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Study of A_{2A} adenosine receptor gene deficient mice reveals that adenosine analogue CGS 21680 possesses no A_{2A} receptor-unrelated lymphotoxicity

[1]Sergey G. Apasov, [2]Jiang-Fan Chen, [1]Patrick T. Smith, [2]Michael A. Schwarzschild, [2]J. Stephen Fink & *,[1]Michail V. Sitkovsky

[1]Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, MD 20892-1892, U.S.A. and [2]Molecular Neurobiology Laboratory, Department of Neurology, Massachusetts General Hospital East and Harvard Medical School, #149 13th Street, Charlestown, Massachusetts, MA 02129, U.S.A.

1 Cell surface A_{2A} adenosine receptor ($A_{2A}R$) mediated signalling affects a variety of important processes and adenosine analogues possess promising pharmacological properties.

2 Demonstrating the receptor specificity of potentially lymphotoxic adenosine-based drugs facilitates their development for clinical applications.

3 To distinguish between the receptor-dependent and -independent lymphotoxicity and apoptotic activity of adenosine and its analogues we used lymphocytes from $A_{2A}R$-deficient mice.

4 Comparison of $A_{2A}R$-expressing (+/+) and $A_{2A}R$-deficient (−/−) cells in cyclic AMP accumulation assays confirmed that the $A_{2A}R$ agonist CGS 21680 is indeed selective for A_{2A} receptors in T-lymphocytes.

5 Incubation of $A_{2A}R$-expressing thymocytes with extracellular adenosine or CGS 21680 *in vitro* results in the death of about 7–15% of thymocytes. In contrast, no death was induced in parallel assays in cells from $A_{2A}R$-deficient mice, providing genetic evidence that CGS 21680 does not display adenosine receptor-independent intracellular cytotoxicity.

6 The A_{2A} receptor-specific lymphotoxicity of CGS 21680 is also demonstrated in a long-term (6-day) *in vitro* model of thymocyte positive selection where addition of $A_{2A}R$ antagonist ZM 241,385 did block the effects of CGS 21680, allowing the survival of T cells.

7 The use of cells from adenosine receptor-deficient animals is proposed as a part of the screening process for potential adenosine-based drugs for their receptor-independent cytotoxicity and lymphotoxicity.
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Keywords: Adenosine; purinergic receptors; T-lymphocytes; cytotoxicity

Abbreviations: ADA, adenosine deaminase activity; Ado, adenosine; $A_{2A}AR$, A_{2A} adenosine receptor; CGS 21680, $A_{2A}R$ agonist; CTL, cytotoxic T-lymphocytes; extAdo, extracellular adenosine; FCS, foetal calf serum; PI, propidium iodide; SCID, severe combined immunodeficiency; TCR, T-cell antigen receptor; ZM 241,385, $A_{2A}R$ antagonist

Introduction

Adenosine and adenosine analogues have attracted significant interest as potential pharmacological agents, based on studies implicating adenosine-triggered signalling through A_1, A_{2A}, A_{2B} and A_3 subtypes of adenosine receptors in a variety of normal and pathological processes, including cardioprotection, and neuro- and immunomodulation (Jacobson *et al*., 1996; 1999; van der Ploeg *et al*., 1996; Olsson, 1996; Apasov *et al*., 1995; 1997). Adenosine and adenosine agonists were shown to protect brain and heart tissues during ischaemia, and these effects of adenosine were explained by functional antagonism with other transmitters (Rudolphi & Schubert, 1996; Olsson & Pearson, 1990; Abbracchio *et al*., 1997; Brambilla *et al*., 1998; Abbracchio, 1996; Kohno *et al*., 1996). Agonists of A_{2A} receptors were also found to accelerate wound healing (Montesinos *et al*., 1997).

Adenosine A_{2A} receptors are considered to be involved in the regulation of normal immune responses (Koshiba *et al*., 1997; Apasov *et al*., 1995; 1997), and these observations point to A_{2A} receptors as attractive molecular targets for immunomodulation and to adenosine receptor agonists and antagonists as potential immunomodulators. However, consideration of adenosine receptor agents as drugs, in general, is complicated by the fact that adenosine and adenosine analogues may cause such diverse and opposing effects as cytoprotection and cell death (Jacobson *et al*., 1999).

The lymphotoxic properties of adenosine attracted significant attention several decades ago in studies of an inherited disease, severe combined immunodeficiency (SCID), in adenosine deaminase (ADA)-deficient patients (reviewed in Hershfield & Mitchell, 1995; Hirschhorn, 1995). The pathogenesis of this disease was explained by both intracellular (Hershfield & Mitchell, 1995; Hirschhorn, 1995) and extracellular mechanisms (Apasov *et al*., 1997; Huang *et al*., 1997), since adenosine was shown to induce cyclic AMP accumulation (Hirschhorn *et al*., 1970; Kizaki *et al*., 1990; McConkey *et al*., 1990; Szondy, 1994; Apasov *et al*., 1997; Huang *et al*., 1997).

It was suggested that the extracellular adenosine may cause T-cell depletion (and therefore the immunodeficiency) by A_{2A} receptor-mediated inhibition of TCR signalling, and thus by preventing TCR-dependent positive and negative selection of thymocytes (Apasov *et al*., 1997; Huang *et al*., 1997; Apasov & Sitkovsky, 1999). Immunodeficiency could also be due to

*Author for correspondence; E-mail: michail_sitkovsky@hih.gov

inhibition of important TCR-triggered effector functions of T cells, including T-cell expansion, T-cell-mediated cytotoxicity and lymphokine production (Koshiba *et al*., 1997; 1999).

