytes were magnetically separated from the spleens of C57Bl/6J mice. The purified lymphocytes were treated with fluorescently labeled IgM-, CD40- , CD16/32 or CD23-specific antibodies and unlabeled α7-specific antibody and examined by flow cytometry. The α7-specific antibody binding interfered with that of anti-CD40 but not of anti-IgM, anti-CD16/32 or anti-CD23 suggesting that α7 nAChRs are located close to CD40. B lymphocyte activation either in vitro with anti-CD40 or in vivo by immunization with cytochrome c resulted in increased α7 nAChR expression. Anti-CD40-induced B lymphocyte proliferation studied by [^{3}H]thymidine incorporation was increased upon α7 nAChR inhibition with methyllicaconitine, choline or antibiotic gentamicin, as well as in the presence of the inhibitor of acetylcholine synthesis hemicholine-3. Mice injected with both cytochrome c and methyllicaconitine responded with IgM anti-cytochrome c antibodies faster than those injected with cytochrome c alone, while the secondary IgG responses were similar. It is concluded that α7 nAChRs negatively control CD40-mediated B lymphocyte proliferation but did not affect the IgM-IgG class switch or memory B cell activation. Endogenous acetylcholine may be regarded as an auto/paracrine regulator of B lymphocyte activation.

Key words: nicotinic acetylcholine receptot, B lymphocytes, CD40, lymphocyte activation.

Functioning of immune cells is regulated by a variety of soluble factors including immune cytokines/chemokines, hormones and neurotransmitters within the triad of nervous, immune and endocrine systems. Acetylcholine is a classical mediator of fast synaptic transmission in neuro-muscular junctions and autonomic ganglia where it activates the ligand-gated ion channels called nicotinic acetylcholine receptors (nAChRs) [1]. The data accumulated during the last decade clearly demonstrate that acetylcholine is a universal mediator produced in many non-excitable cells to regulate their vital functions, such as proliferation, survival, adhesion, motility etc. [2]. Consequently, the nAChRs are found now in plants, invertebrates and even prokaryotes, where their functions are quite different from those in neuronal or neuro-muscular synapses [3–5]. In higher vertebrates, the multiplicity of nAChR functions appears in the cells of different tissues like skin keratinocytes, vascular endothelial and respiratory epithelial or hematopoietic cells [6–9]. Lymphocytes were shown to express several nAChR subtypes involved in the regulation of their development and activation [10–11]. However, the functions of distinct nAChR subtypes are still poorly understood.

Previously we reported that knockout mice lacking α4 or β2 nAChR subunits produced more antibodies in response to immunization compared to the wild-type mice, and B lymphocytes of β2 knockouts responded to anti-CD40 stimulation stronger than those of the wild-type animals [11]. Later, the increased antibody production was observed in α7 knockout mice [12]. It has also been demonstrated that there were α7-containing nAChRs, which regulated the propagation of mature B lymphocytes in the spleens of mice treated with nicotine [10]. The α7 nAChR subtype is of special interest because of its documented role in attenuating inflammatory responses [13]. The aim of the present study was to elucidate the involvement of α7 nAChRs in B lymphocyte activation by using nAChR subtype-specific pharmacological tools. Such an approach has an advantage com-

pared to the use of knockout animals because it does not allow compensatory expression of alternative nAChR subtype(s) *in vivo*.

Materials and Methods

B lymphocytes were separated from the mouse spleen by magnetic separation procedure. The erythrocytes were eliminated from the total splenocyte suspension with the lyses buffer (Sigma, USA). The remaining spleen leukocytes were resuspended in 0.5% BSA-PBS and were treated with magnetic microbeads coupled with B220-specific antibodies. The B220-positive cells were sorted out on MidiMACS columns (Miltenyi Biotec, Germany) according to the manufacturer instructions. The sorted cells purity was checked by flow cytometry using FITC-coupled B220-specific antibody (BD Pharmingen).

