

Mechanisms of inhibition of IgE synthesis by nedocromil sodium: Nedocromil sodium inhibits deletional switch recombination in human B cells

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IgE synthesis requires IL-4 and a T cell-B cell interaction that involves the B-cell antigen CD40 and its ligand expressed on activated T cells. Nedocromil sodium (NS), an effective prophylactic agent in asthma, inhibits IgE synthesis by human B cells. In this report we examined the mechanisms of this inhibition. NS targeted the B cells because it inhibited IgE synthesis induced by anti-CD40 and IL-4 in highly purified B cells (>98% CD19⁺). NS had no effect on the induction of ϵ germline transcripts by IL-4 but strongly inhibited CD40-mediated $S\mu \rightarrow S\epsilon$ deletional switch recombination. The effect of NS was not specific for CD40 because it inhibited IgE synthesis in B cells stimulated with hydrocortisone plus IL-4. Moreover, the effect of NS was not specific for IgE because it inhibited CD40/IL-4-driven IgG₄ synthesis by B cells sorted for lack of surface expression of IgG₄. NS caused only modest inhibition of spontaneous IgE synthesis by B cells from patients with hyper-IgE syndrome, suggesting that it has little effect on B cells that have already undergone isotype switching. These results strongly suggest that NS inhibits IgE isotype switching by inhibiting deletional switch recombination and that NS has a novel potential mechanism for the prevention of asthma and other allergic diseases. (J Allergy Clin Immunol 1996;97:1141-50.)

Key words: Allergy, isotype switching, CD40, IL-4, asthma

Nedocromil sodium (NS), a pyranoquinoline dicarboxylic acid, is an antiinflammatory drug that has been proven to be efficacious in the treatment of patients with reactive airway disease and allergic rhinitis.¹ NS has been shown to act on a wide variety of inflammatory cells associated with both early- and late-phase allergic responses.² The precise mechanism by which this drug exerts its clinical activity is still poorly understood.^{3,4} Several studies have demonstrated the efficacy of NS in diminishing the late asthmatic response.⁵ It has also been shown to downregulate the activity of various effector cells in vitro such as monocytes, mast cells, neutrophils, T cells, and eosinophils.^{3,6}

Induction of IgE synthesis in human B cells

Abbreviations used

AET:	2-Aminoethylisothiuronium bromide
mAb:	Monoclonal antibody
NS:	nedocromil sodium
PBMCs:	Peripheral blood mononuclear cells
PCA:	Polymerase chain reaction
PHA:	Phytohemagglutinin A
PMA:	Phorbol myristate acetate

requires two signals. The first signal is delivered by the cytokines IL-4 or IL-13 and results in ϵ germline transcription.⁷⁻¹⁰ A second signal, normally delivered by T cells,¹¹ is required for isotype switching and IgE synthesis. The T-cell signal can be replaced by monoclonal antibody (mAb) to CD40,^{12,13} Epstein-Barr virus,¹⁴ or hydrocortisone.^{15,16} Interaction between CD40 on B cells and its ligand expressed on activated T cells plays an important role in physiologic T-cell-dependent isotype switching for IgE.^{17,18}

We have previously shown that sodium cromoglycate, an antiallergic drug with clinical effects similar to those of NS, although structurally dissim-

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ilar, inhibits IgE synthesis and deletional switch recombination in human B cells.¹⁹ Recently, Kimata and Mikawa²⁰ reported that NS also inhibits human IgE synthesis. The mechanism of this inhibition is not known. In this report we confirm that NS inhibits IgE synthesis by peripheral blood mononuclear cells (PBMCs) from the majority of normal human subjects, as well as IgE synthesis by highly purified human B cells stimulated with anti-CD40 plus IL4. NS had no effect on the induction of ϵ germline transcripts by IL-4 but strongly inhibited CD40-mediated deletional switch recombination. NS also inhibited isotype switching to IgG₄ in B cells. These results strongly suggest that NS acts on B cells to inhibit immunoglobulin class switching.

METHODS

Cell preparation, cell culture, and IgE assay

Highly purified B cells were prepared as previously described²¹ from the blood of nonatopic subjects after informed consent was obtained for a protocol approved by our institutional review board. Briefly, PBMCs were isolated on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and adhered twice on plastic for 1 hour at 37° C in 10% AB⁺ serum to deplete monocytes; nonadherent cells were rosetted twice for 2 and 16 hours, respectively, with 2-aminoethylisothiuronium bromide (AET)-treated sheep red blood cells to deplete T cells, then treated twice with anti-CD3 mAb (OKT3; Ortho Diagnostics, Raritan, N.J.) plus rabbit complement (Pel-Freez Biologicals, Inc., Rogers, Ark.). The B cell-rich populations thus obtained contained more than 70% CD19⁺ cells, less than 1% CD3⁺ cells, less than 5% CD14⁺ cells, and up to 20% CD16⁺ cells and did not proliferate in response to phytohemagglutinin (PHA; 10 μ g/ml) but strongly proliferated in response to phorbol myristate acetate (PMA; 25 ng/ml) plus ionomycin (0.5 μ mol/L). PBMCs were also isolated from two patients with hyper-IgE syndrome whose serum IgE values were ~4,000 U/ml and ~12,000 U/ml, respectively.

PBMCs and B cells were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum (Hyclone Sterile Systems, Inc., Logan, Utah), 2 mmol/L L-glutamine, 100 U/ml penicillin, and 50 μ g/ml streptomycin (complete medium) in the presence or absence of IL-4 (50 U/ml) and anti-CD40 mAb (5 μ g/ml) or pokeweed mitogen (1 μ g/ml). Control cultures for the evaluation of preformed immunoglobulin were set up in the presence of cycloheximide (100 μ g/ml; Sigma Chemical Co., St. Louis, Mo.). In day 10 of culture, supernatants were assessed for their IgE level by a radioimmunoassay with a sensitivity limit of 150 pg/ml.²² IgG₄ levels were determined by a commercially available ELISA (The Binding Site, Birmingham, U.K.) with a lower limit of sensitivity of 2.1 ng/ml.