More recently, the ability of adenosine analogues to cause necrotic or apoptotic cell death was documented (Abbracchio, 1996; Kohno *et al*., 1996; Sei *et al*., 1997; Barbieri *et al*., 1998), raising the issue of undesirable side effects of adenosine receptor agonists due to their general cytotoxicity. Especially worrisome are observations of receptor-independent intracellular toxicity of adenosine receptor agonists and antagonists (Barbieri *et al*., 1998; Abbracchio *et al*., 1995). Indeed, it has been shown in several cellular systems including embryonic sympathetic neurons (Wakade *et al*., 1995), HL-60 cells (Tanaka *et al*., 1994), leukaemia cells (Ruchaud *et al*., 1995; Hoffmann *et al*., 1996), and thymocytes (Apasov *et al*., 1995; Szondy, 1995) that adenosine analogues kill non-specifically following their intracellular uptake and without the involvement of adenosine receptors. It is believed that intracellular toxicity of adenosine analogues could involve caspases (Porter *et al*., 1997) and that intracellular phosphorylation of nucleosides is required for cell death (reviewed in Jacobson *et al*., 1999).

Overall, these observations suggest that the cytotoxic effect of these agents occurs independently of adenosine receptor recognition, a concept that has hindered the development of clinical applications for adenosinergic agents. However, this issue remains debatable and the ability of selective adenosine receptor agonists to cause receptor-independent cell death should be carefully evaluated.

The development of specific agonists and antagonists of different adenosine receptors (mostly for A_1, A_{2A}, and A_3 subtypes) (Jacobson & van Rhee, 1997) has been instrumental in assessing the potential effects of adenosine. One of the most selective agonists of A_{2A} receptors is the 2-substituted adenosine derivative 2-[4-[(2-carboxyethyl)-phenyl]ethylamino]-5′-N-ethylcarboxamidoadenosine, also known as CGS 21680; while 8-chlorostyrylcaffeine (CSC) and 4-(2-[7-amino-2-(2-furyl) [1,2,4]triazolo[2,3a] [1,3,5]triazinyl-amino]ethyl)-phenol (ZM 241385) are among the most selective A_{2A} receptor antagonists.

Here we utilize thymocytes from A_{2A}R-deficient mice to determine whether the A_{2A}R-specific agonist CGS 21680 is able to cause thymocyte death in the absence of functional adenosine receptors.

Methods

Animals

Mice were maintained in a pathogen-free environment at NIH animal care facilities. Mice were 6–10 weeks old, and two to three animals were used in each experiment. A_{2A}R gene-mutant mice (−/−) were generated by gene targeting, and lacked functional A_{2A} receptors as described by Chen *et al*. (1999). Littermates were genotyped for wild type (+/+), heterozygous (+/−) and homozygous mutant (−/−) A_{2A}R alleles by Southern blot analysis as shown in Figure 1.

Cells and medium

Thymocytes were isolated from adult thymus *ex vivo* and incubated in RPMI-1640 medium (Biofluids, Rockville, MD, U.S.A.) supplemented with 5% dialyzed foetal calf serum (heat-inactivated), or in AIM-V serum free medium (Life Technology, Grand Island, NY, U.S.A.) and 100 u ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, 1 mM sodium pyruvate, 1 mM HEPES, non-essential amino acids, and 5×10^{-5} M 2-mercaptoethanol.

Reagents

Adenosine and adenosine analogues were prepared freshly as 20–100 mM stock solution with pH adjusted to 7.1 and were purchased either from Sigma ImmunoChemicals (St. Louis, MO, U.S.A.) or from RBI (Natick, MA, U.S.A.). MAb were purchased from PharMingen (San Diego, CA, U.S.A.)

Analysis of thymocytes

A single-cell suspension of murine thymocytes was isolated by standard procedures. Cells were washed and incubated at 37°C in a 5% CO_2 incubator. Cells (0.5–10^6 ml^{-1} were cultured in a total of 0.2 ml of medium in 96-well plates. Control incubation was done in parallel at 4°C at the same cell concentration. Adenosine and adenosine analogues were added at various concentrations as indicated in figure legends. After incubation for 16–18 h, or as indicated (4 or 6 days in the thymocyte survival assay), cells were harvested and analysed by flow cytometry.

Flow cytometry quantitation of live, apoptotic, and dead cells was done according to a modified flow cytometry procedure (Darzynkiewicz *et al*., 1992). This assay allowed the quantitation of spontaneous thymocyte death and the determination of the proportion of cells that were killed due to adenosine- or CGS 21680-induced cytotoxicity. Briefly, cells from the short-term cultures were gently pipetted and transferred from 96-well plates (200 ml) directly into polystyrene tubes (12×75 mm; Falcon, Becton Dickinson Labware, Lincoln Park, NJ, U.S.A.), and 200 ml of FACS buffer (PBS with 2% foetal calf serum and 0.05% sodium azide) was added to each sample. Each sample had equal volume and was analysed at the same flow rate in duplicate or triplicate. Propidium iodide solution (1 μg ml^{-1} final concentration) was added to each tube for 10 s prior to FACS analysis. Live, dead, and apoptotic cells were estimated from 2×10^5 cells in each sample by counting cell numbers in appropriate gates using a forward/side scatter dot plot in linear scale and PI staining in log scale (Apasov *et al*., 1997). Data are presented as percentage of surviving cells from total cell input. Statistical analysis of triplicate sample measurements was calculated as described below and as previously reported (Apasov *et al*., 1997). Standard deviations of triplicate measurements within the same experiment were typically lower than 1%.

