Modulation of cytokine release by purine receptors in patients with rheumatoid arthritis

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ABSTRACT

Objective. *Since adenosine receptors are known to modulate the release of some inflammatory mediators in control subjects, we have examined the effects of the mixed A1 and A2 adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA) on basal and lipopolysaccharide (LPS)-induced cytokine release in diluted whole blood cultures from rheumatoid arthritis (RA) patients and healthy volunteers.*

Methods. *Twenty-eight patients with rheumatoid arthritis aged 18-75 years gave their voluntary consent to participate and give a blood sample. Basal levels of tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) were measured by ELISA, and whole blood cultures were prepared to assess the effects of LPS activation.*

Results. *Following a 40-hour incubation, activation of adenosine receptors by NECA, added to the cell cultures from rheumatoid arthritis patients, was found to suppress both the basal and LPS-induced release of TNF-α and IL-1β, while causing an increase in the release of both basal and LPS-induced IL-6. In healthy volunteers basal cytokines were undetectable, but NECA alone induced the release of all three cytokines. Stimulated levels of TNF-α were more than double those in patients. In the control blood cultures, NECA suppressed LPS-induced release of TNF-α and IL-1β, but increased IL-6 release.*

Conclusions. *Adenosine receptor stimulation has a differential effect on the release of pro-inflammatory cytokines, and may induce cytokine release in normal subjects. Stimulated release of TNF-α is substantially lower in patients with rheumatoid arthritis than in control subjects, possibly indicating saturation, exhaustion or down-regulation of the release process.*

Introduction

Pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) are believed to contribute to joint destruction and other tissue dysfunction in rheumatoid arthritis (1). The levels of these cytokines are elevated in the joints of arthritic patients (2). Blood cell cultures from patients with rheumatoid arthritis exhibit high levels of IL-1β, TNF-α and IL-6 compared with control blood as a result of the elevated activity of mononuclear cells (3). Inhibition of the effects of pro-inflammatory cytokines forms the basis for several new anti-arthritic treatments: anti-TNF-α antibody therapy (2), and an IL-1 receptor antagonist (4) are proving to be effective in alleviating the symptoms of rheumatoid arthritis.

There is increasing evidence to suggest the involvement of adenosine in rheumatoid arthritis. The anti-inflammatory effects of the disease-modifying anti-rheumatoid drugs methotrexate and sulphasalazine are thought to be mediated by the release of adenosine (5), and recently it has been reported that synovial fibroblasts from rheumatoid patients have elevated adenosine deaminase activity (6). Adenosine A2A receptors have been shown repeatedly to suppress the production of superoxide from human neutrophils (7,8), an effect which further indicates that adenosine may be a possible candidate in the treatment of inflammatory conditions.

The release of these pro-inflammatory cytokines from activated macrophages and neutrophils can be modulated by purine receptors accessible by extracellular adenosine (8), but so far this modulation has been studied only in healthy control subjects. We have now examined the effects of purine receptor activation in patients with rheumatoid arthritis. We have assessed the ability of adenosine receptors to modulate cytokine release both in the basal state and after lipopolysaccharide (LPS) activation of diluted whole blood cultures.

Methods

Patients and recruitment

Twenty-eight patients (22 female/6 male), aged 18–75 years, were recruited from routine rheumatology clinics by a consultant rheumatologist, and rheumatoid arthritis was diagnosed using the American Rheumatism Association revised criteria (9). One patient was diagnosed with disease activity 2, fourteen patients with an activity score

of 3, ten patients with an activity score of 4 and three patients were diagnosed with disease activity 5. All gave written, informed consent to participation in the study. Since pain varies with time of day (10), and patients with rheumatoid arthritis show a significant circadian variation in the levels of IL-6 (11), blood samples were always collected in the morning. In addition, blood was also collected from ten healthy volunteers for comparison.

Blood sampling and cell culture

Blood for cytokine analysis was collected from patients and healthy volunteers into tubes containing heparin to prevent coagulation. The heparinised whole blood was then diluted 1/10 with RPMI 1640 culture medium with L-glutamine and 25 mM HEPES (Invitrogen) containing 1% gentamycin solution (Invitrogen). The diluted blood was aliquoted into 1 ml fractions in 24-well tissue culture plates. LPS from *Salmonella typhimurium* (Sigma Chemical Company) was added at a final concentration of 100 ng/ml to stimulate cytokine production. Basal cytokine release was measured in diluted whole blood cultures with no LPS added. 5'-N-ethylcarboxamidoadenosine (NECA; Sigma Chemical Company) was added at a final concentration of 2 μM to both basal and LPS-stimulated wells to examine the effects of adenosine receptor activation on cytokine release. NECA and LPS concentrations were selected from preliminary concentration/response experiments. All cultures were covered with lids, mixed gently and incubated for 40 hours at 37°C in a CO_2 incubator set at 5% CO_2. Following incubation, the contents of each well were decanted into eppendorf tubes and centrifuged at 3,000 rpm for 10 minutes. The supernatant was then removed and stored at -70°C until required for cytokine analysis.

