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Induction of interleukin 8 release from the HMC-1 mast cell line: Synergy between stem cell factor and activators of the adenosine A_{2b} receptor

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Abstract

The HMC-1 mast cell line has both adenosine A_3 and A_{2b} receptors on its surface, but only agonists of the A_{2b} receptor are effective at releasing interleukin 8. Object of this study was to look for co-factors for adenosine A_{2b} receptor activation. There was a powerful and statistically significant synergy for release of IL-8, both at the mRNA level (measured after 4 hr) and protein level (measured after 24 hr), between adenosine A_{2b} receptor agonists and stem cell factor (SCF). Suitable concentrations for showing synergy were 100 ng/mL SCF and 3 μM 5′-*N*-ethylcarboxamidoadenosine (NECA). At these concentrations, the IL-8 released into the culture medium after SCF and NECA together was typically 3–5-fold greater in amount than the sum of the amounts of IL-8 released after exposure to the same concentrations of NECA and SCF separately. Since mast cells may be exposed to both adenosine and stem cell factor in the diseased lung, the synergy observed in this model system may have implications for asthma.

Keywords: Mast cell; HMC-1; Adenosine A_{2b} receptor; Stem cell factor; Interleukin 8

1. Introduction

Exposure of human subjects to adenosine is more likely to induce bronchospasm when the subjects are asthmatic [1]. The mechanism appears to involve mast-like cells [2]. Whilst in adenosine-induced bronchospasm, biogenic amines such as histamine or serotonin are likely to play the key role, mast cells also produce many other products for example IL-8, a cytokine which can act as a chemotactic agent for granulocytes (both neutrophils and eosinophils). Recently, there has been increased interest in defining the mechanism of adenosine-induced mast cell activation not only in relation to degranulation and histamine release but also in relation to release of factors such as IL-8 which are not granule derived.

Obtaining sufficient numbers of lung mast cells for biochemical studies is difficult and therefore many workers have used the mast cell leukaemia HMC-1 as a model system. Although HMC-1 is an immature cell, it has many of the characteristics of mature mast cells, even if only to a limited degree. Like lung mast cells, the principal neutral protease expressed is tryptase and not chymase [3]. The HMC-1 cell has been reported to respond to certain adenosine receptor agonists such as 5′-*N*-ethylcarboxamidoadenosine (NECA) by releasing large quantities of IL-8 [4]. In our hands, the response of this cell line was remarkably variable which caused us to look for potential cofactors for adenosine responsiveness whose varying quantity during culture might explain the changes in our cell line's responsiveness. We here report on the activity of the cytokine stem cell factor (SCF), sometimes also termed Steel factor or mast cell growth factor.

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Abbreviations: APNEA, adenosine, *N*6-[2-(4-amino-3-iodophenyl)ethyl]-; CCPA, adenosine, 2-chloro-*N*6-cyclopentyl-; CGS 21680, benzenepropanoic acid, 4-[2-[[6-amino-9-(*N*-ethyl-beta-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]-, monohydrochloride; CPCA, beta-D-ribofuranuronamide, 1-(6-amino-9*H*-purin-9-yl)-*N*-cyclopropyl-1-deoxy-; FAM, 6-carboxyfluorescein; I-AB-MECA, beta-D-ribofuranuronamide, 1-deoxy-1-[6-[[(4-amino-3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-*N*-methyl-; IB-MECA, beta-D-ribofuranuronamide, 1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-*N*-methyl-; FCS, foetal calf serum; IL, interleukin; IMDM, Iscove's modified Dulbecco's medium; NECA, 5′-*N*-ethylcarboxamidoadenosine; NGF, nerve growth factor; (rh)SCF, (recombinant human) stem cell factor; RT-PCR, reverse transcription-polymerase chain reaction; TAMRA, 6-carboxytetramethylrhodamine.

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2. Materials and methods

2.1. Chemicals and cytokines

APNEA was synthesised by C. Woenckhaus, Gustav Embden Zentrum der Biologischen Chemie, Frankfurt, Germany. I-AB-MECA, CCPA and CPCA were purchased from RBI, CGS 21680 and IB-MECA were purchased from Tocris, NECA was purchased from Sigma–Aldrich, recombinant human SCF and IL-6 were purchased from R and D Systems, and interleukin 9 was purchased from Biotrend. CGS 21680 and IB-MECA were stored as stock solutions in DMSO and the stock solution of I-AB-MECA was dissolved in 0.01 N NaOH. Other substances were soluble in water (APNEA only up to 100 μM; for preparing highly concentrated solutions DMSO was used as solvent). The substances in DMSO solution were added to culture medium so that the final DMSO concentration did not exceed 1% and was standardly 0.3%.

2.2. Cell culture

HMC-1 cells (kindly supplied by J.H. Butterfield, Mayo Clinic) were maintained at between 3×10^5 and 1×10^6 cells/mL in Iscove's modified Dulbecco's medium (Bio-Whittaker), supplemented with 25 mM HEPES, 4 mM L-glutamine, 1.2 mM monothioglycerol and 10% foetal calf serum (Cool Calf, Sigma–Aldrich). For stimulation and cytokine measurement the cells were further cultured in 2 mL plastic wells (24-well plate, Costar) at 1×10^6 mL^{-1} in the IMDM as above but without calf serum and with 0.25 U/mL adenosine deaminase (Sigma–Aldrich). For differentiation studies HMC-1 cells were seeded at 2.5×10^5 mL^{-1} and cultured for 10 days in IMDM supplemented with 25 mM HEPES, 4 mM L-glutamine, 30% heat inactivated horse serum (Sigma–Aldrich) and 30% L-cell supernatant. This was collected from murine L-cell fibroblasts (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ no. ACC2) maintained at confluence in RPMI 1640 with ultraglutamine (Bio-Whittaker, Item 12-702F/U) and 10% foetal calf serum (Seromed). For stimulation, the differentiated cells were adjusted to 1×10^6 mL^{-1} in IMDM plus 0.25 U/mL adenosine deaminase, as above.

