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Inhibition of tumour necrosis factor-alpha (TNF-α) release from mast cells by the anti-inflammatory drugs, sodium cromoglycate and nedocromil sodium

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SUMMARY

TNF-α is a cytokine thought to be involved in the pathogenesis of asthma and in several other inflammatory conditions. Given recent evidence that mast cells (MC) are an important source of TNF-α, we investigated the effects of two anti-inflammatory drugs, nedocromil sodium (NED) and sodium cromoglycate (SCG), on rat MC-derived TNF-α. We established that at least 2 h pretreatment with NED or SCG followed by washing was required to inhibit TNF-α-dependent cytotoxicity by rat peritoneal MC (PMC). A maximum inhibition of TNF-α occurred after 6 h treatment. The inhibitory effect of NED and SCG (10^{-5}–10^{-3} M) was concentration-dependent (20–37% for NED and 16–37% for SCG). The time-course analysis and the use of cycloheximide, an inhibitor of protein synthesis, provided strong evidence that new protein synthesis by the MC is required for this inhibitory effect. Furthermore, 24 h treatment with 1 mM NED inhibited the levels of mRNA for TNF-α by 59–83%. In addition to the effect on TNF-α-dependent cytotoxicity by MC, 20 min pretreatment with 10^{-4} M NED and SCG inhibited antigen-stimulated TNF-α release (6 h) by 42% and 48%, respectively. Interestingly, the functionally distinct intestinal mucosal MC (IMMC) is unresponsive to these drugs with regard to histamine secretion. However, as with PMC, 2 h pretreatment with NED or SCG inhibited TNF-α-dependent cytotoxicity by IMMC. These effects may be important in the action of these drugs *in vivo* in the late phase reaction in asthma or other inflammatory conditions.

Keywords mast cells TNF-α nedocromil sodium sodium cromoglycate cytotoxicity

INTRODUCTION

Mast cells (MC) are major effector cells in allergic reactions through their IgE antigen-mediated release of mediators such as histamine, serotonin and arachidonate metabolites [1]. The agents sodium cromoglycate (SCG) and nedocromil sodium (NED) are effective in the treatment of allergic disorders such as rhinitis, conjunctivitis, and asthma [2–4]. They protect against exercise-induced asthma, as well as early and late asthmatic responses to inhaled antigen [4,5]. SCG and NED are thought to act, at least in part, by stabilizing MC [6]. However, it is evident that these drugs have several effects, including inhibition of release of some MC mediators and prevention of release of chemotactic and inflammatory mediators by neutrophils, eosinophils, monocytes, and alveolar macrophages [7,8]. Recently, there has been considerable interest in the possibility that their mechanism of action may involve inhibition of chloride flux [9].

SCG is a potent inhibitor of histamine release from rat connective tissue MC when stimulated by IgE cross-linking or by secretagogues such as endorphins and neuropeptides [10–12]. However, SCG shows marked tachyphylaxis (unresponsiveness to a drug following treatment) for histamine secretion by these MC within 1 min of preincubation [13]. In addition, SCG is ineffective in reducing IgE-dependent histamine secretion from certain MC types, particularly the rat intestinal mucosal MC (IMMC) [10]. This difference was also observed in human MC where SCG and NED inhibited histamine secretion from human lung, tonsillar and intestinal MC, but had no effect on histamine release from human skin MC [14].

Cytokines such as IL-1, IL-6 and TNF-α and their receptors are important in the regulation of allergic responses, but the cytokine network is complex and is incompletely understood [15]. Recently, it has been established that the expression of several cytokines is up-regulated in bronchoalveolar lavage fluids or lung biopsies from patients with asthma [16]. TNF-α is one of the cytokines secreted after IgE triggering of sensitized lung tissue [17]. TNF-α is well known to play a pivotal role in inflammation by stimulating neutrophil phagocytosis and degranulation, eosinophil toxicity, production of cytokines such as IL-1, IL-6, granulocyte-macrophage

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colony-stimulating factor (GM-CSF), interferon, and platelet-derived growth factor [18]. TNF-α can also induce an influx of inflammatory cells into tissues by increasing the expression of adhesion molecules on endothelial cells [19]. Given the biological activities of TNF-α, this cytokine may play an important role in pathogenesis of asthma and other allergic and inflammatory diseases [20].

