Adenosine is an endogenous compound that regulates function of several immune cells including lymphocytes by activating adenosine receptors (ARs). Several reports indicate that stimulation of ARs on lymphocytes affects lymphocyte activation, proliferation and lymphocyte-mediated cytolysis. Unfortunately, most studies focused on T lymphocytes and little information exists on involvement of ARs in B cells regulation. In this study we elucidated the impact of ARs activation on immunoglobulin M (IgM) production by purified human peripheral blood B lymphocytes stimulated in vitro with *Staphylococcus aureus* Cowan I (SAC) plus IL-2. Performed experiments showed that endogenous adenosine that is released/produced by human B lymphocytes is able to induce cAMP accumulation in the cell through activation of A_{2A}-AR however, this takes place only when other ARs are inhibited by selective antagonists. We observed that accumulated intracellular cAMP suppressed IgM production by B cells stimulated with SAC plus IL-2. Our experiments showed that human B cells cultured at 25 mM glucose produced significantly less IgM in response to stimulation with SAC comparing to cells maintained in media containing 5 mM glucose. However, the high glucose effect on IgM production by B cells stimulated with SAC depended on other factor/s than ARs.

**Key words:** adenosine receptors, B lymphocyte, glucose, immunoglobulin M, *Staphylococcus aureus*

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**INTRODUCTION**

Adaptive immune response to microbial pathogens depends on the humoral immunity and cell-mediated immunity. The humoral immunity relay on functioning of B lymphocytes that when stimulated with an antigen proliferate and differentiate into cells producing and secreting antibodies. The function of immune cells including B lymphocytes is subjected to regulation by various signaling molecules released by immune and non-immune cells. Some of these molecules are necessary for initiating the immune response, whereas others control the course of action. Adenosine is an endogenous compound that could outflow from the cell or be generated on cell surface from released ATP (1, 2). Its concentration in extracellular space is low, although it might significantly increase under the metabolic stress conditions like diabetes, hypoxia or ischemia (3-5). Its immunomodulatory effects are recognized universally (6, 7). Adenosine elicits its physiological actions by ligation of cell surface P1 purinergic receptors and activation of the downstream intracellular pathways. There are four types of known adenosine receptors namely A_{1}-AR, A_{2A}-AR, A_{2B}-AR, A_{3}-AR (8). Adenosine can regulate lymphocyte function indirectly by stimulating AR on macrophages, dendritic cells, neutrophils, mast cells and altering production of several cytokines or directly by binding and activating AR receptors on lymphocytes (7, 9). Several recent reports demonstrated that adenosine activating AR on lymphocytes affects lymphocyte activation, proliferation and lymphocyte-mediated cytolysis (9, 10). Studies on AR-knockout mice showed that activation of A_{2A}-AR on CD4+ T cells suppress the production of IL-2, IFN-γ and IL-4 (11-13). It appears that A_{2A}-AR is the major adenosine receptor influencing the function of lymphocytes. Unfortunately, most if not all of this investigations focused on T lymphocytes and little information exists on involvement of adenosine receptors in regulation of B cells function. Finding a link between adenosine receptors and altered function of B cells could increase our understanding of mechanism/s leading to impaired humoral immune response in pathological conditions like bacteremia related diseases (14, 15) and metabolic diseases like diabetes (16, 17). Development of diabetes is associated with altered adenosine metabolism, transport and ARs expression on B cells (10). Therefore, the goal of our study was to investigate the ARs involvement in regulation of an antigen-induced IgM production by human peripheral blood B lymphocytes and to examine the effect of high glucose concentration on these processes.