2.3. Fluorescence-activated cell sorting for surface adenosine receptors

Surface adenosine A_{2b} receptor was identified by a sandwich technique. The first layer was rabbit anti-human adenosine A_{2b} receptor antibody (Alpha Diagnostic). The second layer was fluorescein-conjugated goat anti-rabbit IgG heavy and light chain (Life Technologies). In controls, the first layer was substituted by the same concentration of rabbit myeloma IgG (Serotec) or by an antibody to an unrelated antigen (ovalbumin). Cells were incubated at 4° for 30 min with each layer in turn then fixed (Q-prep) for fluorescence-activated cell sorting.

2.4. Surface adenosine A_3 receptor

Surface adenosine A_3 receptor was identified by a single layer technique. Rabbit anti-human adenosine A_3 receptor antibody was prepared by immunising rabbits with either of the peptides MPNNSTALSLANVTC and CHPSDSLDTSIEKNSE derived from the published amino acid sequence of this receptor. Specific immunoglobulin was separated by affinity chromatography and fluorescein conjugated (fluorescein/protein ratio between 4 and 12). An amount of 40 μg/mL (final concentration) fluorescein conjugated antibody was incubated for 30 min at 4° with the cells, which were then fixed (Q-prep), for fluorescence-activated cell sorting (Coulter). Controls were FITC-antibody that had been previously blocked by incubation for 1 hr at 4° with an excess of the immunising peptide, or 40 μg/mL rabbit myeloma IgG (Serotec). The A_3 receptor was also identified by a double layer technique using the same antibodies as for the single layer technique (but without fluorecein conjugation) and a second layer of FITC-labelled goat anti-rabbit IgG. As control, the anti-A_3 receptor immunoglobulin was replaced by a rabbit myeloma IgG in the first layer.

2.5. Preparation of total RNA from tissues and cell pellets

Cell were snap frozen in liquid nitrogen and stored at −80° prior to isolation of total RNA with the RNeasy Midi-Prep System (Qiagen). The kit method was slightly modified by inclusion of an extra digestion step with RNAase-free DNAase (Qiagen).

2.6. Preparation of cDNA from RNA

For studies (e.g. Figs. 3 and 8) in which the same gene was measured in samples from similar cells grown under different conditions, cDNA was prepared using the same primers and enzymes as used for the PCR reaction in a one step assay (Taqman™ EZ RT-PCR core mix, Applied Biosystems). For studies in which the abundance of RNA's coding for different genes (e.g. A_{2b} receptor, A_3 receptor) was compared in the same samples, reverse transcription and PCR amplification were carried out as two separate steps. The first, reverse transcription step used oligo$(dT)_{12–18}$ primer (Invitrogen) with Omniscript™ reverse transcriptase (Qiagen) in the presence of RNAse inhibitor (Roche, Mannheim, Item 799017), 10 units/20 μL reaction.

2.7. Qualitative PCR and nucleotide sequencing

cDNA was prepared from the isolated RNA by RT-PCR (Titan™ One-Tube Kit, Boehringer Mannheim). For qualitative analysis, the products were separated by agarose

gel electrophoresis and stained with ethidium bromide. For the A_{2b} receptor, the forward primer was 5′-AACAACTGCACAGAACCCTGG-3′ and the reverse primer 5′-AATCCCCACAATCATGGCC-3′. For the A_3 receptor, the forward primer was 5′-AATTTTCATTGGACTCTGCGCC-3′ and the reverse primer 5′-TGGTGACCCTCTTGTATCTGACG-3′. The adenosine A_1 and A_{2a} receptor primers were standard sets from Biognostik. For sequencing the appropriate band was cut out from the gel, and the cDNA purified (Qiaex II kit, Qiagen) and subjected to further PCR-amplification (PCR Core Kit, Boehringer Mannheim). The amplification product was ligated into a pcr™ 2.1 vector (Original TA cloning kit, Invitrogen). The construct was transformed into *E. coli* strain xl_1 blue and DNA was isolated from positive clones using the Qiagen Plasmid Mini Kit. Double-stranded automatic fluorescence sequencing of the insert was performed (ABI Prism Sequencer, MediGenomix). Because the initially sequenced PCR product obtained with the adenosine A_{2b} receptor primers differed by one base from the published sequence, a second PCR product was prepared using the same A_{2b} primers but cDNA prepared using a different reverse transcriptase enzyme (Omniscript™, as in Section 2.6), and sequencing was repeated.