Originally, activated monocytes and tissue macrophages were thought to be the principal cellular source of TNF-α [21]. More recently, however, different types of MC have been shown to secrete different cytokines, including TNF-α [22,23]. Interestingly, a seven-fold increase in the number of MC staining for TNF-α has been observed in asthmatic lung biopsies [24]. Furthermore, IgE-dependent MC activation stimulates the release of TNF-α [25] that has been shown to play a major role in the development of the late-phase inflammatory responses [25,26]. Thus, given the potential role of TNF-α in the pathogenesis of asthma and other allergic diseases and MC as an important source of TNF-α, we investigated the modulation of TNF-α release from MC by two agents known to prevent their activation, namely NED and SCG. In contrast to histamine release, the release of TNF-α by both connective tissue and mucosal MC was inhibited by pretreatment with these drugs, and there was no evidence of tachyphylaxis. Thus, one of the actions of NED and SCG *in vivo* may be to down-regulate the production of TNF-α by MC, an effect potentially important in the inhibition of inflammatory cascades initiated or perpetuated by MC.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were obtained from Charles River Canada Inc. (St-Constant, Canada) and maintained in an isolation room with filter-topped cages to minimize unwanted infections. Rats of 300–400 g were infected 5–6 weeks before MC isolation (both peritoneal MC (PMC) and IMMC) with 3000 third-stage larvae of *Nippostrongylus brasiliensis* by a single s.c. injection. This infection sensitized MC in different anatomic sites to worm antigens, but induced MC hyperplasia, mainly in the intestine [27]. This experimental protocol was approved by our University Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Reagents

NED and SCG were generously provided by Fisons Pharmaceuticals (Loughborough, UK). Actinomycin D, a RNA synthesis inhibitor, was purchased from Calbiochem (La Jolla, CA) and the protein synthesis inhibitor, cycloheximide, was obtained from Sigma Chemical Co. (St Louis, MO).

MC isolation

Peritoneal cells were obtained by lavage of the peritoneal cavity with 15 ml cold HEPES-buffered Tyrode's solution containing 0·1% bovine serum albumin (BSA). Cells recovered were layered on a two-step (30%/80%) discontinuous gradient of sterile Percoll (Pharmacia Ltd, Uppsala, Sweden) as described elsewhere [28]. The purity of recovered PMC was 97–99% and viability always exceeded 97%. IMMC were isolated and enriched using a gradient of Percoll, as described previously [29]. A yield of $2{\cdot}1 \pm 0{\cdot}3 \times 10^6$ IMMC/rat ($n = 35$) is obtained using this procedure [29]. The purity of IMMC in the pellet in 80% Percoll was 67 ± 5% with a viability of 92–95%. The viable contaminating cells were mostly small lymphocytes. However, the viability of the total isolated cells was 68 ± 4% because of the large number of dead epithelial cells.