Reagents

NS, donated by Fisons plc, Pharmaceutical Division (Loughborough, Leicestershire, U.K.) was dissolved in water at 1×10^{-2} mol/L and filtered through a Millipore filter (Millipore Corp., Bedford, Mass.) and was further diluted in complete medium. Human recombinant IL-4 (specific activity 2×10^7 U/mg) was obtained from Amgen Biologicals (Thousand Oaks, Calif.), and the anti-CD40 mAb 626.1 has previously been described.²³ Hydrocortisone, PHA, PMA, concanavalin A AET, and ionomycin were purchased from Sigma Chemical Company. Hydrocortisone was dissolved in ethanol at 1×10^{-2} mol/L, filtered, and stored at -20° C. Hydrocortisone was diluted in complete culture medium before use. Tritiated thymidine and Econofluor were from New England Nuclear (Boston, Mass.). Soluble (s)CD40 and sCD44 were products of fusion of complementary DNA segments encoding the extracellular domain of CD40²⁴ and CD44²⁵ to genomic DNA segments encoding human IgG₁ and were purified on a protein A column.

Cell surface expression of the CD40 ligand

T cells, isolated from PBMCs by rosetting once with AET-treated sheep red blood cells, were left untreated or were stimulated with PMA, 20 ng/ml, and ionomycin, 0.5 μ mol/L. The cells were then suspended in staining buffer and incubated successively for 30 minutes on ice with 20 μ g/ml sCD40 or sCD44 as a control, 10 μ g/ml biotinylated protein G (Calbiochem Novabiochem Corp., San Diego, Calif.), and phycoerythrin-labeled streptavidin (R&D Systems, Minneapolis, Minn.) as previously described.²⁶ Fluorescence was examined by using a FACScan cytofluorometer (Becton Dickinson, Mountain View, Calif.). There was no detectable binding of sCD44 with or without stimulation (data not shown).

Cell sorting

To obtain highly purified B cells, B cell-rich populations of cells were labeled with fluorescein isothiocyanate-conjugated anti-CD19 mAb and sorted by a FACStar Plus flow cytometer (Becton Dickinson). Setting of the markers and analysis of the sorted cells were performed as previously described.¹⁵ Reanalysis of the sorted B cells revealed more than 98% CD19⁺, less than 1% CD14⁺, less than 1% CD16⁺, and less than 1% CD3⁺ cells.

Enriched populations of B cells were depleted of surface IgG₄-bearing B cells by magnetic cell sorting with mouse anti-human IgG₄ mAb (The Binding Site) and magnetic beads coated with goat anti-mouse IgG. Depletion of surface IgG₄⁺ cells was monitored by FACS analysis with mouse anti-human IgG₄ mAb and fluorescein-conjugated goat anti-mouse IgG.

Northern blot analysis

Total cellular RNA was prepared by cell lysis with guanidium isothiocyanate, followed by centrifugation of

the lysate through a cesium chloride step gradient. Northern blot analysis was carried out with 10 µg of total cellular RNA by using phosphorous 32-labeled gel-purified DNA probes. A 0.88 kb *HinfI* fragment, which spans the first two exons of *Cε*, detects both germline and mature *Cε* transcripts. The human cDNA β-actin probe was kindly provided by Dr. Cox Terhorst (Beth Israel Hospital, Boston, Mass.). Prehybridization, hybridization, washing, and autoradiography conditions were as previously described.⁹ Densitometric analysis was performed with an LKB Ultrascan XL densitometer (LKB, Bromma, Sweden).

Polymerase chain reaction primers/amplification of S_μ/S_ε "switch fragments"

High molecular weight DNA was prepared from 5 to 10 × 10⁶ cultured B cells with the A.S.A.P. Genomic DNA isolation kit (Boehringer Mannheim Biologicals, Indianapolis, Ind.). Nested primer polymerase chain reaction (PCR) analysis for S_μ/S_ε "switch fragments" was performed on high molecular weight DNA isolated from cultured B cells with the following four primers as previously described²⁷:

S6:5'-CTGCAGACTCAGAAGGGAGGGGATGCTC
CG-3'
S4:3'-ACTGATCCAAGACAGGAGTGTGGCGGA
TGT-5'
S7:5'-GAGGGTGGTAATGATTGGTAATGCTTTG
GA-3'
S9:3'-GAACCCTGAGGTCCGGTCCCCGCTTCCC
GG-5'

The first round of PCR was performed with primers S6/S4. The second round of PCR was performed on a 5 µl aliquot of the first-round PCR mixture with primers S7/S9. PCR amplification and purification of the amplified fragments were carried out as previously described.²⁷

RESULTS

NS inhibits IL-4-induced IgE synthesis by PBMCs

We first examined the effect of 10⁻⁶ mol/L NS on IgE synthesis induced by IL-4 in PBMCs. We chose this concentration of NS because it is in the range of the peak NS level measured in the serum of patients after inhalation of the drug.²⁸ One micromole NS reproducibly caused strong inhibition (>85% inhibition) of IgE synthesis by PBMCs in 10 of 15 subjects, each tested on at least on two separate occasions. In the remaining five subjects, inhibition of IgE synthesis was less than 50%. Because of this bimodal distribution, we focused the remainder of our studies on subjects whose PBMCs were susceptible to inhibition by NS. Fig. 1 represents the results of five experiments in which

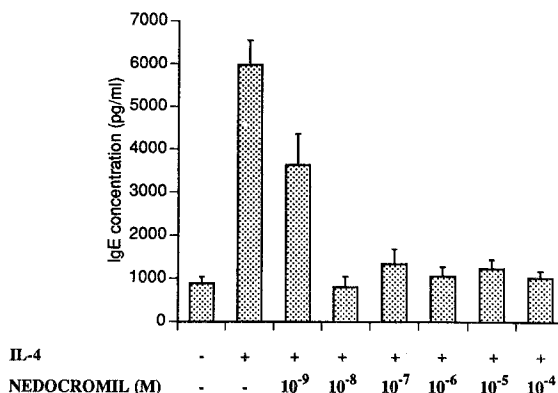


FIG. 1. Effect of NS on IgE production by PBMCs in the presence of IL-4 (50 U/ml). PBMCs at a concentration of 1.5 × 10⁶ cells/ml were cultured for 10 days with different concentrations of NS. Supernatants were harvested after 10 days, and IgE levels were measured by radioimmunoassay. Values represent mean ± SEM net synthesis IgE (picograms per milliliter) of five experiments.

the effect of various concentrations of NS on IgE synthesis was examined. Concentrations of NS of 1 × 10⁻⁸ mol/L and higher caused substantial inhibition of IgE synthesis by PBMCs stimulated with IL-4.