The purified B lymphocytes, 1×10^6 per 50 μl, were stained with biotinylated α7(179-190)-specific antibody, 0.1 mg/ml, for 15 min at room temperature. Then cells were washed with 1 ml of 0.5% BSA-PBS, centrifuged for 5 min at 1600 rpm and treated with Streptavidin-phycoerythrin conjugate (BD Pharmingen) for additional 15 min at room temperature and in dark. After subsequent washing the cells were resuspended in 0.7 ml of 0.5% BSA-PBS and analyzed with EPICS-XL flow cytometer (Coulter-Beckman). For double staining the cells were treated simultaneously with unlabeled α7(179-190)-specific antibody and with either FITC-labeled IgM-specific, CD16/32-specific, CD23-specific antibody or biotinylated CD40-specific antibody (BD Pharmingen). For the latter, additional treatment with Streptavidin-phycoerythrine has been performed.

In another set of experiments, the purified B lymphocytes were treated with unlabeled CD40-specific antibody for 15 min, then the antibody was washed out by centrifugation and the cells were incubated in RPMI 1640 medium which contained 10% FBS at 37 °C for additional 3 h. The cells were stained with biotinylated α7(179-190)-specific antibody immediately after separation, after 3 h incubation and after CD40-specific activation and subsequent incubation and were analysed by flow cytometry as described above.

For proliferation assay magnetically purified B lymphocytes, 1×10^6 per well, were stimulated with anti-CD40 antibody (BD Pharmingen, clone HM40-3, 1 : 5000) in the presence or absence of nAChR agonists/antagonists (25 nM methyllicaconitine, 10 μM choline, 10 μM epibatidine or 0,1 μM dihydro-β-erythroidine), antibiotics (40 μg/ml gentamicin or penicillin-streptomicin mixture) or hemicholine-3. The antibody concentration was selected in preliminary experiments to be within the linear portion of the saturation curve. [^{3}H]-thymidine (Amersham, 1 μCi per well) was added 24 h after the stimulation and cell-incorporated radioactivity was measured 24 h later with the scintillation β-counter.

Two groups of C57Bl/6 mice, 5 animals per group, were immunized intraperitoneally with 100 μg of bovine cytochrome *c* (Sigma) emulsified in complete Freund's adjuvant, 0.5 ml of resulting emulsion per mouse. For one group the emulsion was supplemented with MLA (250 ng per mouse). The second immunization was done after a month by similar procedure and with the same antigen dose. Blood samples were taken from the tail veinvin several terms after immunizations. The blood sera samples separated by centrifugation were analysed by enzyme-linked immunosorbent assay using cytochrome *c*, 20 μg/ml, as coating antigen, as described in [11]. Mice were sacrificed by cervical dislocation (in accordance with the guidelines of the Institute of Animal Care and Treatment Committee) three weeks after the second immunization, the spleens were removed, and B lymphocytes were sorted out to be analysed for the α7 nAChR expression by flow cytometry as described above.

Results and Discussion

In the first set of experiments we were interested, which B lymphocyte surface molecules were in proximity to α7 nAChRs. To address this question, magnetically separated mouse spleen B lymphocytes were incubated with α7-specific antibody (obtained by us previously and shown to bind this nAChR subtype in the neurons of autonomic ganglia, α7-transfected cells and B lymphocytes [14–16]) together with the fluorescently labeled antibodies against several conventional B

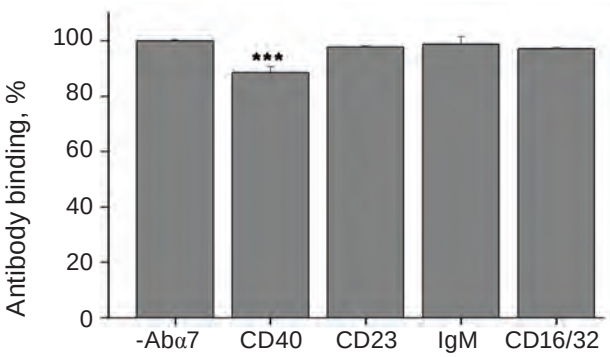

Fig. 1. The binding of CD40-, CD23-, CD16/32- or IgM-specific antibody to mouse B lymphocytes in the presence of α7-specific antibody. The binding of each marker-specific antibody alone is taken as 100%

lymphocyte markers. It was found that α7-specific antibody binding interfered with that of CD40-specific antibody, but not with those of IgM-specific, CD23-specific or CD16/32-specific ones (Fig. 1). These data suggested that α7 nAChRs were located close to CD40, but not to the antigen-specific receptor (IgM), Fcγ-receptor (CD16/32) or CD23. Therefore, we could expect the involvement of α7 nAChRs in CD40-mediated activation events.