2.8. Quantitative PCR

Quantitation of IL-8 and adenosine A_{2b} receptor gene expression (Figs. 3 and 8) was performed by the Taqman real time PCR system ([5]; ABI Prism 7700 Detector, Applied Biosystems). The fluorogenic probes were labelled [5′]6-FAM [3′]TAMRA. IL-8 measurement used as forward probe 5′-GGCCGTGGCTCTCTTGG-3′, reverse probe 5′-CTTTAGCACTCCTTGGCAAAACTG-3′ and fluorogenic probe 5′-AGCCTTCCTGATTTCTGCAGCTCTGTGTG-3′. A_{2b} receptor measurements used as forward probe 5′-TCTTTCCTCGCCTGCTTCGT-3′, reverse probe 5′-CCAGGTATCTGTCGACTGCCA-3′ and fluorogenic probe 5′-CTGGTGCTCACGCAGAGCTCCATCT-3′. Results were expressed as the ratio of specific IL-8 or adenosine A_{2b} receptor mRNA to total 18S ribosomal RNA (determined with ribosomal RNA control reagents (VIC probe)) (Applied Biosystems, Item 4308329).

For comparison of the abundance of A_{2b}, A_{2a}, A_3 and A_1 receptor mRNA, cDNA obtained with oligo$(dT)_{12–18}$ primer was amplified using Taqman universal PCR master mix (Applied Biosystems). The adenosine A_{2b} receptor probes were as above. Adenosine A_{2a} receptor measurements used as forward probe 5′-TGCTTCGTCCTGGTCCTCA-3′, reverse probe 5′-CATTGTACCGGAGCGGGAT-3′ and fluorogenic probe 5′-CAGAGCTCCATCTTCAGTCTCCTGGCC-3′. Adenosine A_3 receptor measurements used as forward probe 5′-CCTGGCTGACATTGCTGTTG-3′, reverse probe 5′-AGAAGTGGATTGTGATGCCCA-3′ and fluorogenic probe 5′-CATGCCTTTGGCCATTGTTGTCAGC-3′. Adenosine A_1 receptor measurements used as forward probe 5′-CTGAAGATCGCCAAGTCGCT-3′, reverse probe 5′-CAGTTGAGGATGTGCAAAGGC-3′ and fluorogenic probe 5′-CTCTTCCTCTTTGCCCTCAGCTGGC-3′.

2.9. Cytokine assay

Interleukin 8 was measured by enzyme-linked immunoassay using microtitre plates coated with a monoclonal anti-IL-8 antibody. The assay used a sandwich technique in which the ligand bound to the plates was detected in a two step process. The first layer was a monoclonal biotinylated anti-IL-8 antibody and the second streptavidin–peroxidase conjugate (Immunotech, kit 2237).

2.10. Tryptase

Tryptase was assayed by hydrolysis of the synthetic substrate *N*-*p*-tosyl-Gly-Pro-Lys-*p*-nitroanilide (Sigma, Item T-6140). Release of *p*-nitroaniline was followed at 405 nm over 5 min at 37° with a ''Cobas Bio'' centrifugal analyser. The reaction mixture contained 70 μL and 100 μL 10 mM Tris buffer pH 7.4 supplemented with 5 mM $CaCl_2$ and 100 mg/L bovine lung heparin (Sigma Item H-4898). The assay was initiated by addition of the peptide substrate to give a final peptide concentration of 0.6 mM.

2.11. Statistical analysis

In all figures except Fig. 8, results are shown as means. Vertical bars represent standard deviations. One object of this study was to test whether different cytokines were able to synergise with an adenosine receptor agonist (as in the experiments shown in Figs. 3 and 4). To calculate significance levels, the comparison of the expected value of the combination of adenosine receptor agonist (e.g. NECA) and cytokine (e.g. SCF) with the expected sum of the values of the cells treated with adenosine receptor agonist or cytokine alone was done with a modification of the Welch test using Satterthwaite's approximation for the degrees of freedom. Since not only two expected values were compared, the modification was necessary to take the variance of all groups into consideration. In addition, in Figs. 3 and 4, the effects of the single cytokine treatments, either in the presence or the absence of NECA were compared to the appropriate control (with or without NECA) by an original one-sided Welch test. Because of the multiple test situation, the *P* values were adjusted according to Bonferroni–Holm to control the level of significance ($\alpha = 0.05$). The data in Fig. 8 was handled differently, because for the observed distribution of values a non-parametric statistic was more appropriate. Values shown are medians with quartiles. Comparison of different treatment groups (in Fig. 8, SCF, NECA and SCF plus NECA) to control was by two-sided Wilcoxon test. To allow for the fact that a multiple test was performed *P* values were adjusted according to Bonferroni–Holm.

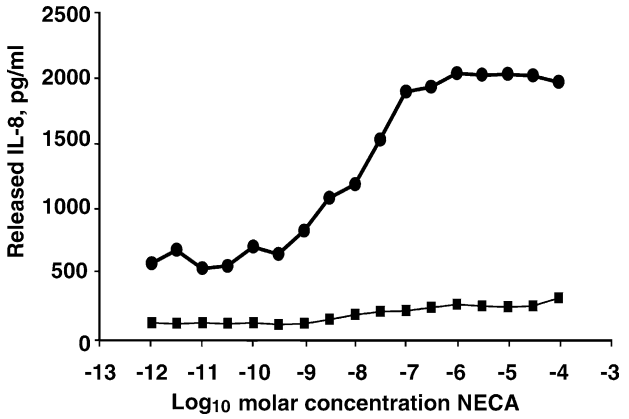

Fig. 1. Interleukin 8 production after 24 hr exposure of HMC-1 cells to the stated concentrations of NECA in the presence of 100 ng/mL SCF (●), and in absence of SCF (■). Each point is the mean of duplicate readings on two separate wells. Values for IL-8 production in the absence of NECA were 80 pg/mL in the absence and 560 pg/mL in the presence of 100 ng/mL SCF. The graph shows one of three experiments, each of which gave similar results.