Induction of IgE synthesis by IL-4 in PBMCs is T cell-dependent and involves interaction between the B-cell antigen CD40 and the CD40 ligand expressed on activated T cells. NS had no effect on T-cell viability, as assessed by trypan blue dye exclusion or on T-cell proliferation to anti-CD3 mAb, PHA, the staphylococcal superantigen TSST-1, and tetanus toxoid antigen (data not shown). More importantly, NS (1 × 10⁻⁶ mol/L) did not interfere with CD40 ligand expression by T cells after stimulation by PMA and ionomycin (Fig. 2). These results suggested that the inhibitory effect of NS on IgE synthesis may be exerted at the B cell level.

NS inhibits IL-4/CD40-induced IgE synthesis in B cells

To determine whether NS can exert its effect independently of T cells, we assessed its effect on IgE synthesis induced by anti-CD40 mAb plus IL-4 in a purified B-cell population depleted of T cells, as evidenced by a content of less than 1% CD3⁺ T cells and by failure to respond to an optimal concentration of PHA. Fig. 3, A shows that anti-CD40 mAb 626.1 plus IL-4 induced vigorous IgE synthesis in these B cells. Addition of NS resulted in a dose-dependent inhibition of IgE synthesis by B cells, with ~50% inhibition of IgE synthesis at a concentration of 1 × 10⁻⁸ mol/L NS.

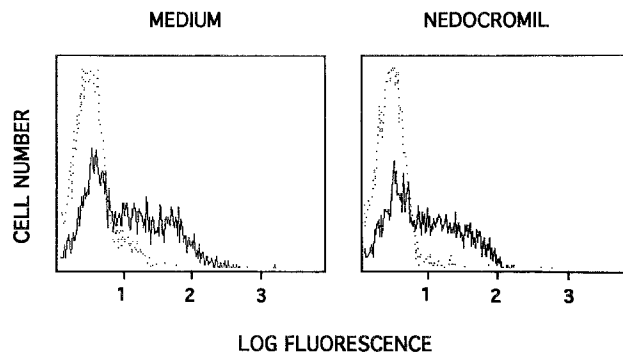


FIG. 2. Cell surface expression of the CD40 ligand. T cells were left untreated (*dotted line*) or stimulated with PMA (100 ng/ml) plus ionomycin (5 μ mol/L) (*continuous line*). CD40 ligand expression with or without NS was assessed at 6 hours.

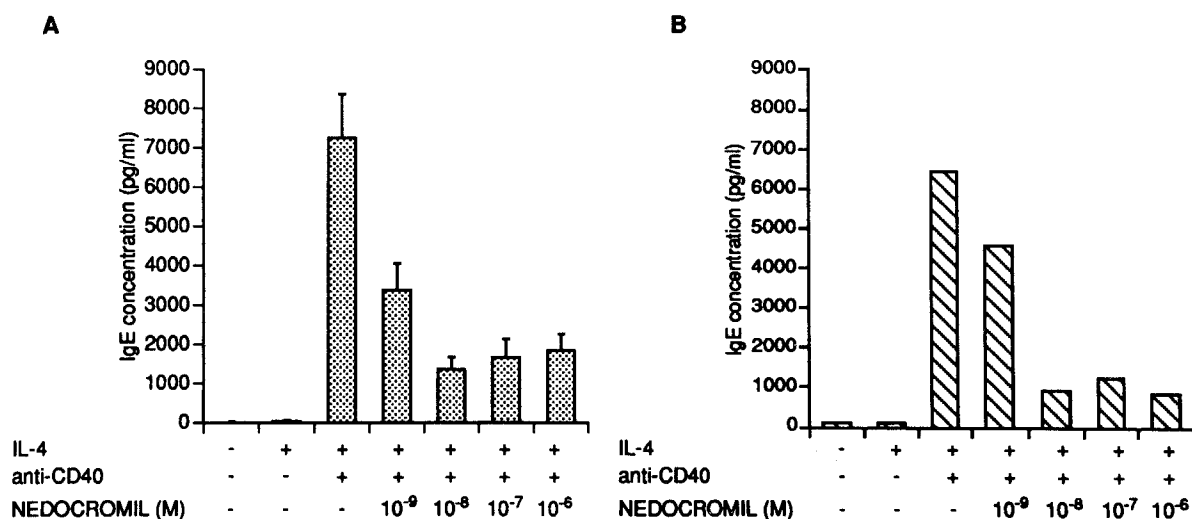


FIG. 3. A, Effect of NS on IgE production by T cell-depleted, B cell-enriched populations of cells in the presence of IL-4 (50 U/ml) and anti-CD40 mAb (5 μ g/ml). Cells were cultured at a concentration of 1×10^6 cells/ml for 10 days with different concentrations of disodium cromoglycate. Supernatants were harvested after 10 days and IgE levels were measured by radioimmunoassay. Results represent mean $\pm$ SEM net synthesis IgE (picograms per milliliter) of three experiments. **B,** Effect of NS on IgE production by highly purified B cells isolated by sorting for CD19 expression. B cells were cultured as described in **A**. Similar results were observed in a second experiment.

To establish unequivocally a direct effect of NS on B cells and to rule out a role for contaminating monocytes and natural killer cells in the standard B-cell preparations, we prepared highly purified B cells by cell sorting for CD19⁺ cells. On reanalysis, these populations consisted of more than 98% CD19⁺ cells and less than 1% CD3⁺, less than 1% CD14⁺, and less than 1% CD16⁺ cells. Fig. 3, *B* shows that NS inhibited IgE synthesis triggered by anti-CD40 plus IL-4 in these B cells. This effect was not due to altered cell viability or to nonspecific toxicity because NS did not inhibit B-cell proliferation to PMA plus ionomycin and to anti-

CD40 plus IL-4 in 4-day cultures; moreover, addition of NS to B cells stimulated with anti-CD40 plus IL-4 to mimic the conditions used for IgE synthesis did not decrease cell viability at the end of the 10-day culture period as assessed by trypan blue dye exclusion (data not shown). These results strongly suggest that B cells are targets for inhibition by NS.