Cytokine analysis

TNF-α was measured in 200 μl aliquots of culture supernatant using a commercial quantitative sandwich enzyme immunoassay kit (R&D Systems). IL-1β and IL-6 were each measured in 200 μl aliquots of culture supernatant using commercial solid phase enzyme amplified sensitivity immunoassay kits (Biosource). All assays were performed in duplicate.

Statistics

Data are expressed as mean ± 1 SEM. Comparisons between paired values at individual time points were made using a non-parametric Wilcoxon matched pairs test. In all cases a significance threshold of 5% ($p < 0.05$) was employed.

Results

Cytokine levels in rheumatoid arthritis

Basal levels of all three cytokines examined were readily measurable in the supernatants from the patients with rheumatoid arthritis after 40 hours in culture (Fig.1). Incubation with LPS (100 ng/ml) produced a modest increase in the release of TNF-α (21%; $p < 0.05$). LPS stimulation induced a marked increase in the release of IL-1β (141%; $p < 0.001$) and IL-6 (72%; $p < 0.001$) compared with basal levels.

The addition of the adenosine mixed A1 and A2 receptor agonist NECA (2 μM) depressed the production of TNF-α by approximately 80% both in the basal samples and in those where white cells had been activated by LPS ($p < 0.001$). NECA was also able to suppress the basal (33%; $p < 0.001$) and LPS-stimulated (20%; $p < 0.001$) release of IL-1β, while adenosine receptor activation increased the basal (17%; $p < 0.05$) and LPS-induced (19%; $p < 0.01$) production of IL-6 (Fig. 1).

Cytokine levels in healthy volunteers

In healthy volunteer control subjects, basal levels of TNF-α, IL-1β and IL-6 could not normally be detected (Fig. 2).

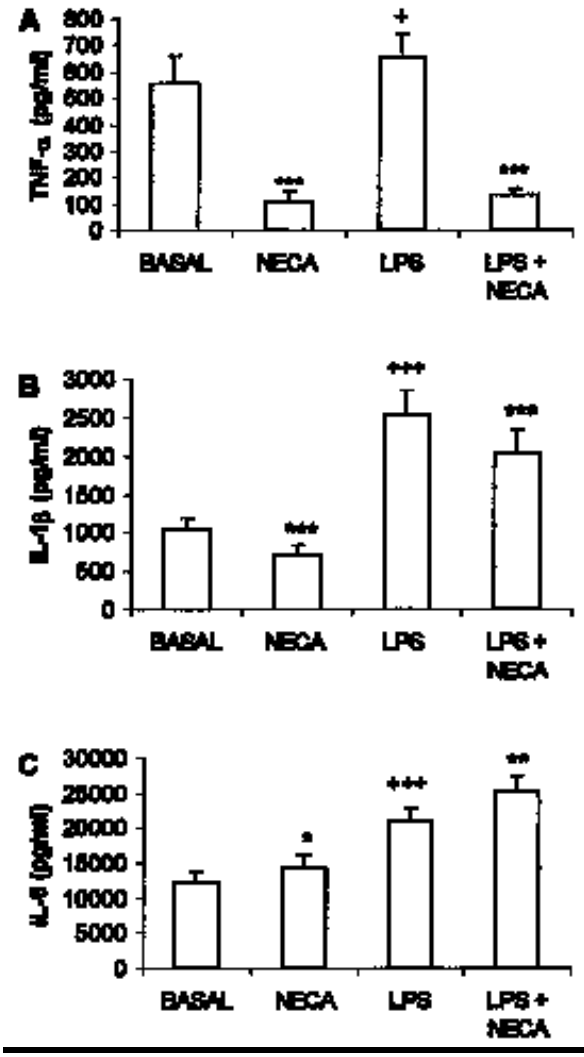

Fig. 1. Levels of cytokines in diluted whole blood cultures from rheumatoid arthritis patients (n = 28) following a 40-hour incubation. Basal and LPS (100 ng/ml) stimulated levels are shown, together with the effects of NECA(2 μM) on basal and stimulated cytokine release. (**A**) TNF-α: ***$p < 0.001$ between basal and NECA, and between LPS and LPS plus NECA; + $p < 0.05$ between basal and LPS. (**B**) IL-1β: *** $p < 0.001$ between basal and NECA, and between LPS and LPS plus NECA; +++ $p < 0.001$ between basal and LPS. (**C**) IL-6 : * $p < 0.05$ between basal and NECA; ** $p < 0.01$ between LPS and LPS plus NECA; +++ $p < 0.001$ between basal and LPS [Wilcoxon matched pairs test].