Cytotoxicity assay

MC cytotoxicity was measured using a ^{51}Cr-release assay. After isolation or treatment with the compounds, MC were suspended in RPMI 1640 supplemented with 5% fetal calf serum (FCS). MC were distributed at different concentrations (25×10^3, $12{\cdot}5 \times 10^3$, and $6{\cdot}25 \times 10^3$ MC) in triplicate in 96-well round-bottomed microtitre plates. TNF-α-sensitive mouse fibrosarcoma target cells ($2{\cdot}5 \times 10^3$ cells), WEHI-164 (obtained from Dr P. Ernst, University of Texas, Galveston, TX), which were labelled previously with 100 μCi of $^{51}CrNaCrO_4$ (Amersham Corp., Arlington Heights, IL) for 90 min and washed three times, were added to the MC in plates. Spontaneous release (SR) corresponds to the ^{51}Cr release from target cells in the presence of RPMI medium without MC. Total releasable (TR) ^{51}Cr was measured by adding 0·01% Triton X-100 to the target cells. Plates were incubated for 16 h, spun (150 ***g***, 5 min), and radioactivity was determined in cell-free supernatants. The percentage of cytotoxicity was calculated by the formula: (ct/min in presence of MC–SR)/(TR – SR) × 100.

Results are expressed in lytic units (LU) for 20% cytotoxicity ($LU_{20}/10^6$ MC) calculated with the program of E. Lattime (obtained from M. Rola-Pleszczynski, University of Sherbrooke, Québec, Canada) using the equations published by Pross *et al.* [30].

Inhibition of RNA and protein synthesis

In experiments with RNA or protein synthesis inhibitors, 0·5 μg/ml actinomycin D (a concentration which completely inhibits RNA synthesis) or 5 μg/ml cycloheximide (90% inhibition of protein synthesis) were added to the cultures 1 h before the addition of NED or SCG. After 2 h treatment with NED or SCG, PMC were washed and incubated for 1 h to recover before being added to the target cells for the cytotoxicity assay. Cell viability of the cultures was determined by trypan blue exclusion test.

Antigen

Antigen used was a collection of soluble excretory and secretory products of adult *N. brasiliensis* prepared by incubating worms in saline buffer at 37°C for 4 h and then collecting the soluble material. Worms were counted and antigen concentration described as worm equivalents (we)/ml.

Antigen-induced release of TNF-α

The effects of NED or SCG were measured on antigen-stimulated TNF-α release from PMC. PMC were incubated in RPMI 1640 medium for 1 h to let them recuperate after the isolation procedure, then they were gently washed and resuspended in fresh medium. Cells were treated with NED and SCG for 20 min before the addition of antigen (five we/ml). After 6 h, cell-free supernatants were collected and the cells were resuspended, frozen, thawed and sonicated on ice (10 s on followed by 20 s off, three times). Both supernatants and cell-associated samples were tested for TNF-α activity using TNF-α-sensitive

target cells, WEHI-164 clone 13 (generously given by Dr T. Mossman, University of Alberta, Canada), in an alamarBlue assay (BioSource International, Camarillo, CA) measuring metabolic activity [31]. Briefly, clone 13 cells were added to different sample dilutions and incubated for 22 h. Then, 20 μl of alamarBlue which incorporates an oxidation-reduction indicator were added to each well and the plate was incubated for an additional 6 h. At the end of the incubation, fluorescent intensity (at 530 nm excitation and 590 nm emission) was measured using a Cytofluor 2350 (Millipore, Nepean, Canada). To quantify the effects of NED and SCG on TNF-α release, dilution curves of samples were compared with a standard curve using human rTNF-α with a probit analysis program [32]. The cytotoxic activity of the samples was inhibited by anti-murine TNF-α neutralizing antibody.

RNA isolation and Northern blot

Total RNA from MC given sham or 24 h pretreatment with NED was prepared as described previously [33]. Total RNA samples (10 μg) were denatured in 50% formamide–6·7% formaldehyde in morpholine propanesulfonic acid (MOPS) buffer (400 mM MOPS pH 7, 100 mM sodium acetate, 10 mM EDTA). After heating for 10 min at 65°C, samples were electrophoresed in a 1·3% agarose gel containing 2·3% formaldehyde in MOPS buffer [34]. RNA was then transferred using a vacuum transfer system (Tyler Research Instruments Corp, Edmonton, Canada) to a Hybond N + membrane (Amersham, Aylesbury, UK) with 50 mM NaOH. Hybridization to a TNF-α 25 mer oligonucleotide labelled with ^{32}P-dATP was performed at 62°C in a solution containing 5 × SSC (0·15 M NaCl, 0·015 M sodium citrate), × 5 Denhardt's solution, 0·1% SDS and 150 mg/ml of denaturated salmon sperm DNA for 16 h [35]. The most stringent wash was performed in 0·1 × SSC, 0·1% SDS for 15 min at 56°C. Filters were then exposed to Kodak X-O-Mat AR film with two intensifying screens for autoradiography. Band intensities were quantified using laser scan densitometry (Abaton 300), corrected for changes in β-actin level and compared with the band intensities obtained from sham-treated cells.