Indeed, CD40-mediated B lymphocyte activation influenced the level of α7 nAChR expression. As shown in Fig. 2, purified B lymphocytes stimulated with anti-CD40 for 15 min and then incubated at 37 °C for additional 3 h increased the number of surface α7 nAChRs compared to non-stimulated cells. Similarly, B lymphocytes of mice immunized with cytochrome *c* bound more α7-specific antibodies than those of non-immunized age-matched controls. The increase of α7 nAChR numbers on the cells of immunized mice could be due to intensified biosynthesis/folding of the nAChR subunits in activated/memory B lymphocytes. In contrast, the short period of B lymphocyte stimulation *in vitro* excluded *de novo* synthesis of nAChR molecules and rather suggested that they were re-distributed between intracellular and membrane-exposed pools. Anyway, B lymphocyte activation was accompanied with the increase of surface α7 nAChRs suggesting their possible role in the activation process.

B lymphocyte proliferation stimulated with anti-CD40 was significantly increased with 25 nM methyllicaconitine (MLA) or 10 μM choline and to much lower extent with 0.1 μM dihydro-β-erythroidine (Fig. 3*A*). MLA taken at 5-25 nM concentration is a potent and specific competitive antagonist of α7 nAChRs, while choline is a selective α7 agonist [1]. However, choline is also a potent desensitizing agent for this nAChR subtype, especially, when it is present constantly in the cell culture [17]. In contrast, epibatidine and dihydro-β-erythroidine are specific ligands (agonist and antagonist, respectively) for heteromeric α4β2/β4 or α3β2/β4 nAChRs. The data obtained indicated that blocking of α7 nAChRs facilitated the B lymphocyte activation induced by CD40 ligation much more than blocking of heteromeric nAChR subtypes.

In the other set of experiments, B lymphocytes were stimulated with anti-CD40 in the medium which contained different antibiotics. As shown in Fig. 3*B*, the highest [^{3}H]-thymidine incorporation was observed in the samples cultured in the presence of gentamicin known to be a non-competitive α7 nAChR blocker [18]. Its potency to increase the CD40-mediated B lymphocyte activation is in accord with the data presented above (Fig. 3*A*).

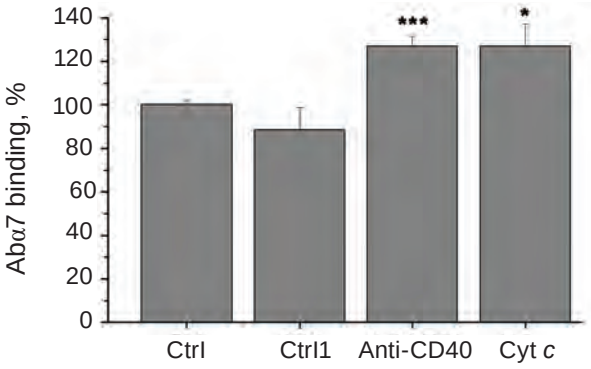

Fig. 2. The increase of α7-specific antibody (Abα7) binding to mouse B lymphocytes activated either in vitro with anti-CD40 or in vivo by immunization with cytochrome c. The binding to non-stimulated cells or to those of non-immunized mice (Ctrl) is taken as 100%. Ctrl 1 — non-stimulated cells incubated for 3 h at 37 °C

It was further shown that culturing B lymphocytes in the presence of hemicholine-3 significantly increased their proliferation in response to anti-CD40 compared to control cells (Fig. 3*B*). Hemicholine-3 blocks acetylcholine synthesis [19], therefore, the data obtained indicated that CD40-mediated B lymphocyte proliferation was negatively regulated by endogenous acetylcholine being produced in these cells.

Mice, which were immunized with cytochrome *c* mixed with MLA, responded by IgM anti-cytochrome *c* antibodies faster than mice immunized with cytochrome *c* alone (Fig. 4*A*). In contrast, the secondary IgG response was similar in these two groups of mice (Fig. 4*B*). These data were in accordance with increased B cell proliferation in the presence of MLA (Fig. 3*A*) and indicated that blocking of α7 nAChRs affected CD40-dependent primary immune response but not IgM-IgG switch and memory immune response.