Fluorimetric measurements of apoptosis in cell culture were also done using the annexin V binding assay as described (Martin *et al*., 1995). Briefly, 0.5–1×10^6 cells from 96-well plate were resuspended in 100 μl of buffer containing (mM): HEPES 10 pH 7.3, NaCl 150, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.8 and incubated with 0.3–1 μg/ml of FITC-conjugated annexin V and with 1–5 μg ml^{-1} propidium iodide for 5 min. After incubation samples were diluted four times with buffer containing 1.8 mM $CaCl_2$ and analysed by Flow cytometry. Annexin V-FITC was purchased from Trevingen (Gaithersburg, MD, U.S.A.) and BioWhitaker (Walkersville, MD, U.S.A.).

Statistical analysis

Statistical analysis of percentage of live, dead and apoptotic cells and flow cytometry data acquisition and analysis were done on FACScan using FACScan research software and

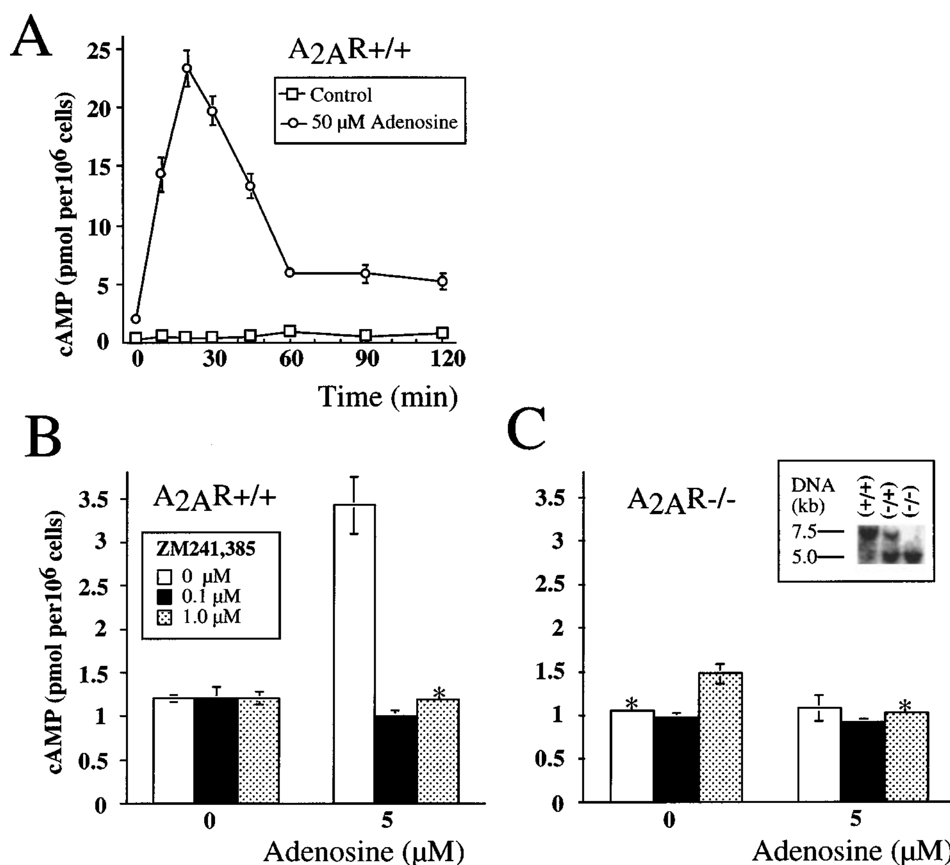

Figure 1 Measurement of cyclic AMP accumulation in murine thymocytes after incubation with adenosine. Results are representative of four independent experiments with $P<0.01$. (*indicates that standard deviations are not visible on the graph). *Ex vivo* thymocytes from $A_{2A}R$ wild type (+/+) (A,B) and from homozygous (−/−) $A_{2A}R$-deficient 'knock-out' mice (C) were incubated with adenosine in the presence or absence of $A_{2A}R$ antagonist ZM 241385 and cyclic AMP accumulation was measured as described in Methods. (A) Transient accumulation of cyclic AMP in thymocytes from $A_{2A}R$ wild type (+/+) mice during 2 h incubation with 50 μM adenosine. Thymocytes from $A_{2A}R$ wild type (+/+) mice (B) or $A_{2A}R$ (−/−) mice (C) were incubated 30 min with 5 μM adenosine in the presence or absence ZM 241385 at indicated concentrations. Inset illustrates Southern blot screening procedure for identification of wild type (+/+), heterozygous (+/−) and homozygous (−/−) mice.

CellQuest programs (Becton-Dickinson, San José, CA, U.S.A.) after acquisition of 20,000 cells in each triplicate or duplicate sample. Standard deviations in estimations of percentage of dead, survived cells were calculated using Stat-View analysis program (Abacus Concept Inc., Berkeley, CA, U.S.A.).