**MATERIALS AND METHODS**

**Antibodies and reagents**

Insulin, penicillin, streptomycin, crude preparation of inactivated *Staphylococcus aureus* Cowan I, IL-2, RPMI-1640 medium, Histopaque-1077, forskolin, adenosine deaminase (ADA), N6-cyclopentyladenosine (CPA), 2-chloro-N6-cyclopentyladenosine (CCPA), 1,3-dipropyl-8-cyclopentylxa-
nthine (DPCPX), 2,3-dihydro-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate (ZM1523), N-(4-cyano-phenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1H-purin-8-yl)-phenoxy]acetamide (MRS 1754), alloxazone, were obtained from Sigma-Aldrich Sp. z o.o. (Poznan, Poland). 5′-N-ethyl-carboxamidoadenosine (NECA), N-[9-chloro-2-(2-furanyl)]/[1,2,4]-triazolo[1,5-c]quinazolin-5-benzeneacetamide (MRS 1220), 2-[p-(2-carbonyl-ethyl)-phenylethylamino]-5′-N-ethylcarboxamidoadenosine (CGS 21680), 1-buty1-8-(hexahydro-2,5-methanopentalen-3a(1H)-yl)-3,7-dihydro-3-(3-hydroxypropyl)-1H-purine-2,6-dione (PSB 36), N′-[3-iodobenzyl]adenosine-5′-N-methyuronamide (IB-MECA), 2-chloro-N′-[3-iodobenzyl]adenosine-5′-N-methyluronamide (Cl-IB-MECA), 4-[2-[7-amino-2-[2-furyl]-[1,2,4]triazolo[2,3-α][1,3,5]triazin-5-yl-amin0][ethyl]phenol (ZM 241385), and 8-(3-Chlorostyryl)caffeine (CSC) were from Tocris Bioscience (Nortpoint, UK). Mouse anti-human β-tubulin antibody, goat polyclonal antibodies to A1-AR, A2A-AR, A2B-AR, A3-AR antibody, and rabbit anti-goat IgG alkaline phosphatase conjugate were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human IgM monoclonal antibody, FITC-conjugated mouse anti-human CD19 monoclonal antibody, phycoerythrin (PE)-conjugated mouse anti-human CD19 monoclonal antibody, PE-conjugated mouse anti-human IgGk monoclonal antibody were from BD Biosciences Pharmingen (Heidelberg, Germany).

**Human peripheral blood cells isolation**

Fresh buffy coats (not more than 6 hours old) were obtained from Regional Blood Bank in Gdansk. Human peripheral blood lymphocytes were isolated by centrifugation of white blood cells suspension through Histopaque-1077 at 700 g for 30 min at room temperature. Isolated lymphocytes were further purified into B cells by negative selection with magnetic nanoparticles coated with specific monoclonal antibodies (MagCellect Human B cell Isolation Kit) according to manufacturer’s protocol. The purity of B cell population was more than 95%.

**Flow cytometric analysis**

Cells were stained in FACS buffer (phosphate buffered saline, 0.5 mM EDTA, 1% FCS) according to standard procedures with anti-CD-19-PE, and anti-IgM-FITC for the surface IgM expression FITC-conjugated or PE-conjugated mouse anti-human IgGk monoclonal antibody was used as an isotype control. The two-color analysis of B cell surface molecules was performed by a FACSScan (Becton Dickinson). The antibody-coated cells were gated on living cells by cell size and granularity and counted by means of flow cytometric analysis.

**B cell stimulation**

Purified human peripheral blood B lymphocytes were maintained under standard conditions (5% CO2–95% air, 98% humidity and 37°C) in RPMI-1640 medium containing glucose concentration as indicated in the Figure legends, supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% heat-inactivated fetal bovine serum (Gibco). Cells were cultured in flat-bottomed culture bottles (Sarsted) at a density of ~5×10⁶ cells/ml. After 48 hours cells were collected and suspended in appropriate medium (low or high glucose). The number of viable cells was determined by Trypan Blue dye exclusion. Only cell cultures with a 95% viability or greater were used. For in vitro IgM synthesis 8×10⁵ cells (in a volume of 500 µL) were stimulated for 5 days with 0.01% SAC plus 20 U/mL IL-2. Compounds tested were added to the cells (concentrations indicated in the Figure legends) 1 hour before SAC plus IL-2 stimulation. Control cultures were kept in media without B cell stimulants. After 120 hours supernatants were collected and stored at -20°C until assayed for IgM content.