NS does not inhibit IL-4 induction of ϵ germline transcripts in B cells

The effect of NS on IgE synthesis could be exerted at the level of induction of ϵ germline

transcription by IL-4, at the level of CD40-mediated switch recombination, or at both levels. Fig. 4 shows that NS did not affect the accumulation of ϵ germline transcripts in B cells treated with IL-4. It should be noted that although the ϵ probe we have used is able to detect both the ϵ germline transcript and the ~ 200 bp longer mature ϵ transcript, the transcript detected in Fig. 4 is the ϵ germline transcript because it was induced early (4 days), and more importantly it was induced by IL-4 alone. In three experiments the mean ratio of ϵ germline transcript to actin of B cells stimulated with IL-4 in the presence NS was 0.92 ± 0.09 times that of B cells stimulated with IL-4 in the absence of NS, as determined by densitometry. NS by itself did not induce detectable ϵ germline transcripts (data not shown).

NS inhibits CD40-mediated induction of switch recombination in B cells

We have previously shown that addition of anti-CD40 mAb to B cells treated with IL-4 results in deletional switch recombination as demonstrated by nested primer PCR amplification of recombined S_{μ}/S_{ϵ} regions. To determine whether NS interferes with switch recombination, we performed nested primer PCR amplification of recombined S_{μ}/S_{ϵ} regions on DNA isolated from B cells stimulated with IL-4 and anti-CD40 in the presence or absence of NS. We have previously documented, by cloning and sequencing, that the PCR bands obtained in the anti-CD40 plus IL-4 system are indeed S_{μ}/S_{ϵ} switch regions.²⁷ Because different B cells switch or recombine at different sites within the S_{μ} and S_{ϵ} regions, the PCR products from different B-cell clones are of different sizes. In the nested PCR semi-quantitative assay a greater number of PCR products is seen with higher concentrations of template DNA, reflecting the fact that a higher number of B-cell clones are represented in the samples with the higher DNA content. Fig. 5 shows that PCR products were readily visualized in samples containing as little as 10 ng of DNA from B cells stimulated with anti-CD40 plus IL-4, which made ~ 7000 pg/ml of IgE. In contrast, NS-treated B cells, which made 847 pg/ml of IgE, produced no detectable signal in the PCR assay. The nested PCR assay we have used was probably not sensitive enough to detect switch junctions in cultures that made less than 850 pg/ml of IgE.²⁹ It should also be noted that the nested PCR assay is inefficient in amplifying switch junctions that are large in size; the theoretic maximal size of these products is ~ 8 kb.

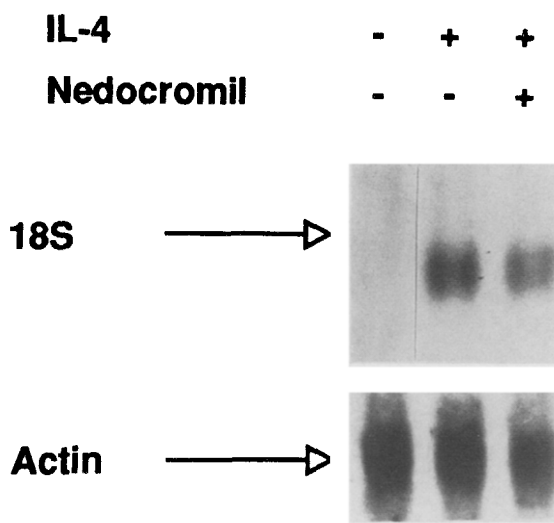


FIG. 4. Effect of NS on the induction of germline ϵ transcripts in B cells stimulated with IL-4. Total RNA (10 μ g) was prepared from normal peripheral blood B cells incubated with medium or IL-4 (50 U/ml) or IL-4 (50 U/ml) and NS (10^{-8} mol/L) for 4 days then electrophoresed on a 1% formaldehyde-agarose gel, transferred to a nitrocellulose membrane, and hybridized to a ^{32} P-labeled 0.88 kb *Hinf*I fragment. Densitometric analysis was performed on germline ϵ transcript mRNA bands versus actin transcript mRNA. The autoradiograph was exposed for 5 days. Similar results were obtained in two additional experiments.

These results strongly suggest that NS inhibits CD40-mediated deletional switch recombination.

NS inhibits hydrocortisone-mediated induction of IgE synthesis in IL-4-treated B cells

It has been previously shown that hydrocortisone plus IL-4 induces IgE synthesis in B cells.²⁸ This is accompanied by deletional switch recombination.³⁰ To determine whether the effect of NS on IgE isotype switching is restricted to CD40, we examined the effect of NS on IgE isotype switching induced by hydrocortisone plus IL-4. Fig. 6 shows that NS inhibited IgE synthesis induced by hydrocortisone plus IL-4 in B cells. The dose-response curve of the inhibition was similar to that seen with anti-CD40 plus IL-4. These results suggest that the inhibitory effect of NS on IgE isotype switching is not specific to CD40.

NS inhibits isotype switching to IgG₄ in B cells stimulated with anti-CD40 and IL-4

Immunoglobulin class switching is thought to involve events that are common to all isotypes, as well as events that are isotype-specific.³¹ It was