Measurements of cyclic AMP

DBA-2 thymuses were harvested, thymocytes isolated and resuspended at 4×10^6 cells ml^{-1} in the culture media (RPMI-1640) at 4°C. Incubations of cells (4×10^5 thymocytes per assay) with various agents were performed in 1.5 ml Eppendorf tubes in a final volume 200 μl containing media alone, or adenosine (0–125 μM), CGS 21680 (0–10 μM), and/or ZM 241,385 (0–1 μM). Controls such as the complete reaction mix at time zero or no cell mixture were used with every experimental set. The reactions were initiated by the addition of adenosine or adenosine analogues and incubation lasted from 0–120 min at 37°C in an Eppendorf Thermomixer Model 5436. Thymocyte reaction mixtures were gently resuspended every 5 min and reactions were terminated by the addition of 25 μl of 1N HCl, followed by freezing of samples on dry ice. The cyclic AMP levels were determined using cyclic AMP enzyme immunoassay (EIA) kit from Amersham according to manufacturer's instructions.

Cell incubations and extractions were performed in the absence of cyclic AMP phosphodiesterase inhibitors IBMX to avoid complications with interpretation of results due to the possibility of their effects on adenosine receptors (Beavo & Refsnyder, 1990).

Results

In our earlier studies (Huang *et al.*, 1997; Koshiba *et al.*, 1997; 1999) we established that A_{2A} adenosine receptors are mostly responsible for adenosine-induced cyclic AMP accumulation in peripheral T-lymphocytes. Figures 1 and 2 demonstrate that the same is true for thymocytes upon exposure to adenosine or CGS 21680. As shown, both CGS 21680 (Figure 2A) and extracellular adenosine (Figure 1A) trigger transient accumulation of cyclic AMP in thymocytes that peaks at 15 or 30 min, followed by a rapid decline, which most likely reflects the desensitization of Gs-coupled A2a-receptors on thymocytes. This ability of the selective $A_{2A}R$ agonist CGS 21680 to trigger cyclic AMP accumulation suggests that signalling through A_{2A} receptors is responsible for the observed effects in thymocytes. This conclusion is supported by the ability of the selective A_{2A} receptor antagonist ZM 241385 to completely inhibit both adenosine- or CGS 21680-induced accumulation of cyclic AMP in thymocytes (Figures 1B and 2B, respectively).

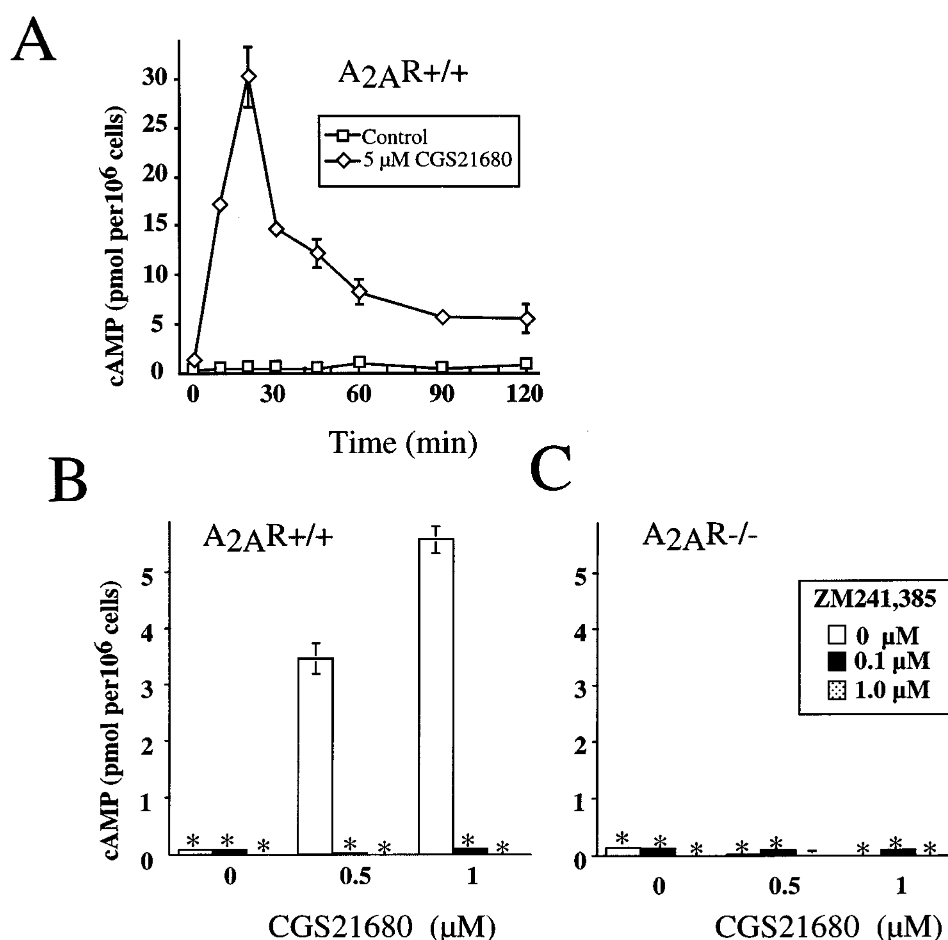

Figure 2 Measurements of cyclic AMP accumulation in murine thymocytes after incubation with adenosine analogue CGS 21680. Results are representative of four independent experiments with $P<0.01$. (*Indicates that standard deviations are not visible on the graph). *Ex vivo* thymocytes from $A_{2A}R$ wild type (+/+) (A,B) and from homozygous (−/−) $A_{2A}R$-deficient 'knock out' mice (C) were incubated with CGS 21680 in the presence or absence of $A_{2A}R$ antagonist ZM 241385 and cyclic AMP accumulation was measured as described in Methods. (A) transient accumulation of cyclic AMP in thymocytes from $A_{2A}R$ wild type (+/+) mice during 2 h incubation with 5 μM CGS 21680. Thymocytes from $A_{2A}R$ wild type (+/+) mice (B) or $A_{2A}R$-deficient (−/−) mice (C) were incubated 30 min with 0.5 μM or 1 μM CGS 21680 in the presence or absence ZM 241385 at indicated concentrations.