Statistical analysis

Analysis of variance, combined with Fisher's PLSD test or Student's tests for paired data were used to compare treatments. Differences were considered significant when $P < 0.05$.

RESULTS

NED and SCG inhibit rat connective tissue MC TNF-α release

To assess the concentration-response characteristics of NED and SCG on MC TNF-α-dependent cytotoxicity, purified rat PMC were treated with different concentrations of NED or SCG for 24 h. After treatment, cells were washed and incubated with TNF-α-sensitive target cells, WEHI-164, for the cytotoxicity assay (Fig. 1). Significant inhibition of TNF-α-dependent cytotoxicity was observed with 10 μM NED (20 ± 3% inhibition) or SCG (16 ± 3% inhibition). NED inhibition at 1 mM (37 ± 2%) was stronger than with the same concentration of SCG (27 ± 2%), but the difference was not statistically significant. The reduction of PMC cytotoxic activity was not due to diminution of the viability of the cells (> 96% after 24 h) as assessed by trypan blue exclusion.

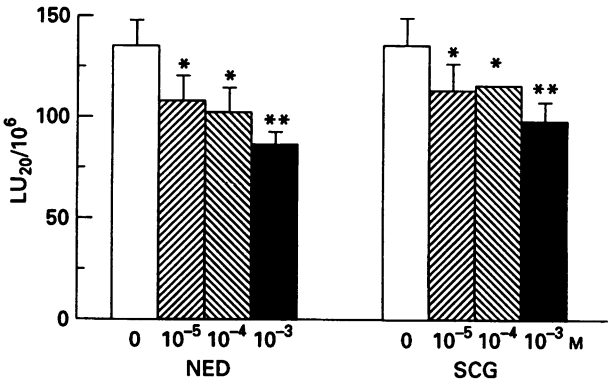

Fig. 1. Inhibition of TNF-α-dependent cytotoxicity by peritoneal mast cells (PMC) (against TNF-α-sensitive target cells, WEHI-164) by anti-inflammatory drugs, nedocromil sodium (NED) and sodium cromoglycate (SCG). PMC were incubated with NED or SCG for 24 h, washed, and added to target cells. A significant inhibition (*$P < 0.05$; **$P < 0.01$) was observed at 10^{-5}–10^{-3} M for both drugs. Each point represents the mean ± s.e.m. of four experiments with three replicates in each experiment. LU, Lytic units.

To investigate the time-course of modulation of PMC TNF-α-dependent cytotoxicity, PMC were preincubated with 10^{-4} M NED or SCG for 2, 4, 6, and 24 h and washed before being added to the target cells (Fig. 2). A significant inhibition was observed after 2 h pretreatment with NED (15 ± 2%) or SCG (16 ± 4%), with a maximum inhibition after 6 h pretreatment (30 ± 11% and 25 ± 10%, respectively). However, the presence of NED and SCG throughout the cytotoxicity assay (time 0) with (data not shown) or without 5 min pretreatment did not significantly inhibit PMC TNF-α-dependent cytotoxicity. Thus, at least 2 h pretreatment of PMC with the compounds was needed to reduce PMC cytotoxic activity.