3. Results

3.1. Response of HMC-1 cells to NECA in the presence and absence of SCF

In the conditions of our assay, and in the absence of SCF, NECA had only a small effect on IL-8 release from HMC-1 cells. However, in the presence of 100 ng/mL SCF, NECA was able to dose-dependently increase production of this cytokine (Figs. 1 and 2). SCF alone was able to cause some stimulation of IL-8 from HMC-1 cells, but when NECA was present the concentrations required for stimulation were considerably lower and the maximum attainable effect was

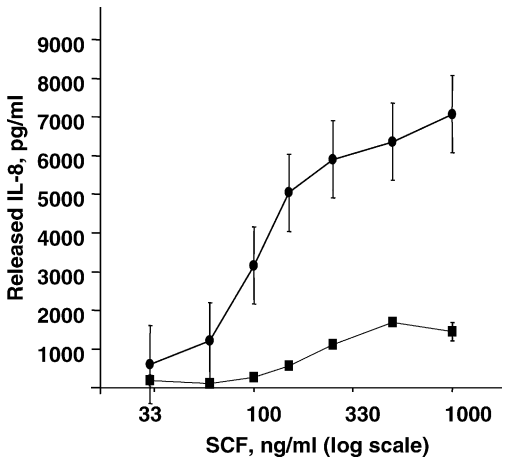

Fig. 2. Interleukin 8 production after 24 hr exposure of HMC-1 cells to the stated concentrations of SCF in the presence of 3 μM NECA (●), and in the absence of NECA (■). Each point is the mean (with standard deviation) of four readings. Values for IL-8 production in the absence of SCF were 62 pg/mL in the absence and 307 pg/mL in the presence of 3 μM NECA. The graph shows one of two experiments, each of which gave similar results.

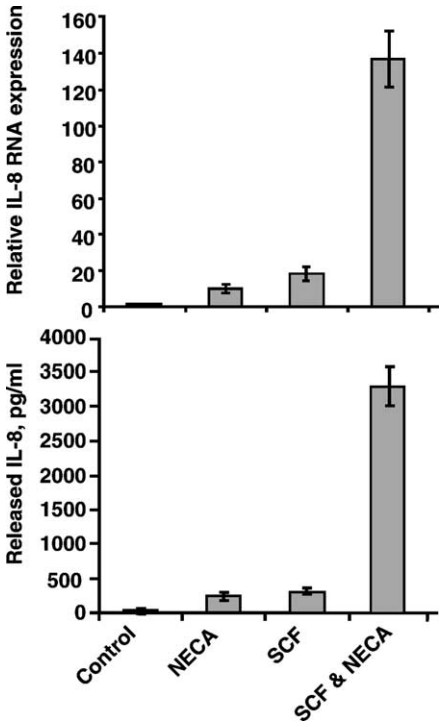

Fig. 3. Production of IL-8 protein (measurement at 24 hr) and cell content of IL-8-specific mRNA (measurement at 4 hr) in HMC-1 cells incubated with 3 μM NECA, 100 ng/mL SCF or a combination of the two. Each bar is the mean with standard deviation of measurements on five wells. The difference between IL-8 protein or specific RNA produced when NECA plus SCF were given together and the sum the values when NECA or SCF were given alone was significantly different from zero, $P < 0.001$ in both cases, modified Welch test. The graph shows one of two experiments, each of which gave similar results.

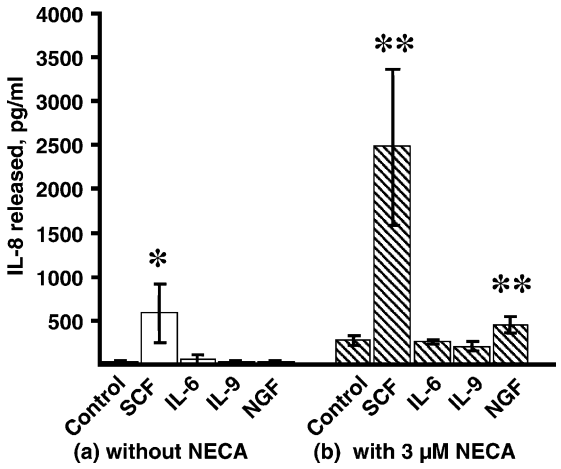

Fig. 4. IL-8 production following 24 hr incubation of HMC-1 cells (a) without NECA (open bars) or (b) with 3 μM NECA (shaded bars) in the absence of added cytokine (control) or in the presence of 100 ng/mL SCF, IL-6, IL-9 or NGF. Each bar is the mean, with standard deviation, of duplicate determinations on eight separate wells. Comparisons were made between treatment with cytokine and treatment with vehicle alone, and for treatment with cytokine plus NECA, with treatment with NECA alone $^{*}P < 0.05$, $^{**}P < 0.01$ (Welch test). In a further test (for synergy) the difference between IL-8 produced when NECA plus SCF were given together and the sum of the values when NECA or SCF were given alone was significantly different from zero ($P < 0.01$, modified Welch test). The graph shows one of two experiments, each of which gave similar results.