Similar results were obtained after thymocytes were incubated with 10, 25 or 50 μM adenosine (data not shown).

The definitive genetic evidence for the selectivity of CGS 21680 for A_{2A}Rs was provided by experiments using cells from $A_{2A}R$-deficient mice (Figures 1C and 2C). It is shown that while both extracellular adenosine and CGS 21680 were able to trigger cyclic AMP accumulation in cells from wild type mice, $A_{2A}R$-deficient cells were refractory to both adenosine or CGS 21680 (up to 100 μM, data not shown). Moreover, the inability of adenosine or CGS 21680 to trigger cyclic AMP accumulation in $A_{2A}R$-deficient thymocytes even at high concentrations also indicates the lack of compensation by other adenosine receptors.

The above experiments allowed us to address the issue of whether CGS 21680 exerts $A_{2A}R$-independent lymphotoxicity in long-term functional assays. This was done by culturing thymocytes in the presence or absence of adenosine or CGS 21680 and estimating the proportion and number of surviving thymocytes using several independent assays. Flow cytometry analysis allowed us to detect proportions of live, apoptotic, and dead thymocytes following exposure to adenosine or CGS 21680 *in vitro*. Figure 3 shows that both adenosine and CGS 21680 produce a moderate decrease in thymocyte survival after 16 h cultures. Corresponding results were obtained by evaluating cell morphology as indicated by the forward (size) and side (granularity) scatter profiles (Figure 3A) and by specific Annexin-V/PI staining in separate experiments (Figure 3B). While CGS 21680 and adenosine both caused lymphotoxicity, a much higher concentration of adenosine (~400 fold) was required to achieve similar proportions of cell death. The effects of CGS 21680 were observed at a concentration as low as 100 nM (data not shown) with maximum effect around 1 μM.

The maximum number of dead cells following incubation with CGS 21680 varied between 7–15% among different mouse strains (Figure 3C). Interestingly, thymocyte from MHC classI/II deficient mice exhibited the highest susceptibility to the effect of CGS 21680 (Figure 3C) presumably due to the higher proportions of CD4/CD8 double positive thymocytes, which are more prone to the CGS 21680-induced cell death (not shown, manuscript in preparation). Evidently, CGS 21680 is much more potent than adenosine in the induction of both signalling and death of thymocytes.

To determine whether signalling through $A_{2A}R$ was indeed responsible for adenosine-induced thymocyte death, we used a sensitive Annexin V assay to detect changes in dying cells that are characteristic of apoptosis (Figure 4). We hypothesized that if CGS 21680-induced cell death was receptor-independent, then the same degree of cell death should be observed after incubation of thymocytes from wild type and from $A_{2A}R$