To determine if NED and SCG modulate antigen-induced release of TNF-α, PMC were incubated for 20 min with the

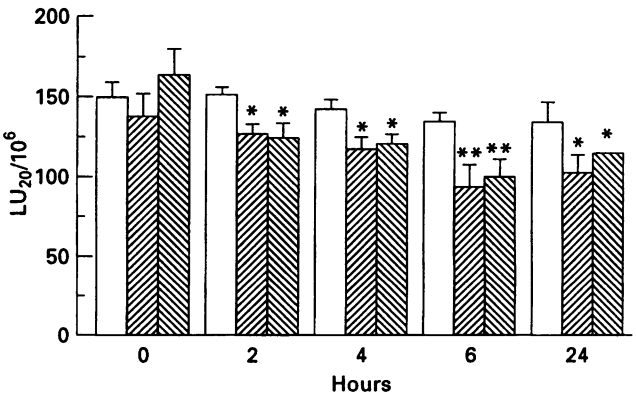

Fig. 2. Time-course of the effect of nedocromil sodium (NED) and sodium cromoglycate (SCG) on peritoneal mast cell (PMC) cytotoxicity. PMC were treated for different periods of time with NED (▨) or SCG (▧) (10^{-4} M), washed and added to target cells. The presence of NED and SCG throughout the assay (time 0) did not inhibit PMC cytotoxicity. A significant (*$P < 0.05$) inhibition was observed after 2 h pretreatment, with a maximum effect at 6 h (**$P < 0.01$). Each point represents the mean ± s.e.m. of three to five experiments with three replicates in each experiment. □, Control. LU, Lytic units.

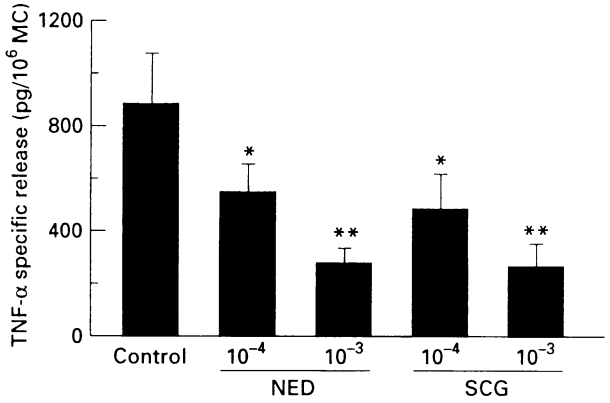

Fig. 3. Inhibition of antigen-induced TNF-α production from peritoneal mast cells (PMC) by nedocromil sodium (NED) (10^{-3} and 10^{-4} M) and sodium cromoglycate (SCG) (10^{-3} and 10^{-4} M) treatment. The spontaneous release of TNF-α has been subtracted from each group. PMC were treated for 20 min before being stimulated with antigen (five worm equivalent ml) for 6 h. Cell-free supernatants were assessed for TNF-α activity. Significant inhibition ($*P < 0.05$; $**P < 0.02$) of antigen-stimulated TNF-α release was observed with NED and SCG treatment. Each point represents the mean ± s.e.m. of five experiments with duplicates in each experiment.

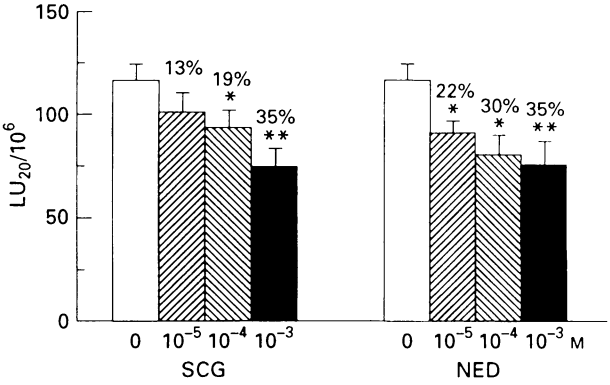

Fig. 4. Inhibitory effect of nedocromil sodium (NED) and sodium cromoglycate (SCG) on intestinal mucosal mast cell (IMMC) TNF-α-dependent cytotoxicity. IMMC were treated for 2 h with different concentrations of anti-allergic drugs, washed, and added to the target cells. A significant inhibition ($*P < 0.05$; $**P < 0.01$) was observed at 10^{-5} 10^{-3} M for NED and 10^{-4} 10^{-3} M for SCG. Each point represents the mean ± s.e.m. of four experiments with three replicates in each experiment. LU, Lytic unit.