considerably greater. Synergy between SCF and NECA for IL-8 production could be demonstrated not only at the level of protein but also at the level of mRNA (Fig. 3). IL-6 or IL-9 tested at 100 ng/mL did not synergise with 3 μM NECA as 100 ng/mL SCF did (Fig. 4), nor could a biologically significant effect be seen when doses of IL-6 or IL-9 from 10 to 2000 ng/mL were tested (data not shown). NGF did show a small effect at 100 ng/mL, but this did not approach the magnitude of the effect seen with SCF, and when concentrations of NGF from 10 to 2000 ng/mL were tested, no greater effect was seen at any of the other concentrations. HMC-1 cells could not be made to show increased response to NECA by prior incubation for 10 days with the L-cell supernatant even though this supernatant did cause cell differentiation as evidenced by more than doubling of the mean tryptase content/cell. There was no significant difference ($P > 0.05$, Wilcoxon, two studies each $N = 5$ treatment group) between L-cell supernatant pre-treated and non-L-cell supernatant pre-treated cells when cell-associated IL-8-specific RNA relative to ribosomal RNA was measured after 4 hr exposure to 3 μM NECA. IL-8 protein release after 24 hr exposure was also not significantly increased by L-cell supernatant. However, when the same, L-cell supernatant-differentiated cells which had shown a poor response to NECA were also exposed to 100 ng/mL SCF, a good response to NECA was observed.

3.2. Identification of the adenosine receptor type interacting with SCF

PCR suggested mRNA for all four adenosine receptor subtypes (A_1, A_{2a}, A_{2b} and A_3) were expressed in HMC-1 cells (Fig. 5). The band for the A_1 receptor was much weaker than that for the A_{2b} receptor. The bands for the A_{2a} and A_3 receptors appeared marginally weaker, but from the gel alone differences were not easy to distinguish. Quantitative real time PCR provided a more precise way to compare RNA copy numbers. This indicated that A_{2b} receptor RNA was about eight times more abundant than A_3 receptor RNA (Fig. 6). It further suggested that A_{2a} receptor RNA was present in about the same amount as the A_3 receptor RNA, whilst A_1 receptor RNA was only present in trace amounts. The existence of A_{2b} and A_3 receptors was confirmed by sequencing the amplified cDNA, and by fluorescence-activated cell sorting using antibodies against human adenosine A_{2b} and A_3 receptors.

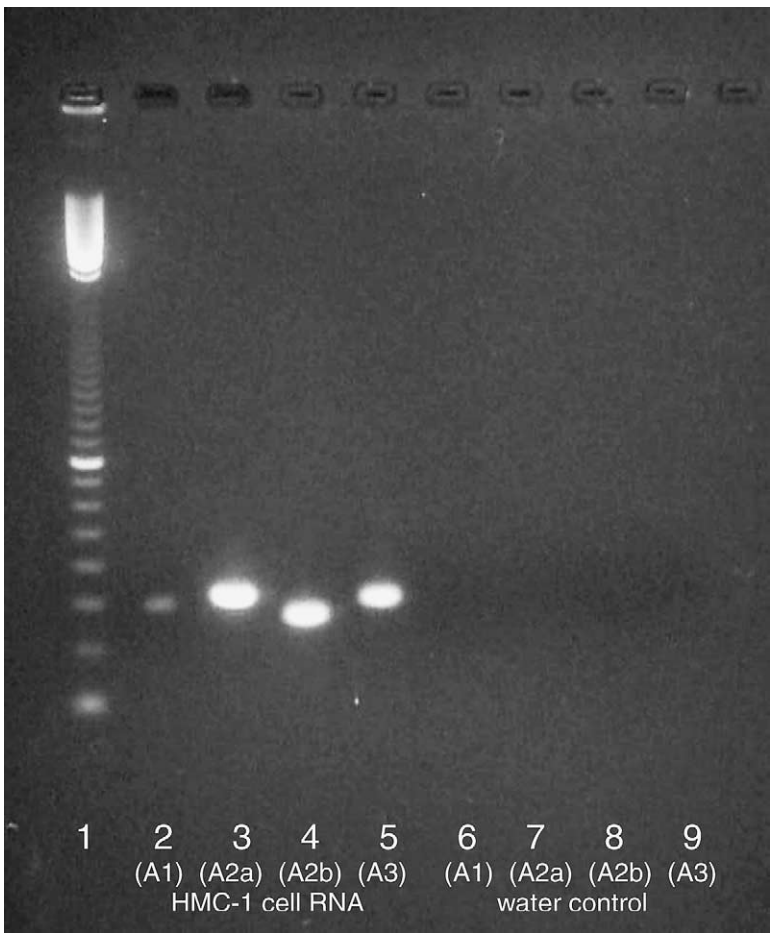

Fig. 5. Gel, ethidium bromide staining. Lane 1: 100 base ladder; lane 2: 3 μg cDNA from HMC-1 cell RNA, amplified with A_1 receptor primers; lane 3: the same amount of HMC-1 cell cDNA as lane 2, but amplified with A_{2a} receptor primers, lane 4: A_{2b} receptor primers; lane 5: A_3 receptor primers, lanes 6–9 as lanes 2–5 but replacement of the HMC-1 cell RNA during PCR amplification by water (control).