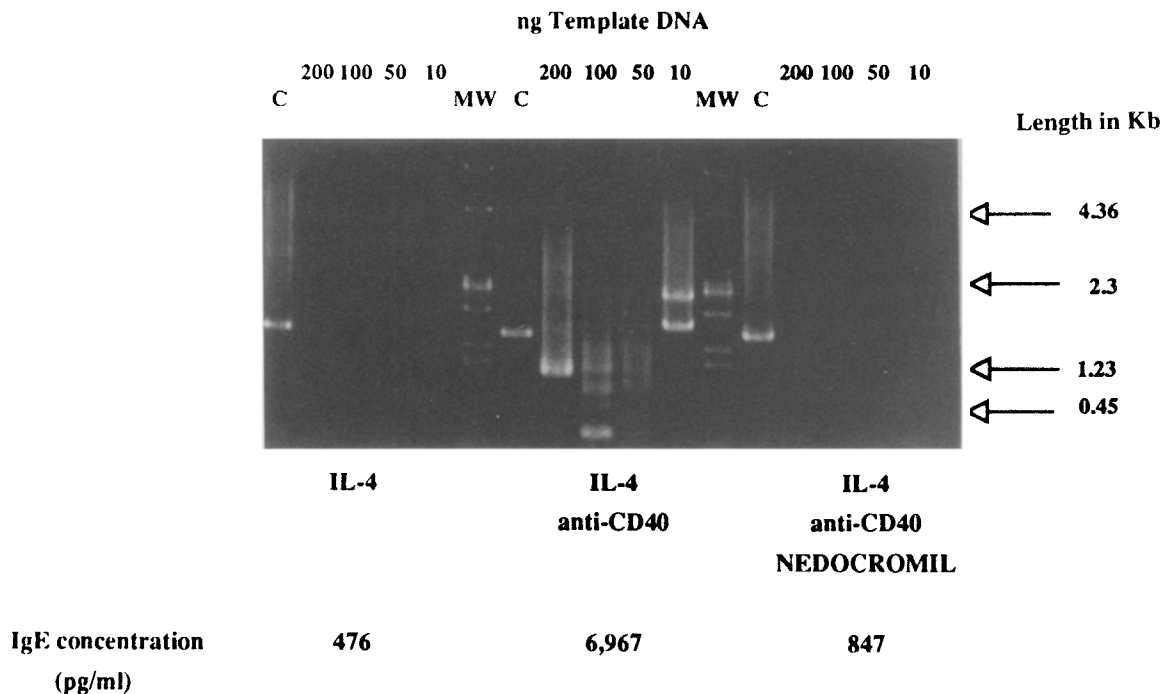


FIG. 5. Effect of NS on the generation of $S_{\mu} \rightarrow S_{\epsilon}$ switch fragments. Serially diluted aliquots of total cellular DNA from B cells stimulated with IL-4, IL-4 plus anti-CD40 mAb, or IL-4 plus anti-CD40 mAb and NS (10^{-8} mol/L) were amplified by nested PCR. The serially diluted template DNA sample amounts used in the first round of PCR are noted above the gel. The second round or nested round of PCR used 10% of the original PCR mixture as DNA template. Final PCR products were subjected to agarose gel electrophoresis. PCR-amplifiable DNA was present in all three samples, as evident by the control (C) band for which primers specific for the human IL-1 β promoter (-1323 ± 72) yield a 1.4 kb fragment by PCR. The higher intensity of the two bands seen in the 10 ng of DNA from control B cells compared with the lower intensity of the numerous bands seen with 50 to 200 ng of DNA from control B cells is probably due to a technical artifact, which may have included lesser competition for the primers in the 10 ng DNA sample. MW, Molecular weight markers.

therefore important to determine whether the inhibitory effect of NS on isotype switching was restricted to IgE or also extended to other isotypes. Because IL-4 also directs switching to IgG₄, we examined the effect of NS on IgG₄ synthesis in B cells stimulated with IL-4 and anti-CD40. To ensure that we were measuring isotype switching, and not merely amplification of immunoglobulin secretion by B cells that had already undergone isotype switching, we used B cells that were magnetically sorted for lack of IgG₄ surface expression. There was no detectable surface expression of IgG₄ in the negatively sorted B cells (data not shown). To ensure that the effect of NS was not exerted at the level of IL-4 induction of γ_4 germline transcription, we preincubated the B cells for 3 days with IL-4 to allow for optimal expression of germline transcripts, then stimulated the cultures with anti-CD40 with or without NS. Fig. 7 shows that unstimulated sIgG₄-B cells secreted no detectable

IgG₄ or IgE into their supernatants. Anti-CD40 plus IL-4 induced the secretion of IgG₄ and IgE, whereas neither of these two agents alone caused IgG₄ or IgE synthesis (data not shown). Because of the low number of B cells recovered after sorting, we could test the effect of only a single concentration of NS, 10^{-6} mol/L. NS inhibited the secretion of both IgG₄ and IgE. In two experiments the mean $\pm$ SD inhibition was $63\% \pm 11\%$ for IgG₄ synthesis and $72\% \pm 13\%$ for IgE synthesis. These results suggest that inhibition of isotype switching by NS is not restricted to IgE.

Effect of NS on IgE synthesis by B cells from patients with hyper-IgE syndrome

PBMCs from patients with hyper-IgE syndrome synthesize large quantities of IgE,³² and their circulating B cells have undergone deletional switch recombination.³³ Table I shows that addition of up to 10^{-6} mol/L NS to PBMCs from

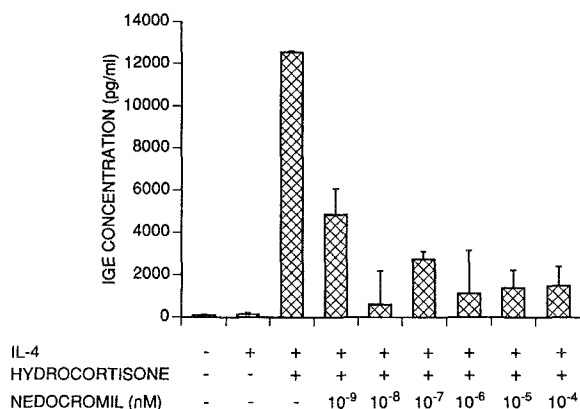


FIG. 6. Effect of NS on IgE production by B cells in the presence of IL-4 (50 U/ml) and hydrocortisone (10^{-6} mol/L). B cells at a concentration of 1×10^6 cells/ml were cultured for 10 days with different concentrations of NS. Supernatants were harvested after 10 days, and IgE levels were measured by radioimmunoassay. Results represent mean $\pm$ SEM net synthesis IgE (picograms per milliliter) of five experiments.

patients with hyper-IgE syndrome resulted in only modest inhibition ($\sim 25\%$) of spontaneous IgE production. These results suggested that NS may only have a modest effect on IgE synthesis by B cells that have already undergone isotype switching.

DISCUSSION

In this article we show that the antiinflammatory drug NS inhibits IgE synthesis in human B cells and that this inhibition resides at the level of deletional switch recombination. These results suggest that NS has a novel potential mechanism for the prevention of allergic disease.