drugs before being stimulated with antigen (five we/ml) for 6 h. After antigen exposure, PMC were spun down and cell-free supernatants were tested for their TNF-α content. Figure 3 shows the antigen-specific release of TNF-α by PMC where the spontaneous release (505 pg/10^6 MC) was subtracted from the antigen-stimulated release (1448 pg/10^6 MC). Specific TNF-α release from PMC was significantly inhibited by treatment with 10^{-4} and 10^{-3} M NED (42% and 68%, respectively) or SCG (48% and 66%, respectively) in a concentration-dependent manner. Furthermore, the total cell content of TNF-α (released and cell-associated TNF-α) after antigen stimulation (2531 pg/10^6 MC) was significantly reduced by NED or SCG treatment (1652 pg/10^6 MC and 1347 pg/10^6 MC, respectively). Thus, NED and SCG inhibit PMC TNF-α-dependent cytotoxicity and antigen-induced TNF-α production.

Modulation of mucosal MC-derived TNF-α-dependent cytotoxicity by NED and SCG

Because these drugs do not inhibit IgE-dependent histamine release from IMMC, we investigated the ability of NED and SCG to modulate TNF-α release from IMMC. Based on the results with PMC and the fragility of isolated IMMC in prolonged culture, IMMC were incubated for 2 h with different concentrations of NED and SCG (Fig. 4). After treatment the drugs were washed out and IMMC were added to the target cells WEHI-164. The viability of IMMC (assessed by trypan blue exclusion) in presence or absence of NED and SCG was not different from sham-treated IMMC (82 ± 8%). A concentration-dependent inhibition of TNF-α activity was observed with both drugs, with statistical significance beginning at 10^{-5} M for NED (23 ± 6%) and 10^{-4} M for SCG (17 ± 5%). The magnitude of inhibition of TNF-α from IMMC was similar to the effect of the drugs on PMC.

Inhibition of RNA and protein synthesis

The time required by NED and SCG to inhibit MC cytotoxicity suggested that RNA and/or protein synthesis were required. Inhibitors of RNA (actinomycin D) and protein (cycloheximide) synthesis were added to PMC or IMMC 1 h before the addition of NED or SCG. After incubation of 2 h with the drugs, MC were washed and incubated for 1 h before being added to the target cells (Fig. 5). The presence of actinomycin D or cycloheximide reduced the cytotoxic activity of PMC (Fig. 5a) and IMMC (Fig. 5b), but no further inhibition with NED and SCG was observed. Thus, the inhibitory effect of NED and SCG on MC TNF-α activity requires new RNA and protein synthesis.

Modulation of levels of mRNA for TNF-α by NED

To explore further the mechanisms by which NED inhibits PMC TNF-α release, PMC were incubated for 24 h with and without NED (1 mM). Total RNA was isolated, purified, and analysed by Northern blot for TNF-α (Fig. 6). NED treatment inhibited by 59% and 83% in two different experiments the levels of mRNA for TNF-α. The results were corrected for change in β-actin level, which was 10% and 1%, respectively.