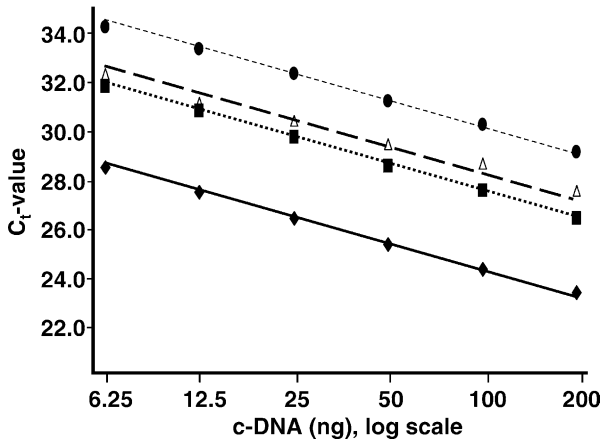

Fig. 6. Quantitative PCR to compare RNA specific for the different adenosine receptor subtypes in cDNA prepared from HMC-1 cell RNA. The parameter C_t (threshold cycle) describes the PCR cycle at which an increase in reporter fluorescence above baseline signal can first be detected. Each point on the graph represents the mean of three measurements of C_t: (◆) A_{2b}; (■) A_3; (△) A_{2a}; (●) A_1.

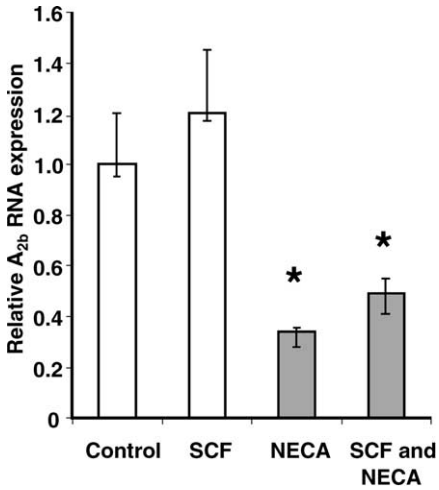

Fig. 8. Cell content of adenosine A_{2b} receptor-specific mRNA in HMC-1 cells incubated for 4 hr with 3 μM NECA, 100 ng/mL SCF or a combination of the two. Each bar is the median (with quartiles) of measurements on five wells (NECA-treated groups shaded). (*) significant difference to control, $P < 0.05$, Wilcoxon. Results show one of two experiments, each of which gave similar results.

The adenosine A_3 sequence amplified in our study was identical to that described in the literature [6–9]. The adenosine A_{2b} sequence amplified using the Titan™ One-Tube Kit differed by one nucleotide from the published sequence [10], there being a cytosine at position 722 instead of the thymine in the published sequence (accession number M97759.1). However, when the sequencing run was repeated using a PCR product amplified from a different cDNA, that had been synthesised with another reverse transcriptase (Omniscript), the sequence amplified by the A_{2b} receptor PCR primers was identical to the published sequence. In the FACS study (histograms, Fig. 7), the A_3-specific fluorescence was relatively modest. The mean fluorescence obtained with FITC-labelled antibody was 40% greater than with same antibody after blocking with specific peptide (N = 3). Using a sandwich technique with first layer rabbit anti-human A_3 receptor and second layer FITC-labelled goat anti-rabbit IgG, the mean fluorescence was 34% above the isotype control (N = 3). The fluorescence with antibody directed against the A_{2b} receptor peptide was 53% higher (mean, N = 6) than with antibody against an unrelated antigen (control, ovalbumin). A 24 hr exposure to SCF had little effect on expression of either adenosine A_3 receptor protein or adenosine A_{2b} receptor protein (mean change in specific cell fluorescence <5% in both cases). The increase in the expression of adenosine A_{2b} receptor mRNA following SCF treatment of undifferentiated HMC-1 cells was only very small and not statistically significant (Fig. 8). The effect of NECA was actually to reduce adenosine A_{2b} receptor mRNA expression. When a series of adenosine agonists with different receptor specificity were tested in

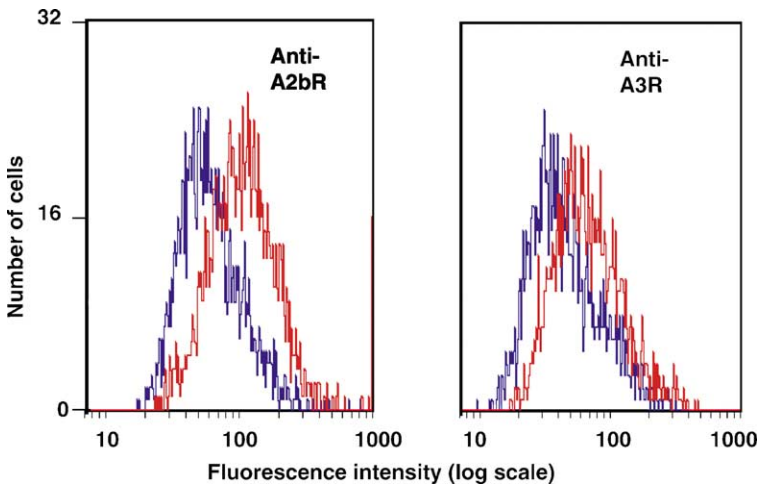

Fig. 7. Typical fluorescence intensity histograms of HMC-1 cells stained as a first layer with (left hand panel, red) a rabbit antibody to the A_{2b} receptor or (right hand panel, red) a rabbit antibody to the adenosine A_3 receptor. The second layer was an FITC-labelled antibody to rabbit IgG. The blue line shows the isotype control.