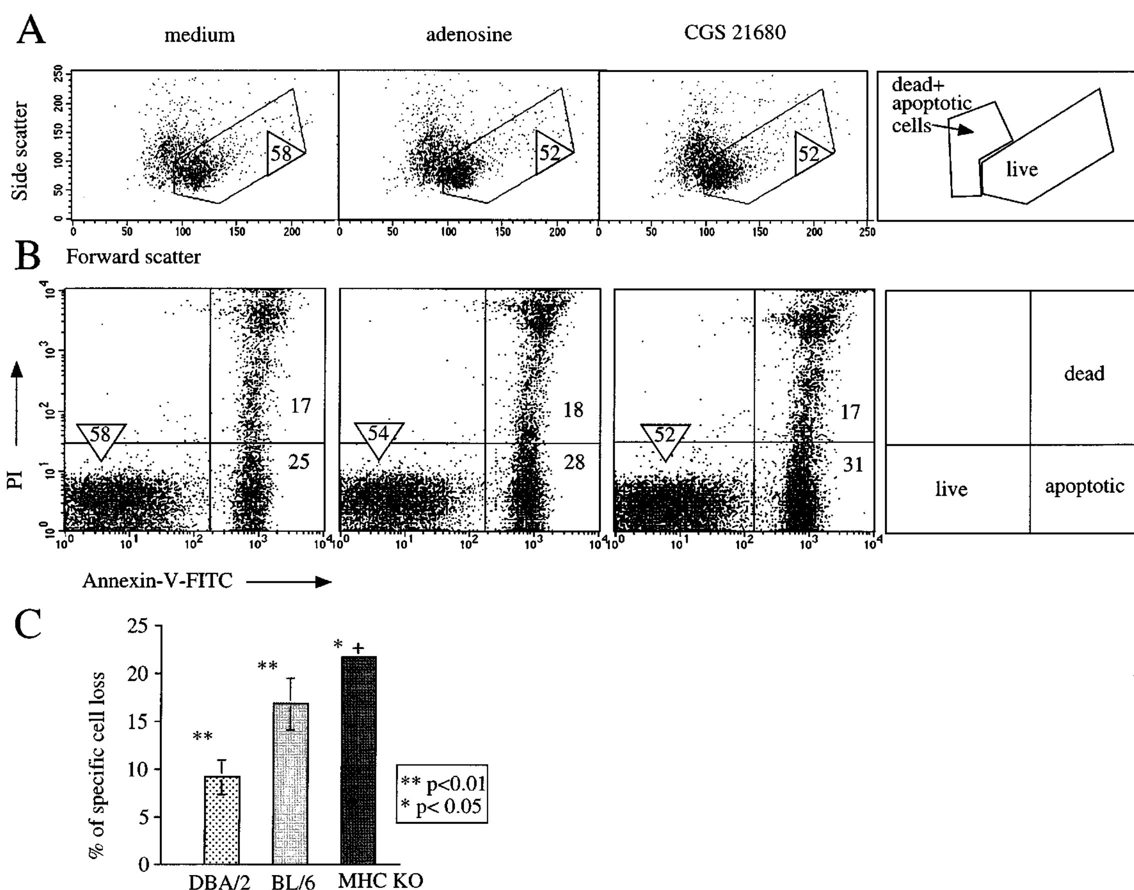

Figure 3 Extracellular adenosine and CGS 21680 induce hymocytes death. *Ex vivo* thymocytes from DBA/2, wild type ($A_{2A}R+/+$) mice were incubated with media alone (control) and with adenosine (125 μM) or CGS 21680 (300 nM, (A,B) or 1 μM, (C) for 16 h in parallel assays. The survival of thymocytes after 16 h of incubation was measured by flow cytometry assays using Side scatter *vs* Forward scatter analysis (A) or Propidium Iodide (PI) staining *vs* Annexin V (B) to discriminate between live, dead and apoptotic cells as described in Methods and illustrated on cartoons. The percentage of survived, live cells (gated) is indicated by a circled number. The data are representative of eight separate experiments. The selection of gates was based on results of multiple earlier experiments where flow cytometry parameters were correlated with other methods of determination of cell death, including trypan blue and DNA fragmentation. (C) CGS 21680 induces death in a small proportion of thymocytes. Demonstration of variability in susceptibility of thymocytes from different strains of mice. DBA/2 (three experiments); BL/6 (three experiments) and double MHC class I and II gene deficient mice (two experiments). *Indicates $P<0.05$; **indicates $P<0.01$; +indicates that standard deviation is not visible on the graph.

deficient mice in parallel experiments. If, however, $A_{2A}R$ was required, we would expect to see a higher proportion of apoptotic cells in wild type cultures than $A_{2A}R$-deficient cultures. Indeed, $2_{2A}R$-deficient thymocytes were resistant to the apoptosis-inducing affects of CGS 21680. Figure 4 shows that thymocytes from $A_{2A}R$-deficient mice survived while $12\pm0.2\%$ of $A_{2A}R+/+$ thymocytes were killed following CGS 21680 (Figure 4A) treatment. In general, CGS 21680 was able to reproducibly induce cell death in about 8–15% of total thymocytes from wild type $A_{2A}R+/+$ mice and no increases in the proportion of dead A_{2A} $R+/+$ thymocytes were observed after incubation of thymocytes with increasing concentrations of CGS 21680 (up to 50 μM; data not shown), suggesting that this number correctly reflects the proportion of thymocytes that express apoptotic signal-transducing A_{2A} receptors among total thymocytes. Furthermore, the ability of $A_{2A}R$ antagonist ZM 241385 to prevent the CGS 21680-induced thymocyte death (Figure 4A) provides pharmacological evidence that the effect of CGS 21680 is, in fact, mediated by signalling through A_{2A} receptors. Overall, these results prove that the lymphotoxic effects of CGS 21680 are $A_{2A}R$-dependent.

Figure 5 summarizes the results of three separate experiments in which CGS 21680-induced $A_{2A}R+/+$ thymocyte death in the presence or absence of ZM 241385 was assessed by PI *versus* Annexin V assays. These data confirm that a small, but significant, proportion of thymocytes can be targeted by CGS 21680. In general, the number of thymocytes in the murine thymus that are susceptible to extracellular adenosine-induced death ranged from 7–15% (based on 10 independent experiments) depending on the age and sex of the animal. These thymocytes are identified as part of the larger thymocyte subset of double positive CD4+/CD8+ cells (data not shown).

Additional evidence for the absence of intracellular lymphotoxicity by CGS 21680 was obtained in long-term (6 day) *in vitro* assays of thymocytes. The design of the experiments in Figure 6 was based on the ability of anti-TCR/CD3 mAb (to the antigen receptor complex) to rescue thymocytes from spontaneous death in which thymocytes die in the absence of activating stimuli unless selected by appropriate levels of TCR-mediated signalling (Surch & Sprent, 1994). Accordingly, we incubated thymocytes *ex vivo* with immobilized mAb to TCR/CD3 complex in the presence or absence of CGS 21680 and/or ZM 241385 and evaluated cell survival after 6 days by flow cytometry (Figure 6). It is shown that there were virtually no surviving thymocytes in the absence of anti-CD3 mAb (medium alone). However, up to 15% of thymocytes could be rescued from death by anti-CD3