DISCUSSION

It is well accepted that inflammation in the airways is an important component of the pathogenesis of asthma [36]. Drugs such as NED and SCG are an effective treatment of asthma in many patients, but their mechanism of action is unclear. As mentioned above, these drugs show some anti-inflammatory activities such as the inhibition of the release of inflammatory mediators by different cell types, but this is the first time that NED and SCG have been shown to inhibit the release from MC of a potent inflammatory cytokine, namely TNF-α. This inhibition is concentration- and time-dependent, with a maximum effect after 6 h pretreatment, and requires new protein and RNA synthesis. Moreover, NED and SCG inhibit antigen-stimulated TNF-α release from PMC as well as TNF-α-dependent cytotoxicity by MC. Further investigation demonstrated that treatment of PMC with NED reduced the levels of

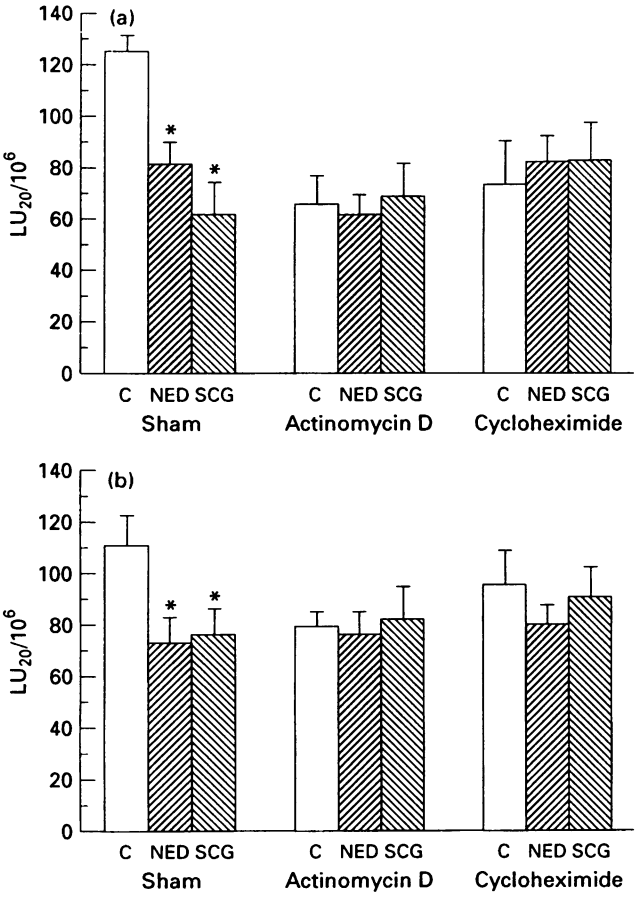

Fig. 5. Effect of RNA and protein synthesis on the modulation of TNF-α by nedocromil sodium (NED) and sodium cromoglycate (SCG). The treatment with RNA (actinomycin D) and protein (cycloheximide) synthesis inhibitors lasted for 3 h (1 h before the addition of anti-allergic drugs and 2 h with the drugs). Cells were washed, rested for 1 h, and added to target cells. No significant inhibition of TNF-α release from peritoneal mast cells (PMC) (a) (n = 5) and intestinal mucosal mast cells (IMMC) (b) (n = 3) was observed with NED (1 mM) and SCG (1 mM) in the presence of either RNA or protein inhibitors. *$P < 0{\cdot}01$. Each experiment was done with three replicates. LU, Lytic unit.