![Fig. 1. Effect of adenosine receptors agonists on IgM production by human B cells stimulated in vitro](image)
Western blot analysis

The cell extract was obtained as described previously (18). The proteins from cell extracts were separated by 12% SDS-polyacrylamide gel electrophoresis, and electrophoretically transferred to Immobilon poly(vinylidene difluoride) transfer membrane. The membrane was incubated at 4°C (overnight) with 3% BSA in Tris-buffered saline (TBS). The membrane was then cut horizontally at appropriate position (based on position of prestained molecular mass marker) and incubated with appropriate primary antibodies. Next, the membrane strips were washed with TBS and incubated with alkaline phosphatase-conjugated secondary antibodies. Membrane bound antibodies were visualized with 5-bromo-chloro-3-indoylphosphate and nitroblue tetrazolium. The β tubulin was used as a reference protein.

Determination of cAMP

Cells (~2×10^6) were separated from the culture media by centrifugation and the resulting pellet was treated with 0.1 M

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**Fig. 2.** Accumulation of cAMP in B cells. Purified human peripheral blood B lymphocytes were cultured for 48 hours in RPMI-1640 medium containing 5 mM glucose, exposed to appropriate compounds for 15 min, and the intracellular cAMP level was determined by an ELISA assay as described under "Materials and Methods". (A) Changes in the intracellular cAMP level in response to treatment with forskolin or A2A-AR agonist (CGS 21680) in the presence and absence of A1-AR antagonist (DPCPX), or nonselective AR agonist (NECA). (B) Accumulation of cAMP in B cells exposed to increased concentrations of selective A1-AR agonists in the presence of fixed concentrations (1 µM) of CGS 21680 (A2A-AR agonist) and DPCPX (A1-AR antagonist). (C) Concentration-dependent inhibition of A1-AR by DPCPX and resulted elevation of cellular cAMP level in B cells incubated in the presence of 1 µM CGS 21680. The data represent the mean ±S.D. from three experiments. *, P<0.05 vs. control (no additives).
HCL. After a 10 min incubation, the lysate obtained was centrifuged and the supernatant was used directly for cAMP determination, according to the manufacturer's protocol for the Direct cAMP Enzyme Immunoassay Kit (Sigma-Aldrich Sp. z o.o., Poznan, Poland).

**IgM assay by ELISA**

IgM level in cell culture media was determined using enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's (Dunn Labortechnik GmbH, Asbach, Germany) protocol. The quantity of IgM in the test sample was interpolated from the standard curve and corrected for sample dilution. Results are expressed as nanograms per milliliter of IgM produced in stimulated cultures minus control cultures.

**Statistical analysis**

Values are reported as means ± S.D. Unpaired Student's t-test was applied to assess differences between groups. P values below 0.05 were considered as significant.

**RESULTS**

In order to assess the possible effect of adenosine receptor (AR) activation on IgM production by B cells stimulated *in vitro* with SAC plus IL-2 we cultured highly purified human peripheral blood B lymphocytes with specific AR agonists. Our experiments showed that IgM production by B cells stimulated with SAC was not significantly affected by addition to the incubation medium any of AR agonists used (Fig. 1). However, significant decrease of IgM level could be observed when 5 µM forskolin was included in the incubation medium. This indicated that a rise in cAMP level suppress SAC-induced IgM production by B cells. Since, activation of A2A-AR and A3-AR should result in stimulation of adenyl cyclase and concomitant increase in intracellular cAMP, we determined cAMP level in B cells exposed to agonists of these receptors. Performed measurements showed only moderate increase of cAMP level in B cells exposed to 1 µM CGS 21680 an agonist of A2A-AR and 10 µM NECA a nonselective AR agonist (Fig. 2). However, blockade of A1-AR by DPCPX (1 µM) significantly altered the CGS 21680-induced raise in cAMP level. Competitive experiments with selective A1-AR agonists (CPA, CCPA) and A1-AR antagonist DPCPX confirmed the major role of A1 receptor in attenuation of CGS 21680-induced accumulation of cAMP in B cells (Fig. 2). On the other hand, the relative weak response to A2A-AR stimulation could result from low expression level of this receptor in B lymphocytes comparing to other AR. We have found that the relative expression levels of AR in human B cells (Fig. 3) is very similar to that determined in rat B lymphocytes (18, 19). Relatively low specificity and potency of CGS 21680 in humans (20) is another factor that might have an impact on lack of A2A-AR stimulation effect noted in our experiments. Moreover, it has been reported that CGS 21680 binds also to sites unrelated to A2A-AR (21, 22). Therefore, under normal conditions the adenosine receptors on human peripheral blood B lymphocytes remain at the mutual levels in such a fashion, that when stimulated by endogenous adenosine the effect of A2A-AR stimulation is counterbalanced by A1-AR and A3-AR activity.