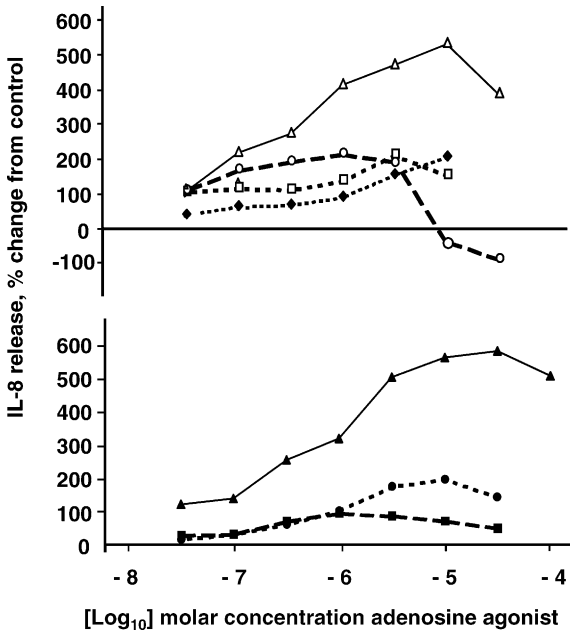

Fig. 9. Change in interleukin 8 production by HMC-1 cells in response to exposure to different concentrations of adenosine receptor agonists over 24 hr. Cells were exposed in the presence of 100 ng/mL SCF. Interleukin 8 release into the culture supernatant is expressed as a percentage of interleukin release with medium containing 100 ng/mL SCF and the same amount of vehicle (DMSO, 0.10 N NaOH or water) used to solubilise the agonist. Each point is calculated from measurements on four test and four control readings (duplicate measurements on two separate wells). For solubility reasons, not all concentrations could be tested with all agonists. For reasons of clarity, the results are divided into two graphs. Lower graph: (▲) NECA, (●) APNEA, (■) CCPA; upper graph: (△) CPCA, (□) CGS 21680, (○) IB-MECA, (◆) I-AB-MECA.

the presence of 100 ng/mL SCF, only NECA and CCPA were potent releasers of IL-8 (Fig. 9).

4. Discussion

This work confirms and extends previous reports that activation of the adenosine A_{2b} receptor on the HMC-1 cell line can lead to increased release of IL-8 [4,11]. In our hands good response was seen only when an appropriate cytokine environment (in particular an adequate concentration of SCF) was present. The failure to see this requirement in the study published earlier by Feoktistov and colleagues may be a reflection of a difference in the batches of serum used for cell maintenance, since sera can contain endogenous cytokines.

At concentrations of 3 μM or above, the effect upon IL-8 production of further increasing NECA concentration was small (Fig. 1), and it was therefore, this optimal concentration was used in all subsequent studies. The concentration of SCF used in most of the subsequent studies was 100 ng/mL which was not maximal, but selected because it was the concentration at which good effects on IL-8 production could be observed in the absence of NECA but only modest effects without NECA (Fig. 2).

Our work further extends the previous study by demonstrating (Fig. 3) that increased IL-8 release is mirrored by markedly increased levels of IL-8 mRNA (implying regulation at the level of transcription or RNA stability). We identified A_{2b} receptors not only functionally, by cytokine release (only agonists with reasonable A_{2b} receptor affinity were good stimulators of release), but also at the protein and mRNA levels. In one of the two sequencing studies, we did find a different base at position 722 from that described in the literature for A_{2b} receptors from brain. As sequencing was double stranded, this is unlikely to be a sequencing error. Probably, a replication error occurred during the reverse transcription used to provide the first batch of cDNA.

We observed (Fig. 9) that CPCA and NECA were good stimulators of IL-8 release but, for example, CCPA and IB-MECA were poor stimulators. In binding studies (unpublished results, CEREP), CPCA and NECA showed relatively strong binding to the human A_{2b} receptor (the values of K_i ca. 0.3 μM) whereas in the same test system binding of e.g. CCPA or IB-MECA to the A_{2b} receptor was too weak to measure even at the highest concentration tested (10 μM). However, at the A_1 receptor CCPA (K_i 53 nM) had a slightly higher binding affinity than NECA (K_i 85 nM) and a much higher affinity than CPCA (ca. 3 μM). Further, at the A_3 receptor IB-MECA (K_i 3 nM) had far greater affinity than CPCA (K_i 200 nM) or NECA (K_i 22 nM). The adenosine receptor agonists that were the best stimulators of IL-8 release were the agonists with the strongest binding to the A_{2b} receptor and not the agonists with the strongest binding to the A_1 or A_3 receptors. We, therefore, agree with a previous report [11], that the primary adenosine receptor involved in the activation of HMC-1 mast cells to release IL-8 is the A_{2b} receptor. Our observations also accord with the recent observation that a potent adenosine A_{2b} receptor antagonist, 3-isobutyl-8-pyrrolidinoxanthine, is a good inhibitor of IL-8 release from HMC-1 cells [12].

This observation, however, contrasts with studies in rats and rat mast cell lines where the A_3 receptor has been proposed to have an important role [13–16]. Our studies showed that A_3 receptors were present in HMC-1 cells. They could be identified both at the mRNA level (by PCR, cloning and sequencing) and at the protein level (by immunofluorescence). The failure of adenosine human A_3 receptor agonists to significantly stimulate the HMC-1 cell line must either reflect a quantitative difference (too few A_3 receptors in comparison to A_{2b} receptors) or the A_3 receptors present must for some reason not be adequately functional. The very weak staining of the A_3 receptor on the HMC-1 line in the FACS study could be a result of a low level of protein expression, or reflect a lack of antibody potency. In favour of the former explanation is the observation of much better staining (213% increase relative to blocked control) with the same FITC-labelled antibody mixture as used in this study, and the eosinophil cell line, EOL-3 (unpublished result). It may seem paradoxical that the principle receptor involved in HMC-1 cell activation is

the A_{2b} receptor, even though this has a much lower affinity than the A_3 receptor for NECA and CPCA. However, binding affinity is not the only criterion for determining receptor activity. Receptor density and receptor coupling are also important.