Susceptibility of normal subjects to the inhibitory effect of NS followed a bimodal distribution. In two thirds of the individuals tested 10^{-6} mol/L NS resulted in vigorous inhibition of IgE synthesis by IL-4-stimulated PBMCs. Inhibition in the remaining third of the subjects was weaker. The reason for this bimodal distribution is at present unclear. It may reflect genetic differences in the ability of cellular receptors to bind NS or in the ability of the cells to metabolize the drug. Experiments are in progress to determine whether sensitivity of IgE synthesis to inhibition with NS correlates with clinical responsiveness to NS. It is of interest to note that inhibition of IgE synthesis by sodium cromoglycate also follows a bimodal distribution.¹⁹ The cellular receptors for NS and sodium cromoglycate have not yet been identified. However, there is reason to believe, on the basis of the

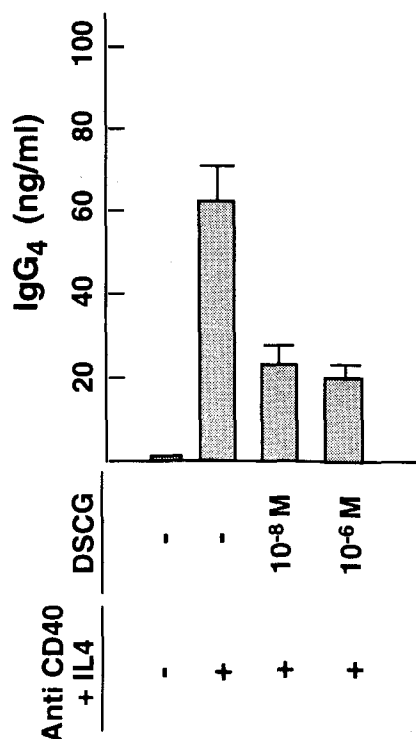
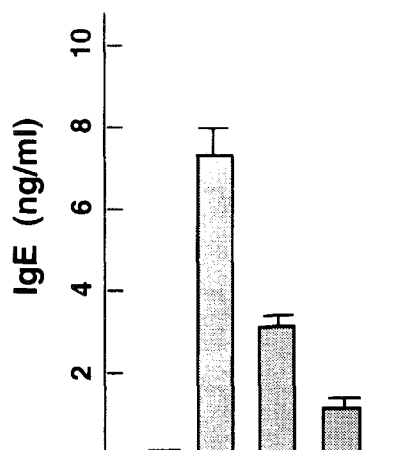


FIG. 7. Effect of NS on IgG₄ production induced in sIgG₄- B cells by IL-4 (50 U/ml) and anti-CD40 mAb (5 μ g/ml). Cells (10^6 cells/ml) were cultured for 3 days with IL-4. IgG₄ and then anti-CD40 were added with or without NS for 10 days. IgG₄ and IgE were measured in the supernatants by ELISA and radioimmunoassay, respectively.

structure of these two compounds, that their receptors may differ. It will therefore be important to determine whether sensitivity of IgE synthesis to inhibition with NS and sodium cromoglycate correlate with each other, which would suggest that

TABLE I. Effect of NS on spontaneous IgE synthesis by PBMCs from patients with hyper-IgE syndrome

NS (mol/L)	Net IgE synthesis (pg/ml)	
	Patient 1	Patient 2
—	12,793	4,051
10^{-9}	12,632	3,912
10^{-8}	9,614	3,106
10^{-7}	9,390	3,313
10^{-6}	9,516	2,921

PBMCs at a concentration of 1.5×10^6 cells/ml were cultured for 10 days in the presence or absence of NS (1×10^{-9} to 1×10^{-6} mol/L). Supernatants were harvested after 10 days, and IgE levels were measured by radioimmunoassay. IgE values represent net IgE synthesis derived by subtracting the IgE values in supernatants of cycloheximide-treated B-cell cultures from the IgE values in supernatants of untreated B-cell cultures. Results for each experiment represent the mean of duplicate cultures.

these drugs may share downstream effector pathways.

Peak plasma levels after inhalation of 4 mg of NS were found to be 3.3 ± 0.7 ng/ml in healthy volunteers and 2.8 ± 0.5 ng/ml in patients with asthma (i.e., $\sim 10^{-7}$ mol/L).²⁸ No information is available on NS levels in bronchoalveolar lavage fluid; however, it is likely that NS levels in bronchoalveolar lavage fluid would exceed blood levels. Given the fact that inhibition of IgE synthesis *in vitro* occurred at concentrations of NS achievable *in vivo*, it is also important to determine whether prophylactic treatment with NS decreases serum IgE and IgG₄ levels or inhibits the development of IgE antibodies.

In the majority of our experiments there was a steep increase in the efficacy of NS in inhibiting IgE synthesis between the 10^{-8} mol/L and 10^{-9} mol/L, rather than a gradual dose-response curve. The reason for this is not clear, and its explanation requires elucidation of the mechanism of action of NS on its cellular targets.

NS did not inhibit the expression of CD40 ligand by T cells stimulated by PMA plus ionomycin (Fig. 2). NS targeted the B cells because it inhibited IgE synthesis induced by anti-CD40 and IL-4 in purified B-cell populations (Fig. 3). These results differ from those of Kimata and Mikawa,²⁰ who found that inhibition of IgE synthesis by NS required the presence of T cells and monocytes. The discrepancy between our results and those of Kimata and Mikawa²⁰ cannot easily be ascribed to contamination of our B-cell preparations with T cells and

monocytes because our standard B-cell preparations contained less than 1% CD3⁺ cells, and our highly purified B cells contained more than 98% CD19⁺ cells, less than 1% CD14⁺ cells, and less than 1% CD3⁺ cells. However, we cannot completely rule out a role for a very small number of contaminating monocytes or T cells in the inhibition of IgE synthesis by NS.

NS had no effect on the induction of ϵ germline transcription in B cells by IL-4 (Fig. 4). In contrast, NS strongly inhibited the CD40-mediated generation of S μ →S ϵ switch fragments in B cells treated with IL-4 (Fig. 5). These data suggest that NS inhibits IgE synthesis in B cells by inhibiting CD40-mediated deletional switch recombination.

NS did not inhibit all CD40-mediated signals. NS did not affect either CD40-mediated B-cell proliferation or CD40-mediated aggregation of B cells (data not shown). These observations suggest that inhibition of IgE synthesis by NS is not specific for the CD40 receptor but may rather target a step involved in IgE isotype switching. This was confirmed by the observation that NS inhibited IgE synthesis in B cells stimulated with hydrocortisone and IL-4 (Fig. 6).

Common and isotype-specific mechanisms are thought to be involved in class switching to different immunoglobulin isotypes.³¹ The presence of common mechanisms for isotype switching is supported by the finding that patients with X-linked hyperimmunoglobulinemia M syndrome, who have been recently shown to have deficient expression of the CD40 ligand on their T cells, fail to switch to all isotypes.³⁴ In agreement with Kimata and Mikawa,²⁰ we found that NS inhibited isotype switching to IgG₄ induced by CD40 plus IL-4 (Fig. 7). This inhibition is unlikely to be due to the effect of NS on IL-4-induced $\gamma 4$ germline transcription because NS inhibited IgG₄ synthesis when added together with anti-CD40 to B cells that were preincubated for 3 days with IL-4 to allow for optimal germline messenger RNA expression. Taken together, these results are consistent with the notion that NS inhibits a common step required for deletional switch recombination.