![Fig. 3. The protein level of adenosine receptors in human peripheral blood B lymphocytes. The cell extract of purified human peripheral blood B lymphocytes were prepared as described under "Materials and Methods". The proteins were separated on 12% SDS-PAGE and immunoblotted with appropriate antibodies. The blots presented are representative of those obtained in at least three experiments performed on pooled B cells from three separate donors.](image)

Our previous studies showed that the expression levels of adenosine receptors are significantly changed in several tissues of diabetic animals (3, 23, 24). We observed that exposition of B lymphocytes to high glucose resulted in a significant diminution of A1-AR, A3-AR and A2B-AR with no changes in A2A-AR expression (18). Therefore, under high glucose concentration A2A-AR becomes the predominant AR on B cells. Thus, at high glucose concentration B cells might be more sensitive to A2A-AR stimulation. In our current experiments we observed that human B cells cultured at 25 mM glucose produced significantly less IgM in response to stimulation with SAC comparing to cells maintained in media containing 5 mM glucose (Fig. 6). However, inclusion of forskolin (5 µM) in the incubation medium resulted in a decrease of IgM to the same level in both high and low...
Exposition of B cells incubated in 25 mM glucose to selective A2A-AR antagonists (ZM 241385 or CSC), resulted in ~25% increase of SAC-induced IgM production. Although, blockade of A2A-AR with ZM 241385 do not altered the forskolin effect on IgM production (Fig. 6). The presence of 1 µM NECA or 1 µM CGS21680 in high glucose media have no effect on SAC-induced IgM production by B cells.

**DISCUSSION**

Accumulated to date evidence clearly shows crucial role of adenosine receptors in modulation of immune cells function including lymphocytes (25, 26). Most of these studies concerned T lymphocytes with few devoted to B lymphocytes. The pharmacological studies and work with adenosine-receptor-knockout mice revealed that adenosine-induced suppression of T cells function is mediated mainly by A3-AR (12, 13, 27, 28).
Investigations performed on chicken B cell lymphoma DT40 and murine splenic B cells showed that adenosine blocked BCR-induced NF-κB activation through the engagement of the A2 adenosine receptors (29). The ability of exogenous adenosine to induce cAMP accumulation was also reported in B lymphocytes isolated from patients with chronic lymphocytic leukemia (30). This corresponds with our results presented in this contribution; however, for the first time we documented that the adenosine-induced cAMP alters the IgM production by human peripheral blood B cells stimulated with a bacterial antigen. We showed that endogenous adenosine that is released/produced by normal human B lymphocytes is able to induce cAMP accumulation through activation of A2A-AR however, this takes place only when other ARs are inhibited by selective antagonists. This means that under normal conditions stimulation of A2A-AR on B cells by endogenous adenosine is