Although the adenosine A_{2a} receptor is structurally related to the adenosine A_{2b} receptor, it is coupled to other transducing mechanisms. CGS 21680, though a high affinity ligand of the A_{2a} receptor (K_i 62 nM), showed no measurable binding to the A_{2b} receptor, and was unable to stimulate the HMC-1 cells to release IL-8 even in the presence of SCF.

Our results not only showed the importance of the A_{2b} receptor, but also that specifically the cytokine SCF greatly enhanced the adenosine A_{2b} receptor-mediated release of IL-8 from the human cell line HMC-1. We observed (Fig. 4) that the effect of SCF was very much greater than the effects of IL-6, IL-9 or NGF. We selected these other cytokines to test, because in previously published studies they had been shown to modulate various mast (or HMC-1) cell functions, including differentiation [17–19]. IL-6 and IL-9 were completely inactive under our test conditions. The effect of NGF was only marginal, and perhaps because the effect was so close to background variability, we were not able to demonstrate a clear dose–response relationship for this cytokine. It is questionable whether the effect we observed with NGF has any biological significance. Our observed effect of SCF is the first time that synergy between a human adenosine receptor and a human cytokine on mast-like cells has been reported. It is of particular importance because bronchial smooth muscle cells produce SCF [20], and because in individuals sensitised to common allergens there is an infiltration by mast cells of the bronchial smooth muscle [21]. Proximity of the two cell types could perhaps alter reactivity of the mast cells from asthmatics to adenosine *in vivo*. A requirement for SCF may possibly at least in part explain why previous *in vitro* studies with lung cells isolated with the aid of proteases have found it difficult to demonstrate reactivity of isolated human mast cells to adenosine unless some kind of co-stimulus was also present, whereas cells isolated without the use of proteases from bronchoalveolar lavage fluid were reactive [22].

Not only did we measure IL-8 production (at 24 hr after challenge) but also IL-8-specific mRNA production (at 4 hr). The particular time points were chosen because, in preliminary experiments (not shown), we got good production of either IL-8 protein or IL-8 mRNA at these times, without the system having reached a ''plateau.'' The synergy, observed at the IL-8 protein level 24 hr after exposure of the HMC-1 cells to the activating stimuli was mirrored by a similar synergy at the IL-8 mRNA level measured 4 hr after exposure. This suggests control at the transcriptional level. Synergy is not simply a function of an overall effect of SCF on maturation and differentiation processes in the HMC-1 cell line. In a previous publication, 10 days treatment with supernatant from the murine L-cell fibroblast line (but not SCF alone) had been reported to induce differentiation in HMC-1 cells [23]. Treatment with this L-cell supernatant, whilst promoting differentiation as evidenced by increased levels of tryptase, did not enhance reactivity to NECA. Exposure to 100 ng/mL SCF had no significant effect on expression of the adenosine A_{2b} receptor assessed at either the mRNA level (measured at 4 hr) or the protein level (measured at 24 hr). We also think it unlikely that the effect of SCF in our system is an effect on cell-survival, since the viability of the HMC-1 cells (even after 24 hr) was high, yet the effect on IL-8 mRNA production occurred after only 4 hr. SCF and the adenosine A_{2b} receptor have common features in their signal transduction pathways, in particular the kinases they activate (e.g. both activate p38 MAP kinase) [24,25]. There may be a possibility of cross-talk, which could contribute to the synergy. Any interpretation in terms of interaction at the kinase level must take into account that the SCF-receptor (c-kitR) in the HMC-1 cell line has a mutation which permits constitutive phosphorylation [26] and thereby activation of at least some c-kit functions.

The particular cytokine we measured in this study (IL-8) is probably more important as a neutrophil rather than an eosinophil chemoattractant and the cell infiltration in the asthmatic lung is characterised by a high proportion of eosinophils. However, IL-8 can act as a chemoattractant for eosinophils from allergic subjects [27,28], and neutrophil infiltration (and IL-8) may play a role in severe persistent asthma [29].

Further, we are cautious in extrapolating from results with an immature mast cell line to mature mast cells. It is, however, attractive to speculate that an interaction between adenosine receptors and SCF could have not only a pathological but also even a physiological role. Whilst mast cell activation in asthma is undesirable, the mast cell activation does play a critical role in immunity to parasites (as also does SCF). Mast cells not only respond to adenosine but also produce the same nucleoside [30] so reactivity to adenosine can provide a means of amplifying a signal initiated by the interaction of an allergen with IgE receptors on the relatively small number of mast cells likely to be able to interact with specific IgE–allergen complexes. The pronounced inhibition, by NECA, of synthesis of new mRNA for the adenosine A_{2b} receptor, as observed in this study, could provide a means of temporal limitation of the proposed signal amplification process. The involvement of SCF in allergic inflammation has been shown in mouse models of allergic asthma in which SCF not only induced mast cell activation as evidenced by histamine release but also eosinophil recruitment into the lung [31,32]. SCF has been identified as a co-factor for IgE-mediated mast cell activation [33]. In man, allergic rhinitis is associated with an increased number of nasal ciliated epithelial cells expressing SCF [34]. Our work suggests a further possible pathway whereby SCF might contribute to the amplification of allergy-related mast cell responses.

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