Spontaneous IgE production by B cells from patients with hyper-IgE syndrome was only modestly inhibited ($\sim 25\%$) by NS (Table I). This suggests that NS may exert an inhibitory effect on immunoglobulin secretion by differentiated B cells that have undergone isotype switching, in addition to its strong inhibitory effect on B-cell isotype switching.

In a previous study we showed that cromolyn

sodium inhibits IgE isotype switching to IgE and IgG₄ in IL-4-treated B cells.¹⁹ The mechanisms of this inhibition paralleled those observed in this study by NS. Both T cell-dependent and anti-CD40-mediated, as well as hydrocortisone-driven IgE synthesis, were blocked by cromolyn sodium. Furthermore, NS inhibited deletional switch recombination but not IL-4 induction of ϵ germline mRNA. Therefore both of these drugs appear to interfere with the pathway necessary for isotype switching. We have shown that switch recombination requires tyrosine kinase activation because it is inhibited by tyrosine kinase inhibitors and by cross-linking of CD40 to the transmembrane phosphatase CD45.^{35,36} It would be of interest to examine the effect of NS and sodium cromoglycate on cellular tyrosine kinases and tyrosine phosphatases.

Recently, it has become evident that NS exerts inhibitory activity on a wide variety of inflammatory cells involved in the allergic response. In vivo, NS inhibited IL-5-induced airway hyperresponsiveness to platelet activating factor and eosinophil migration into the airways.³⁷ In vitro, NS not only inhibits anti-IgE-induced histamine release from mast cells³⁸ and IgG₄-induced eosinophil-dependent basophil degranulation,³⁹ but also inhibits the chemotactic response of human eosinophils pretreated with granulocyte-macrophage colony-stimulating factor to formyl-methionyl-leucyl-phenylalanine and IL-8, as well as the generation of the arachidonic metabolites, leukotriene B₄ and 5-hydroxyeicosatetraenoic acid, by human alveolar macrophages.^{40,41} These in vitro inhibitory effects are exerted at concentrations of NS similar to those we found to inhibit IgE synthesis.

Because NS inhibits events triggered by multiple receptors, it is likely that it interferes with a postreceptor event common to the signaling cascades triggered by these receptors. The inhibitory effect of NS in rat mast cells is associated with rapid phosphorylation of a 78 kd protein⁴² that coincides with the termination of the secretory process.⁴³ It remains to be seen whether a similar protein is phosphorylated by NS in B cells and inflammatory cells.

NS is a water-soluble salt of organic acid with a pK_a value between 2 and 3.²⁸ Thus at physiologic pH values, it is highly ionized and is consequently unable to penetrate cells. For this reason, it is possible that the action of NS involves its association with a membrane receptor. Further study will determine whether NS and sodium cromoglycate, both of which inhibit IgE synthesis in B cells use a

common receptor and/or common biochemical pathways.

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REFERENCES

1. Bauer CP. The protective effects of nedocromil sodium in exercise-induced asthma. *Eur J Respir Dis* 1986;69 (suppl 147):252-4.
2. Gonzalez JP, Brogden RN. Nedocromil sodium: a preliminary review of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy in the treatment of reversible obstructive airways disease. *Drugs* 1987;34:560-77.
3. Church M, Polosa P, Rimmer S. In: Kaliner M, Barnes P, Persson C, eds. *Asthma: its pathology and treatment*. New York: Marcel Dekker, Inc., 1991:561-93.
4. Fink JN, Forman S, Silvers WS, Soifer MM, Tashkin DP, Wilson AF. A double-blind study of the efficacy of nedocromil sodium in the management of asthma in patients using high doses of bronchodilators. *J Allergy Clin Immunol* 1994;94:473-81.
5. Debelic M. Nedocromil sodium and exercise-induced asthma in adolescents. *Eur J Respir Dis* 1986;69 (suppl 147):266-7.
6. Bruijnzeel PLB, Hamelink ML, Kok PTM, Kreunkniet J. Nedocromil sodium inhibits the A23187- and opsonized zymosan-induced leukotriene formation by human eosinophils but not by human neutrophils. *Br J Pharmacol* 1989;96:631-6.
7. Del Prete GF, Maggi E, Parronchi P, et al. IL-4 is an essential factor for the IgE synthesis induced in vitro by human T cell clones and their supernatants. *J Immunol* 1988;140:4193-8.
8. Gauchat JF, Lebman DA, Coffman RL, Gascan H, de Vries JE. Structure and expression of germline ϵ transcripts in human B cells induced by interleukin 4 to switch to IgE production. *J Exp Med* 1990;172:463-73.
9. Jabara HH, Schneider LC, Shapira SK, et al. Induction of germ-line and mature C ϵ transcripts in human B cells stimulated with rIL-4 and EBV. *J Immunol* 1990;145:3468-73.
10. Cocks BG, de Waal Malefyt R, Galizzi JP, de Vries J, Aversa G. IL-13 induces proliferation and differentiation of human B cells activated by the CD40 ligand. *Int Immunol* 1993;5:657-63.
11. Vercelli D, Jabara HH, Arai KI, Geha RS. Induction of human IgE synthesis requires interleukin 4 and T/B cell interactions involving the T cell receptor/CD3 complex and MHC class II antigens. *J Exp Med* 1989;169:1295-307.
12. Jabara HH, Fu SM, Geha RS, Vercelli D. CD40 and IgE: synergism between anti-CD40 mAb and IL-4 in the induction of IgE synthesis by highly purified human B cells. *J Exp Med* 1990;172:1861-4.
13. Zhang K, Clark E, Saxon A. CD40 stimulation provides an IFN- γ -independent and IL-4-dependent differentiation signal directly to human B cells for IgE production. *J Immunol* 1991;146:1836-42.
14. Thyphronitis G, Tsokos GC, June CH, Levine AD, Finkelman FD. IgE secretion by Epstein-Barr virus-infected purified human B lymphocytes is stimulated by interleukin