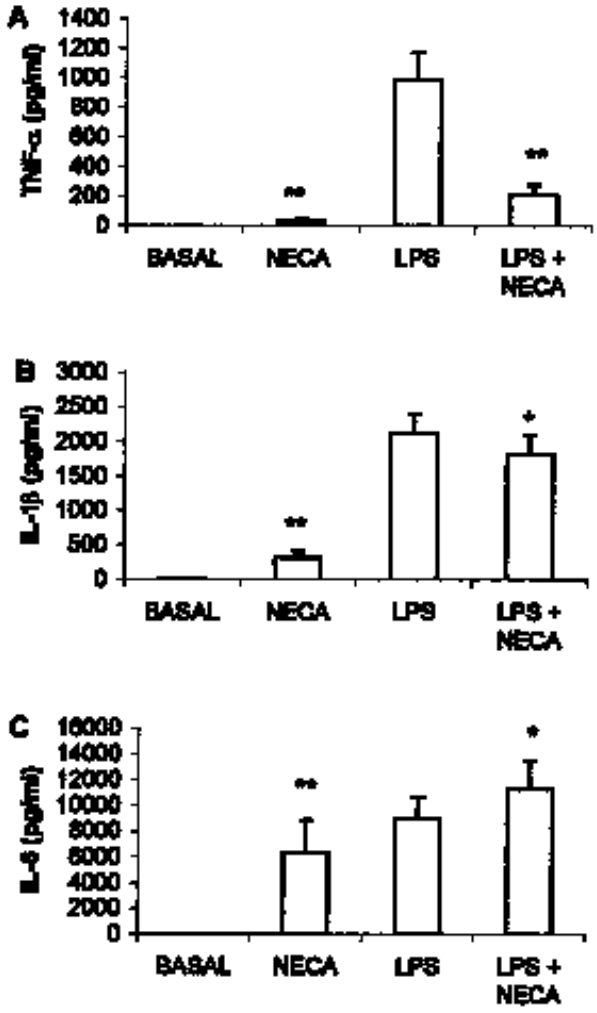

Fig. 2. Levels of cytokines in diluted whole blood cultures from healthy volunteer control subjects (n=10) following a 40-hour incubation. Basal and LPS (100 ng/ml) stimulated levels are shown, together with the effects of NECA (2 μM) on basal and stimulated cytokine release. (**A**) TNF-α: **p<0.01 between basal and NECA, and between LPS and LPS plus NECA. (**B**) IL-1β: **p<0.01 between basal and NECA; *p < 0.05 between LPS and LPS plus NECA. (**C**) IL-6: **p<0.01 between basal and NECA; *p < 0.05 between LPS and LPS plus NECA [Wilcoxon matched pairs test].

However, following incubation with LPS, TNF-α reached levels almost double those seen in patients with rheumatoid arthritis, suggesting an ongoing disease process producing persistent stimulation of this cytokine close to its threshold for activation. Similarly, a marked release of IL-1β and IL-6 was observed following a 40- hour incubation with LPS.

As in the patient samples, NECA at a concentration of 2 μM reduced the LPS-induced secretion of TNF-α (79%; $p < 0.01$) and IL-1β (14%; $p < 0.05$). In the healthy controls NECA increased the LPS-induced release of IL-6 (25%; $p < 0.05$) (Fig.2). Somewhat surprisingly, in these controls but not in the RA patients, NECAalone induced a release of all three of the cytokines studied ($p < 0.01$). The level of IL-6 release by NECA alone represents almost 70% of the levels generated by LPS. At an earlier time point of 5 hours incubation, NECAdid not induce any release of the cytokines, indicating that the effect was not attributable to contamination by LPS (data not shown).

Discussion

The ability of purine receptors to regulate cytokine release is of interest for several reasons. Firstly, inflammatory conditions tend to raise the extracellular levels of adenosine, which might then modify the generation of cytokines and play a role in modulating symptom severity. This in turn may be influenced by dietary factors such as the intake of caffeine, which is an antagonist at both the A1 and A2 subtypes of adenosine receptor. Finally, it is important to know whether adenosine receptors can influence disease symptomatology since there is much interest in developing adenosine receptor agonists, or compounds which increase endogenous adenosine levels such as adenosine kinase inhibitors, as potential therapeutic agents in inflammatory and autoimmune conditions.