**Fig. 5.** Impact of A2A-AR stimulation by endogenous adenosine on SAC-induced IgM production by human B cells in the presence of other active adenosine receptors. Purified human peripheral blood B lymphocytes were cultured for 48 hours in RPMI-1640 medium containing 5 mM glucose and stimulated with SAC plus IL-2 as described under "Materials and Methods". One hour before B cells stimulation with SAC plus IL-2, ARs were blocked by addition of A1-AR selective antagonist (DPCPX, 1 µM), A2A-AR selective antagonist (ZM 241385, 1 µM), A2B-AR selective antagonist (MRS 1754, 1 µM), A3-AR selective antagonist (MRS 1523, 1 µM) in a fashion that leave active ARs indicated in the figure. On fifth day IgM levels in cell culture media were determined by an ELISA assay as described under "Materials and Methods". The data represent the mean ±S.D. from three experiments. * P<0.05 vs. control (no additives).

**Fig. 6.** Effect of glucose concentration and A2A-AR on IgM production by human B cells stimulated in vitro with SAC plus IL-2. Purified human peripheral blood B lymphocytes were cultured for 48 hours in RPMI-1640 medium containing 5 mM or 25 mM glucose concentration and stimulated with SAC plus IL-2 as described under "Materials and Methods". Forskolin, A2A-AR selective antagonists (ZM 241385, CSC), nonselective AR agonist (NECA), or A2A-AR agonist (CGS 21680) were added one hour before B cells stimulation with SAC plus IL-2. On fifth day IgM levels in cell culture media were determined by an ELISA assay as described under "Materials and Methods". The data represent the mean ±S.D. from five experiments on cells from two separate donors. On cells from each donor parallel incubations in 5 and 25 mM glucose were performed. *, P<0.05 vs. control (no additives).
counterbalanced by simultaneous activation of other ARs namely A2A-AR and A3-AR.

It could be assumed that conditions changing the expression level of ARs in a way that alters their mutual levels should also result in altered response of the cell to adenosine. Such a situation takes place in diabetes when the expression level of adenosine receptors changes in several tissues including B lymphocytes (3, 10, 23, 24). Studies performed on animals showed that insulin and glucose levels are the major factors responsible for altered ARs expression (18, 19). In humans, clinical observations indicate that development of diabetes is associated with impairment of humoral immunity. Patients with diabetes are generally more prone to certain specific infections, and some occur almost exclusively in them. Diabetes was identified as a risk factor for skin infection, urinary tract infections and for upper and lower respiratory tract infections (31). Moreover, infections caused by some microorganisms like Staphylococcus aureus and Mycobacterium tuberculosis occur with increased frequency whereas other pathogens (Streptococcus pneumonia, influenzavirus) are associated with increased mortality and morbidity (32, 33). Evidences obtained from animal and in vitro studies indicate that diabetic mice experimentally infected with B streptococcal bacteria had reduced clearance of bacteria and higher mortality rates (34). This could be related to the lowered functionality of B cells since impaired humoral immune responses in patients with poor long-term glucose control have been also observed (35, 36). This assumption is in line with our observations indicating that IgM production by B lymphocytes isolated from diabetic rats and stimulated with a bacterial antigen is lowered comparing to B lymphocytes isolated from healthy animals (unpublished). Similar observations were reported from study on streptozotocin-induced diabetic rats (37). Results presented in this report show that high concentration of glucose impairs the IgM production by human B cells stimulated with a bacterial antigen. It could be assumed that this is the result of high glucose-induced changes in ARs expression on B cells in such a way that A3-AR becomes the major adenosine receptor. However, inhibition of A3-AR by a selective antagonist resulted only in marginal increase of IgM production. This means that altered response to SAC stimulation of B cells cultured in high glucose is caused by other factor than changes in the expression level of ARs. Our recent studies showed that P2 purinoceptors, are involved in regulation of IgM production by human B lymphocytes (38).

Concluding, presented evidence indicates that at low glucose concentration B cell IgM production in response to a bacterial antigen stimulation is maintained by cooperative action of all ARs and could be altered by pharmacological manipulation of adenosine receptors activities and cAMP accumulation level. We assume that high glucose-induced decrease of B cell immunoglobulin production depends on other factors than altered activity of ARs.

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