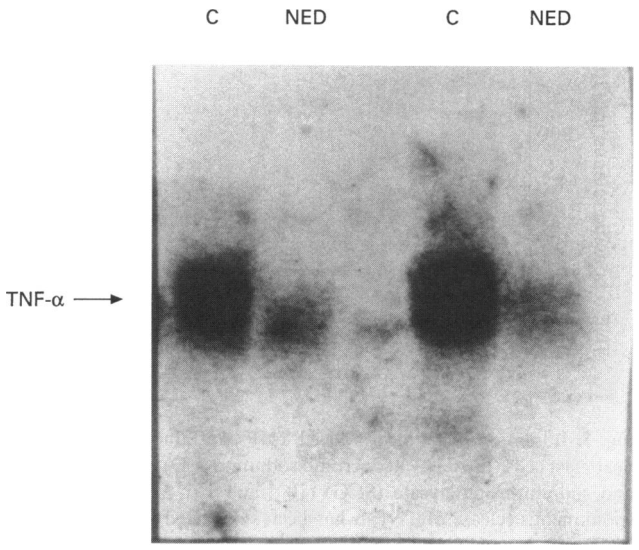

Fig. 6 Inhibition of levels of mRNA for TNF-α by nedocromil sodium (NED). Peritoneal mast cells (PMC) were treated with 1 mM NED for 24 h. Levels of mRNA for TNF-α of treated PMC (NED) were lower than the control (C), but mRNA levels for β-actin did not change. The amount of total PMC RNA per lane was 10 μg (n = 2).

mRNA for TNF-α. Thus, the inhibition of TNF-α release by NED is mediated at least in part by reduction in the steady state levels of mRNA for TNF-α (Fig. 6). TNF-α synthesis may be another target of drug action.

The modulation of TNF-α release by NED and SCG differs considerably from their effects on histamine release. In contrast to the well known tachyphylaxis of these drugs for histamine secretion, no tachyphylaxis for TNF-α was observed following 2–24 h of treatment with NED and SCG. Thus, histamine and TNF-α secretion by MC are modulated differently by NED and SCG. Histamine is a preformed mediator which is released within 10 min after stimulation. The presence of either NED or SCG concurrently with antigen is necessary for inhibition of histamine secretion, establishing that their effects on histamine release are immediate. By contrast, pretreatment with NED and SCG is required to modify the release of TNF-α from PMC. The TNF-α-dependent cytotoxicity of MC involves both stored TNF-α and TNF-α that is newly synthesized following MC activation. In the presence of inhibition of protein synthesis, SCG and NED did not inhibit TNF-α-dependent cytotoxicity of MC, thus suggesting that these drugs act on TNF-α through a mechanism dependent on protein synthesis, perhaps by inhibiting the synthesis of TNF-α itself.

NED and SCG also inhibit antigen-stimulated TNF-α release from PMC. The time course of release of TNF-α when PMC are stimulated with antigen is different than that of histamine. The latter is released within 10 min after antigen stimulation, whereas no significant difference was observed in the release of TNF-α in presence or absence of antigen after 2 h stimulation (data not shown). However, at 6 h a significant increase in TNF-α production and release was observed, and both were inhibited by NED and SCG. These results may explain in part the differences of NED and SCG effects on histamine and TNF-α release by PMC.

The heterogeneity of rat MC is well documented, and it has been shown that IMMC and PMC respond differently to anti-allergic drugs regarding histamine secretion [37]. However, for TNF-α-dependent cytotoxicity, NED and SCG inhibited both IMMC and PMC. We have similar data with interferon pre-treatment of PMC and IMMC, where the release of both histamine and TNF-α from rat PMC were inhibited by IFN treatment, but the same treatment inhibited the release of TNF-α from rat IMMC, but not histamine release [38]. In addition, the release of histamine from rat PMC does not necessarily correlate with TNF-α release [39]. Thus, the modulation of cytokine release from MC by NED and SCG appears to be different from the modulation of histamine release, and the drugs studied appear to act equally well on TNF-α release from MC subtypes. Further investigations are required to confirm these suggestions using MC populations from other tissues and species.

Given the importance of cytokines in the pathogenesis of asthma and other inflammatory diseases, and the observations

that MC are a source of several cytokines [24], therapies which target cytokine release from MC may be useful in the control of inflammatory conditions such as asthma. Interestingly, it has been recently suggested that some of the clinical efficacy of corticosteroids and cyclosporin A may be mediated by the inhibition of MC TNF-α production [40]. Our results suggest that some of the therapeutic effects of NED and SCG depend on their ability to reduce the production and release of TNF-α by MC.

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