Previously we reported that nicotine stimulated, while α7-specific antagonist α-cobratoxin inhibited proliferation of mouse hybridoma cells [20]. Similarly, choline stimulated, while MLA inhibited proliferation of chicken pre-B lymphoma DT40 cells [21]. Moreover, the absence of α7 nAChRs in mature spleen B lymphocytes of knockout mice prevented their lymphocytes propagation upon nicotine consumption [10]. According to these and some other literature data [22], α7 nAChRs mediate pro-proliferative signals in tumor/transformed cells and in non-stimulated B lymphocytes. In contrast, blocking of α7 nAChRs with pharmacological agents described here resulted in enhanced B lymphocyte proliferation stimulated through CD40. This result is in accord with the

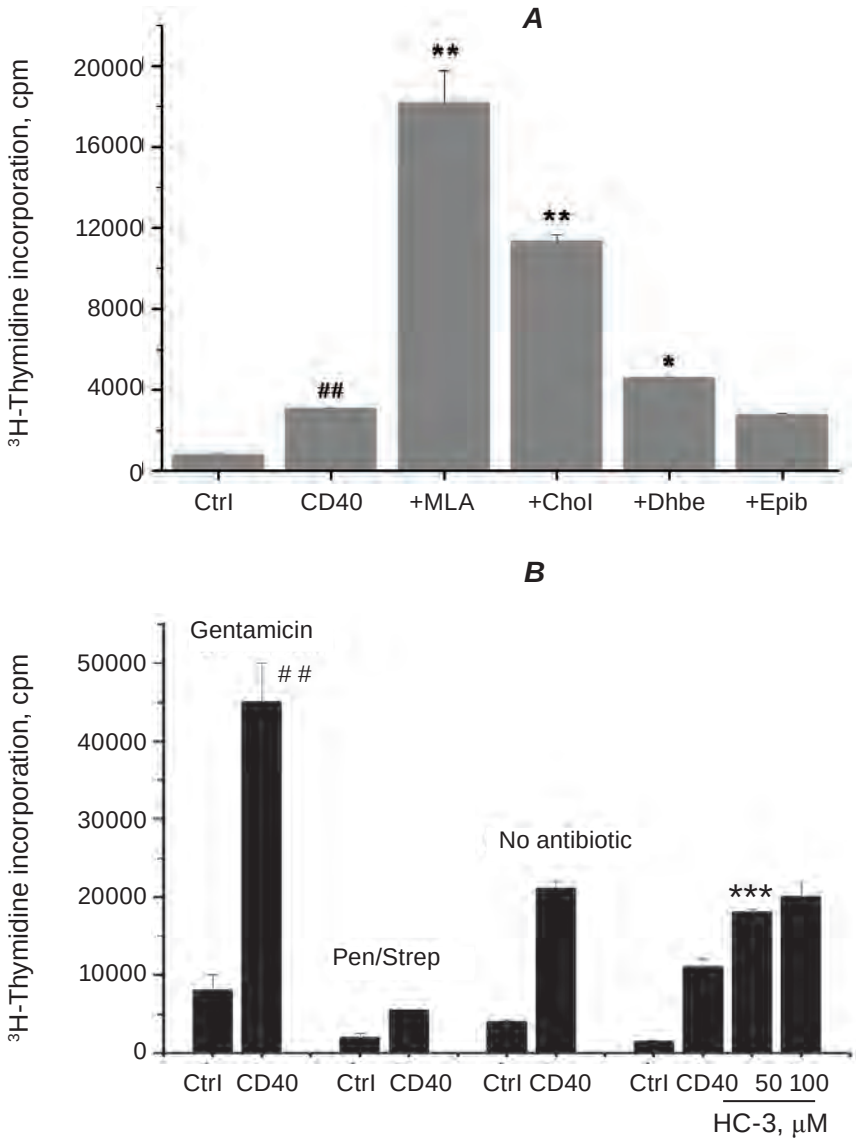

*Fig. 3. Proliferation of sorted mouse B lymphocytes stimulated with anti-CD40 in the presence of methyllicaconitine (MLA), choline (chol), dihydro-β-erythroidine (Dhβe) or epibatidine (Epib) – A, antibiotics or hemicholine-3 (HC-3) – B. ## $P < 0.005$ compared to non-stimulated cells; *,**,*** – $P < 0.05$, < 0.005, < 0.0005 compared to anti-CD40-stimulated cells*

previously reported increased antibody production in α7 knockout mice [12]. It means that, depending on the circumstances and/or cell functional state/phenotype, the signals mediated by α7 nAChRs may be either pro- or anti-proliferative and, therefore, may be realized through different signaling pathways. Most probably, the resulting effect depends on the activity of adjacent plasma membrane molecules.