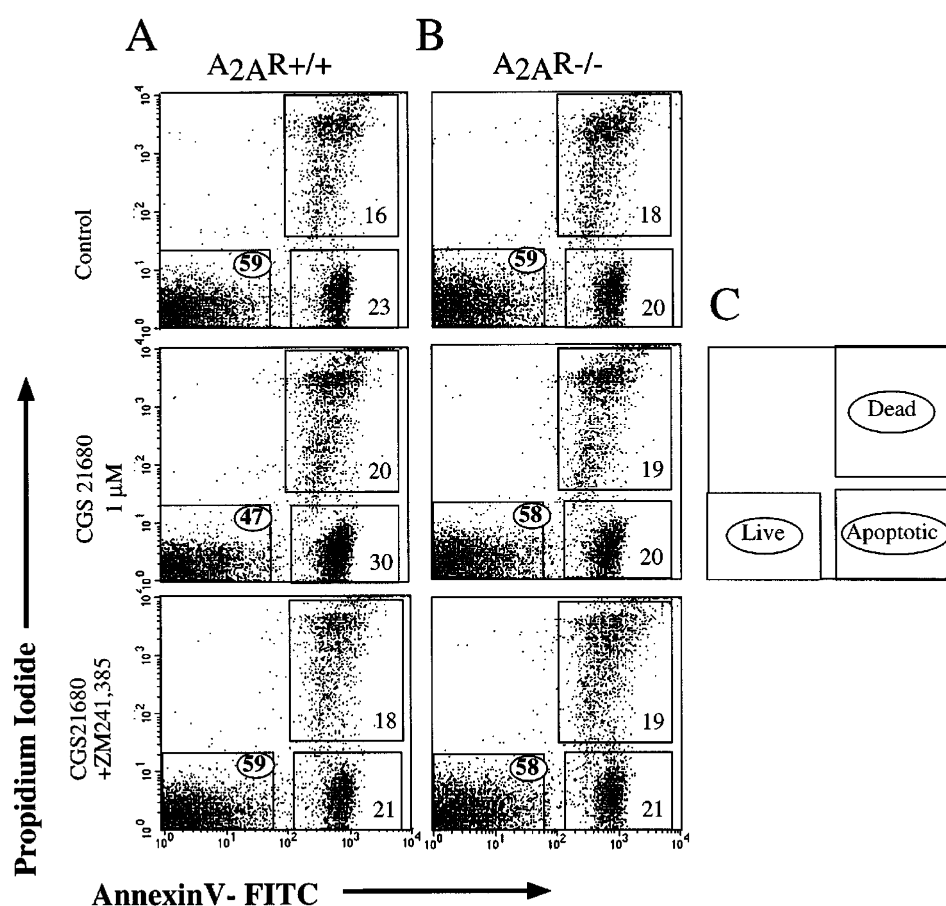

Figure 4 Extracellular adenosine and CGS 21680 are not lymphotoxic with $A_{2A}R$ deficient thymocytes. *Ex vivo* thymocytes from wild type (A, +/+) and from $A_{2A}R-/-$) mice (B) were incubated in parallel assays with media alone (control) and with adenosine or CGS 21680. The survival of thymocytes after 16 h of incubation was measured by flow cytometry using Propidium Iodide *vs* Annexin V staining analysis to discriminate between live, dead and apoptotic cells as described in Methods and illustrated in C. Results are representative of four independent experiments with an analysis of 20×10^3 cells in each sample. The percentage of survived, live cells (gated) is indicated by a circled number.

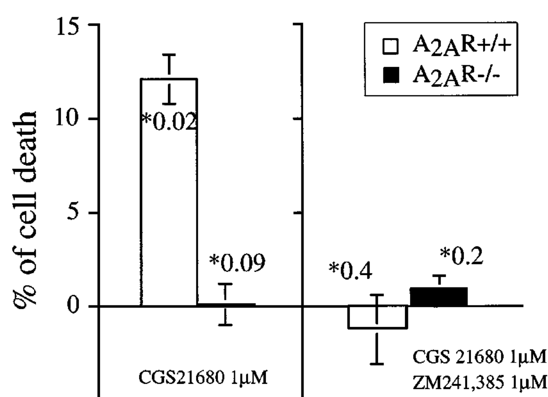

Figure 5 CGS 21680 does not possess $A_{2A}R$ unrelated lymphotoxicity. Results of three parallel independent cell death assays are summarized to demonstrate the presence of a thymocyte subset which is susceptible to CGS 21680-induced death in $A_{2A}R+/+$ but not in $A_{2A}R-/-$ mice. Concentrations of CGS 21680 and ZM 241385 are indicated on the graph. *Number indicates *P* value for each point.

mAb. As expected, and in agreement with our earlier observation (Huang *et al*., 1997; Apasov & Sitkovsky, 1999), the addition of CGS 21680 abolished signalling through the TCR and prevented the rescue of anti-TCR mAb-treated thymocytes. As expected, the $A_{2A}R$ antagonist ZM 241385 prevented the effects of CGS 21680, and the combined addition of both CGS 21680 and of ZM 241385 allowed the survival of the same number of thymocytes on the immobilized anti-CD3 mAb. According to these results, the combined presence of CGS 21680 and ZM 241385 was not lymphotoxic, but together with anti-TCR/CD3 complex mAb, allowed the survival of thymocytes which would otherwise have died. These observations demonstrate that even after 6 days the survival of thymocytes was not affected by the presence of CGS 21680 and ZM 241385, even though control experiments confirm that these drugs were signalling under these conditions, as evidenced by the ability of CGS 21680 to prevent anti-CD3 mAb induced survival and the abrogation of such event by ZM 241285 (Figure 6).