In this study we elected to use NECA as the adenosine receptor agonist of choice since it has approximately equal activity at A1 and A2 receptors, and should therefore reproduce the effects of the natural endogenous ligand, adenosine, but without the problems of metabolism and cellular uptake which complicate studies with adenosine itself. The 40-hour incubation was adopted to ensure that the release of all three of the cytokines of interest had reached a stable plateau (12).

The observation that LPS at a concentration of 100 ng/ml gave rise to higher levels of TNF-α in the controls than in the patients suggests that a maximal level of activation had already been achieved in the patients with rheumatoid arthritis. It may be that the lower release in patient samples reflects a compensatory down-regulation of TNF-α production or release as a physiological response to limit disease activity and tissue damage. It is also possible that in the disease state, as well as increased circulating levels of pro-inflammatory mediators, there is a compensatory increase in the release of anti-inflammatory compounds and cytokine receptor antagonists, which limit the TNF-α response. In contrast to TNF-α, however, LPS activation yielded higher levels of IL-1β and IL-6 in the patients compared with the healthy volunteers.

It has previously been shown that adenosine receptor activation can reduce TNF-α production in healthy controls (13, 14) and the present control data is in agreement with this finding. The present results indicate that even under the inflammatory conditions of rheumatoid arthritis, purine receptors are able to induce a profound suppression of basal TNF-α and IL-1β release. In patients with rheumatoid arthritis, serum IL-1β is thought to reflect disease activity, whereas TNF-α may be related to circulating activated monocytes (15). TNF-α and IL-1β are central to the disease process in RA, and compounds which modify their release have major therapeutic potential. The

reduction by NECAof both of these cytokines supports its potential therapeutic value in the treatment of rheumatoid disease.

All three of the pro-inflammatory cytokines studied here are found in rheumatoid synovial membrane. However, only TNF-α and IL-1β have additionally been located at the cartilage-pannus junction where they could exert further damage and possibly hamper repair mechanisms (16). TNF-α is regarded as the controlling element of the cytokine network in rheumatoid arthritis (16). TNF-α induces IL-1 release, which in turn induces other anti- and pro-inflammatory cytokines, including IL-6 (2). It is also worth considering that when TNF-α is blocked no other pro-inflammatory cytokine takes over the dominant role of TNF-α in rheumatoid arthritis (2). The increase in both basal and stimulated pro-inflammatory IL-6 release by NECA suggests the existence of a complex relationship between purine receptor activation and cytokine production. The relevance of the increase in IL-6 seen is unclear. It is possible that IL-6 levels are increased in response to the reduction in TNF-α and IL-1β, in an attempt to maintain a balance of pro-inflammatory molecules. Nevertheless, potential for the use of adenosine compounds in the treatment of RAis evident.

Studying monocytes from normal healthy volunteers, (5) reported that adenosine would inhibit the LPS-induced release of IL-6 and IL-8, although it was less effective than when inhibiting TNF-α release. This difference from the present data may result from our use of diluted whole blood cultures rather than the purified monocytes used by (5). We chose to use diluted whole blood cultures as whole blood is representative of the natural environment and may be the most appropriate milieu in which to study cytokine production *in vitro*. (12) reported that variations in response to LPS stimulation were always higher in separated peripheral blood mononuclear cells in culture than in whole blood cultures.

The finding that NECA could induce the release of all the cytokines in healthy adults after 40 hours of incubation was unexpected. This seems to be an action which either requires time for the establishment of a link between the adenosine receptors and the cytokine release process, or which only operates once the stimulated release of cytokines has reached a stable plateau, since it was not observed at the 5-hour time point. One possible explanation for our data may lie with NECA demonstrating equal potency for adenosine A1 and A2 receptors. A1 adenosine receptor activation has been shown to induce pro-inflammatory responses, whereas A2 receptor activation has a role in mediating the anti-inflammatory effects of adenosine (5). It is possible, therefore, that there is a shift in the balance of activity of these receptor populations in inflammatory conditions, such that the inhibitory A2A receptor activity predominates over the A1 population, to produce comparable suppression of TNF-α release in both rheumatoid arthritis patients and controls as a protective, homeostatic mechanism.

In conclusion, in patients with rheumatoid arthritis and in healthy controls, activation of adenosine receptors by NECA suppressed both the basal and LPS-induced release of TNF-α and IL-1β. IL-6 release was increased by NECA in both basal and stimulated cultures from the rheumatoid patients, and in stimulated cultures from the healthy controls. When applied alone to control blood in culture NECA induced the release of all three cytokines. This study supports the view that adenosine receptor agonists may have a potential therapeutic role in the treatment of rheumatoid arthritis.

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