In contrast to nAChRs expressed in muscles and autonomic ganglia, those found in the brain and in non-excitable tissues do not create membrane currents themselves, but rather regulate the activity of other receptors or channels. For example, in hippocampus, the α3β4 and α4β2 nAChRs regulate glutamate and N-methyl-D-aspartate excitatory postsynaptic currents [23], while in outer hair cells of the ear, α7-like α9 nAChRs activate nearby Ca^{2+}-sensitive K^+ channels [24]. In immune cells α7 or α7-like nAChRs were shown to modulate the intracellular Ca^{2+} fluxes evoked by adjacent purinergic (in monocytes) or antigen-specific (in T lymphocytes) receptors [25–26]. Our data suggest that α7 nAChRs interfere with CD40-triggered signaling pathways in activated B lymphocytes.

CD40 is a TNF receptor-related co-stimulatory molecule found in B lymphocytes, monocytes and dendritic cells [27]. Upon binding with the

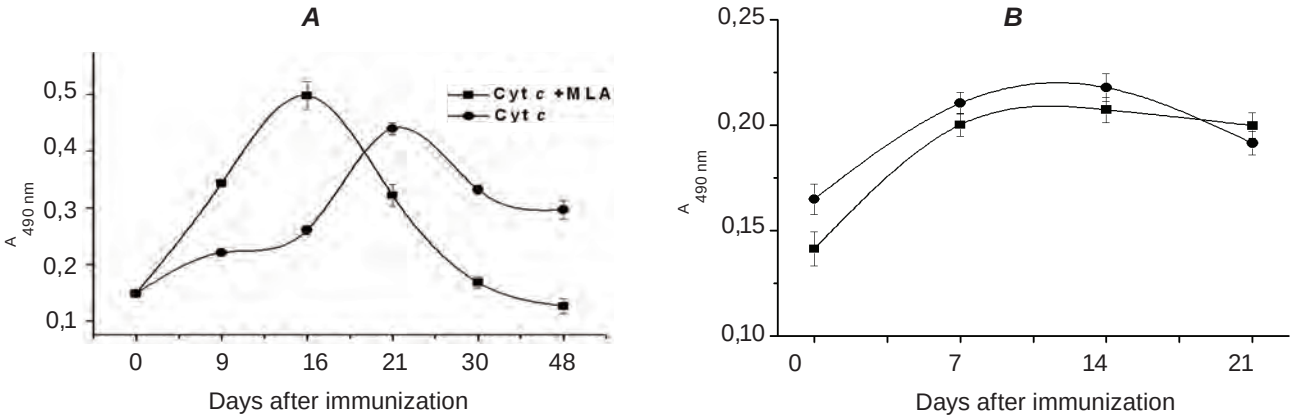

Fig. 4. Primary IgM (A) or secondary IgG (B) antibody response of mice immunized with bovine cytochrome c in the presence or absence of MLA

CD40 ligand (CD154) expressed on T lymphocytes and endothelial cells, CD40 triggers several intracellular signaling cascades resulting in cell proliferation, cytokine production or immunoglobulin class switch [28]. Our data allow suggesting that α7 nAChR signaling interferes with that of CD40 by competing for (sharing) the signaling components involved in mitogenic response but not in IgM-IgG switch. Importantly, the nAChRs expressed in B lymphocytes are affected by endogenous acetylcholine produced by these cells (Fig. 3*B*). Therefore, acetylcholine may be regarded as a negative auto/paracrine regulator of B lymphocyte activation. Previously it was found that activated T lymphocytes or monocytes produced more acetylcholine than the resting ones [25, 29], while activation of nicotinic receptors in these cells attenuated the production of inflammatory cytokines and inhibited T lymphocyte responses [13, 30]. These data allow suggesting that endogenous acetylcholine and the nAChRs expressed in haematopoietic cells form a negative feedback to control the intensity of cell activation. Blocking of this regulatory loop results in increased cell activation and fast primary antibody response. Pro-proliferative effect of nAChR stimulation found in non-activated or transformed cells may mean that nAChR's attachment to the CD40 signaling pathway occurs only upon B lymphocyte activation.