The experiments shown here may provide the best means to evaluate the absence of lymphotoxicity by these compounds, since the readout entails the enumeration of live rather than dead cells. Taken together, these data support the conclusion that adenosine receptor agonist CGS 21680 and antagonist ZM 241385 are not lymphotoxic in long-term *in vitro* assays.

Discussion

The issue of non-specific or receptor-independent cytotoxicity by pharmacological agents and, particularly, of adenosine receptor agonists (reviewed in Jacobson *et al*., 1999), is the subject of significant interest due to the need to minimize drug

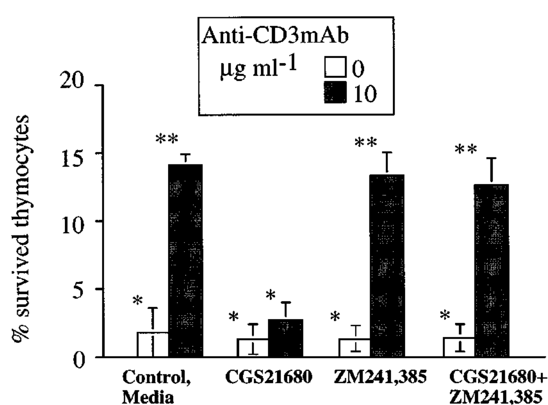

Figure 6 Selectivity of CGS 21680 and ZM 24385 mediated effects on thymocytes. CGS 212680 and ZM 241385 are not lymphotoxic in long thymocyte survival assay. *Ex vivo* thymocytes from wild type ($A_{2A}R+/+$) DBA/2 mice were incubated in parallel assays alone or with thymocyte-activating anti-CD3/TCR mAb (10 μg ml^{-1}) in the presence of media (control) or CGS 21680 (1 μM), ZM 241385 (1 μM) alone or both ZM 241385 and CGS 21680 as indicated on the graph. The survival of thymocytes after 5–6 days of incubation was measured by flow cytometry as described in Methods. The results represent a summary of five independent experiments. *Indicates $P<0.4$; **indicates $P<0.07$.

side effects. The rationale for considering the use of adenosine and adenosine analogues as agonists of distinct adenosine receptors to modulate *in vivo* physiology has been weakened by concerns over the well-known lymphotoxic effects of adenosine. These effects include inhibition of pyrimidine synthesis, inhibition of transmethylation, inhibition of ribonucleotide reductase, DNA nicking and apoptosis (Hershfield & Mitchell, 1995; Hirschhorn, 1995; Liu *et al.*, 1996).

The hope that so-called slow hydrolyzable adenosine analogues and agonists of adenosine receptors may not have intracellular cytotoxic effects was dispelled by studies in different cellular systems (Abbracchio, 1996; Kohno *et al.*, 1996; Sei *et al.*, 1997; Barbieri *et al.*, 1998; Abbracchio *et al.*, 1995; Wakade *et al.*, 1995; Tanaka *et al.*, 1994; Ruchaud *et al.*, 1995; Hoffmann *et al.*, 1996; Apasov *et al.*, 1995; Szondy, 1995; Porter *et al.*, 1997; reviewed in Jacobson *et al.*, 1999). Thus, there is a need to evaluate every promising adenosine receptor agonist for its ability to induce non-receptor mediated intracellular toxicity. However, due to the intrinsic pharmacological limitations of earlier experiments, it was not possible to conclusively discriminate adenosine receptor-mediated toxicity from intracellular toxicity of adenosine or adenosine analogues.

The ability to definitively address receptor specificity in this case has now become possible with the development of $A_{2A}R$-deficient mice (Ledent *et al*, 1997; Chen *et al.*, 1999) and with a better understanding of thymocyte differentiation and the signals that drive T-cell development in long-term differentiation *in vitro* assays (Cibotti *et al.*, 1997). The use of cells from $A_{2A}R$-deficient mice in cyclic AMP accumulation assays confirms the pharmacological selectivity of the $A_{2A}R$ agonist CGS 21680 (Figures 1 and 2), and provides a convenient system with which to detect adenosine-independent lymphotoxic effects.

We show here that adenosine and CGS 21680 cause cell death in a relatively small proportion of thymocytes after 16 h incubation. Moreover, only $A_{2A}R$-expressing thymocytes are susceptible to these direct cytotoxic effects (Figures 3 and 4). Differences in susceptibility of thymocytes to CGS 21680-induced death were found between DBA-2, C57BL/6, and genetically engineered MHC class I and II deficient mice (Figure 3C). It is noteworthy that the larger proportion of thymocytes killed by CGS 21680 was in MHC class I and II double knock-out mice, which predominantly express double positive $CD4^+CD8^+$ thymocytes (data not shown, manuscript in progress). This is consistent with our observation that $CD4^+CD8^+$ double positive T cells (precursors of single positive $CD4^+$ and $CD8^+$ T cells) are the main targets of A_{2A} receptor-mediated effects. An important goal of our ongoing studies is to identify and understand the functional role of $A_{2A}R$ on thymocyte development and T-cell differentiation.

Taken together, the observation that $A_{2A}R+/+$, but not $A_{2A}R-/-$, thymocytes are susceptible to cell death by CGS 21680 shows that the lymphotoxic effects of such pharmacological adenosine analogues is a receptor-dependent process. Furthermore, the fact that the A_{2A} receptor-specific antagonist ZM 241385 could abrogate the CGS 21680-induced death indicates that A_{2A} receptors are specifically involved in this process. Overall, these data support a model in which A_{2A} adenosine receptors are directly involved in CGS 21680-mediated lymphotoxicity.

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