- 4 and suppressed by interferon γ . *Proc Natl Acad Sci USA* 1989;86:5580-4.
15. Jabara HH, Ahern DJ, Vercelli D, Geha RS. Hydrocortisone and IL-4 induce IgE isotype switching in human B cells. *J Immunol* 1991;147:1557-60.
16. Wu CY, Sarfati M, Heusser C, et al. Glucocorticoids increase the synthesis of immunoglobulin E by interleukin 4-stimulated human lymphocytes. *J Clin Invest* 1991;87:870-7.
17. Armitage RJ, Fanslow WC, Strockbine L, et al. Molecular and biological characterization of a murine ligand for CD40. *Nature* 1992;357:80-2.
18. Fanslow WC, Anderson DM, Grabstein KH, Clark EA, Cosman D, Armitage RJ. Soluble forms of CD40 inhibit biologic responses of human B cells. *J Immunol* 1992;149:655-60.
19. Loh RKS, Jabara HH, Geha RS. Disodium cromoglycate inhibits $S\mu \rightarrow S\epsilon$ deletional switch recombination and IgE synthesis in human B cells. *J Exp Med* 1994;180:663-71.
20. Kimata H, Mikawa H. Nedocromil sodium selectively inhibits IgE and IgG₄ production in human B cells stimulated with IL-4. *J Immunol* 1993;151:6723-32.
21. Vercelli D, Jabara HH, Arai K, Geha RS. Induction of human IgE synthesis requires interleukin 4 and T/B cell interactions involving the T cell receptor/CD3 complex and MHC class II antigens. *J Exp Med* 1989;169:1295-307.
22. Umetsu DT, Leung DY, Siraganian R, Jabara HH, Geha RS. Differential requirement of B cells from normal and allergic subjects for the induction of IgE synthesis by an alloreactive T cell clone. *J Exp Med* 1985;162:202-14.
23. Gruber MF, Bjorndahl JM, Nakamura S, Fu SM. Anti-CD45 inhibition of human B cell proliferation depends on the nature of activation signals and the state of B cell activation. *J Immunol* 1989;142:4144-52.
24. Stamenkovic I, Clark EA, Seed B. A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. *EMBO J* 1989;8:1403-10.
25. Stamenkovic I, Amiot M, Pesando JM, Seed B. A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. *Cell* 1989;56:1057-62.
26. Fuleihan R, Ramesh N, Loh R, et al. Defective expression of the CD40 ligand in X-chromosome linked immunoglobulin deficiency with normal or elevated IgM. *Proc Natl Acad Sci USA* 1993;90:2170-3.
27. Shapira S, Vercelli D, Jabara H, Fu SM, Geha R. Molecular analysis of the induction of IgE synthesis in human B cells by IL-4 and engagement of CD40 antigen. *J Exp Med* 1992;289:292.
28. Neale MG, Brown K, Foulds RA, Lal S, Morris DA, Thomas D. The pharmacokinetics of nedocromil sodium, a new drug for the treatment of reversible obstructive airways disease in human volunteers and patients with reversible obstructive airways disease. *Br J Clin Pharmacol* 1987;24:493-501.
29. Shapira SK, Thienes C, Jabara HH, et al. Deletional switch recombination occurs in IL-4 induced isotype switching to IgE expression by human B cells. *PNAS USA* 1991;88:7528-32.
30. Jabara HH, Loh R, Ramesh N, Vercelli D, Geha RS. Sequential switching from μ to ϵ via $\gamma 4$ in human B cells stimulated with IL-4 and hydrocortisone. *J Immunol* 1993;151:4528-33.
31. Esser C, Radbruch A. Immunoglobulin class switching: molecular and cellular analysis. *Annu Rev Immunol* 1990;8:717-35.
32. Vercelli D, Jabara HH, Cunningham-Rundles C, et al. Regulation of immunoglobulin (Ig)E synthesis in the hyper-IgE syndrome. *J Clin Invest* 1990;85:1666-71.
33. Mills FC, Thyphronitis G, Finkelman FD, Max E. Ig $\mu \rightarrow \epsilon$ isotype switch in IL-4-treated human B lymphoblastoid cells: evidence for a sequential switch. *J Immunol* 1992;149:1075-85.
34. Ramesh N, Fuleihan R, Geha RS. Molecular pathology of X-linked immunoglobulin deficiency with normal or elevated IgM (HIGM-X). *Immunol Rev* 1994;138:87-104.
35. Loh RKS, Jabara HH, Ren CL, Fu SM, Geha RS. Role of protein tyrosine kinases in CD40/interleukin-4-mediated isotype switching to IgE. *J Allergy Clin Immunol* 1994;94:784-92.
36. Loh R, Ren C, Fu SM, Jabara H, Geha R. Role of protein tyrosine kinases and phosphates in isotype switching: crosslinking CD45 to CD40 inhibits IgE isotype switching in human B cells. *Immunol Lett* 1994;45:99-106.
37. Pretolani M, Lefort J, Vargaftig BB. Inhibition by nedocromil sodium of recombinant human interleukin-5-induced lung hyperresponsiveness to platelet-activating factor in actively sensitized guinea pigs. *J Allergy Clin Immunol* 1993;91:809-16.
38. Leung KBP, Flint KC, Brostoff J. Effects of sodium cromoglycate and nedocromil sodium on histamine secretion from human lung mast cells. *Thorax* 1988;43:756-61.
39. Beauvais F, Hiebolt C, Benveniste J. Effect of sodium cromoglycate and nedocromil sodium on anti-IgE-induced and anti-Ig4-induced basophil degranulation. *Drugs* 1989;37(suppl. 1):4-8.
40. Damon M, Chavis C, Daures JP, Crastes de Paulet A, Michel FB, Godard P. Increased generation of the arachidonic metabolites LTB₄ and 5-HETE by human alveolar macrophages in patients with asthma: effect *in vitro* of nedocromil sodium. *Eur Respir J* 1989;2:202-9.
41. Warringa RAJ, Mengelers HJJ, Maikoe T, Bruijnzeel PLB, Koenderman L. Inhibition of cytokine-primed eosinophil chemotaxis by nedocromil sodium. *J Allergy Clin Immunol* 1993;91:802-9.
42. Sieghart W, Theoharides TC, Douglas WW, Greengard P. Phosphorylation of a single mast cell protein in response to drugs that inhibit secretion. *Biochem Pharmacol* 1981;30:2737-8.
43. Wells E, Mann J. Phosphorylation of a mast cell protein in response to treatment with anti-allergic compounds. Implications for the mode of action of sodium cromoglycate. *Biochem Pharmacol* 1983;32:837-42.