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РОЛЬ НИКОТИНОВЫХ АЦЕТИЛХОЛИНОВЫХ РЕЦЕПТОРОВ СУБТИПА α7 В АКТИВАЦИИ В-ЛИМФОЦИТОВ

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Исследована роль никотиновых ацетилхолиновых рецепторов (нАХР) субтипа α7 в активации В-лимфоцитов. В-лимфоциты выделяли магнитной сортировкой из селезенки мышей C57Bl/6J и обрабатывали флуоресцентно мечеными антителами против IgM, CD40, CD16/32 или CD23 и немечеными α7-специфичными антителами с последующим анализом проточной цитофлуориметрией. α7-Специфичные антитела препятствовали связыванию антител анти-CD40, но не анти-IgM, анти-CD16/32 или анти-CD23, что говорит о близком расположении α7 нАХР и CD40. Активация В-лимфоцитов антителами против CD40 in vitro или иммунизацией цитохромом *c* in vivo увеличивала экспрессию α7 нАХР. По данным включения [^{3}H]-тимидина, пролиферация В-лимфоцитов, индуцированная анти-CD40, усиливалась при ингибировании α7 нАХР метилликаконитином, холином или антибиотиком гентамицином, а также в присутствии гемихолина-3,

ингибитора синтеза ацетилхолина. Мыши, которым вводили цитохром *c* вместе с метиллликаконитином, продуцировали IgM быстрее, чем те, которым вводили только цитохром *c*, в то время, как в случае с вторичным IgG ответ оставался таким же. Это свидетельствует о том, что α7 нАХР негативно контролируют пролиферацию В-лимфоцитов, опосредованную CD40, но не влияют на переключение классов иммуноглобулинов или на активацию В-клеток памяти. Эндогенный ацетилхолин может рассматриваться как ауто/паракринный регулятор активации В-лимфоцитов.

Ключевые слова: никотиновый ацетилхолиновый рецептор, В-лимфоциты, CD40, активация лимфоцитов.

РОЛЬ НІКОТИНОВИХ АЦЕТИЛХОЛІНОВИХ РЕЦЕПТОРІВ СУБТИПУ α7 В АКТИВАЦІЇ В-ЛІМФОЦИТІВ

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Досліджено роль нікотинових ацетилхолінових рецепторів (нАХР) α7 субтипу в активації В-лімфоцитів. В-лімфоцити виділяли магнітним сортуванням із селезінки мишей C57Bl/6J і обробляли флуоресцентно міченими антитілами проти IgM, CD40, CD16/32 або CD23 і немміченими α7-специфічними антитілами з наступним аналізом протоковою цитофлуориметрією. α7-Специфічні антитіла перешкоджають зв'язуванню анти-CD40, але не анти-IgM, анти-CD16/32 чи анти-CD23 антитіл, що свідчить про близьке розташування α7 нАХР і CD40. Активація В-лімфоцитів анти-CD40 антитілами in vitro або імунізацією цитохромом *c* in vivo збільшує експресію α7 нАХР. За даними включення [^{3}H]-тимідину, проліферація В-лімфоцитів, індукована анти-CD40 антитілами, посилюється у разі інгібування α7 нАХР метиллікаконітином, холіном або антибіотиком гентаміцином, а також у присутності геміхоліну-3, інгібітора синтезу ацетилхоліну. Миші, яким вводили цитохром *c* разом з метиллікаконітином, продукували IgM швидше, ніж ті, яким вводили тільки цитохром *c*, в той час, як вторинна IgG відповідь залишалася незмінною. Це свідчить про те, що α7 нАХР негативно контролюють проліферацію В-лімфоцитів, опосередковану CD40, але не впливають на переключення класів імуноглобулінів або на активацію В-клітин пам'яті. Ендогенний ацетилхолін може розглядатися як ауто/паракринний регулятор активації В-лімфоцитів.

Ключові слова: нікотиновий ацетилхоліновий рецептор, В-лімфоцити, CD40, активація лімфоцитів.

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