

Immunodeficiency and other clinical immunology

Nedocromil sodium inhibits T-cell function in vitro and in vivo

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Nedocromil sodium, a topical antiinflammatory agent recommended as a prophylactic regimen for asthma, is known to inhibit both allergic and nonallergic inflammatory processes in which an essential role for T cells has been implicated. Therefore the direct effects of this drug on several aspects of T-cell activity were analyzed in the present study. By using murine lymphocytes we found that NS at concentrations of 10^{-8} to 10^{-6} mol/L inhibited the mitogen- or antigen-induced proliferative responses of these cells. It is interesting to note that higher concentrations were ineffective. Preincubation of immune lymph node cells from contact sensitized mice with the drug abrogated their ability to transfer contact sensitivity to naive recipients, an effect that was found to be specific for the treated cells. Nedocromil sodium also interfered with the mitogen-induced interleukin-2 and tumor necrosis factor production by T cells and with their ability to adhere to the bound protein components of the extracellular matrix laminin and fibronectin. All these effects may be attributed to the inhibition of the increase of cytosolic calcium, which accompanies the early phase of T-cell activation and which is an essential step in inducing the aforementioned phenomena. (J ALLERGY CLIN IMMUNOL 1993;91:817-24.)

Key words: Nedocromil sodium, T lymphocytes, IL-2, TNF- α , extracellular matrix, contact sensitivity

Nedocromil sodium (NS) exhibits clinical efficacy as a prophylactic regimen in asthma, but its mode of action remains unsolved. Several studies in vivo have shown the particular efficacy of this drug in diminishing the late phase asthmatic responses.¹⁻³ Also, it has been demonstrated that NS has the capacity to downregulate activation of various effector cells in vitro, such as monocytes, mast cells, neutrophils, and eosinophils.⁴ However, its effect on T-cell activity has not been fully elucidated.⁵ Several lines of evidence support the hypothesis that the events occurring in late

Abbreviations used

CS:	Contact sensitivity
DNFB:	Dinitrofluorobenzene
ECM:	Extracellular matrix
[³ H] TdR:	Methyl [³ H] thymidine
I-LNCs:	Immune lymph node cells
LPR:	Late phase reaction
MC:	Mast cell
NS:	Nedocromil sodium
Ox:	Oxazolone
EAN:	Experimental allergic neuritis
TNF:	Tumor necrosis factor
HBSS:	Hank's balanced salt solution
ConA:	Concanavalin A
CPM:	Counts per minute
CTL:	Cytotoxic T-cell line
[Ca ²⁺] _i :	Cytoplasmic free calcium content

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phase reactions (LPRs) may be relevant to the pathogenesis of asthma.⁶⁻⁸ Recently the participation of T cells in LPR in the skin and in the lungs has been described.⁹⁻¹¹ Moreover, activated T lymphocytes

were identified in both bronchial biopsies and bronchoalveolar lavage obtained from stable atopic asthmatic patients.^{12, 13} Accordingly it has been proposed that these cells and their products may contribute to the inflammatory process that characterizes these reactions. It is therefore reasonable to assume, on the basis of these studies, that inhibition of T-cell activity may suppress the evolution of the LPR and the inflammation that underlies the asthmatic process. Furthermore, NS was shown to inhibit the progression of experimental allergic neuritis (EAN), a known T-cell dependent inflammatory demyelinating process of the peripheral nervous system.¹⁴ Although the authors claimed that the drug inhibited the disease by interfering with mast cell (MC) degranulation, its effect on other cells, crucial for the pathogenesis of EAN, had not been evaluated. Moreover, so far the role of MCs in inflammatory processes such as EAN has not been fully elucidated. We therefore decided to evaluate directly the effect of NS on several aspects of T-cell activity. We have found that NS inhibited both mitogen- and antigen-induced proliferative responses of T cells and their ability to transfer contact sensitivity (CS) and to produce interleukin-2 (IL-2) and tumor necrosis factor- α (TNF- α). In addition, the drug inhibited the ability of T cells to adhere to extracellular matrix (ECM) components. These findings may be attributed to the interference with cytosolic free calcium changes associated with the initial steps of T-cell activation.

MATERIAL AND METHODS

Mice and reagents

BALB/c female mice were purchased from the animal breeding facility at the Sackler School of Medicine (Tel-Aviv). All mice were used at 8 to 12 weeks of age. The guidelines prepared by the Institute of Laboratory Animal Resources for the care and use of the animals were followed. 2,4-Dinitrofluorobenzene (DNFB) was obtained from Aldrich Chemical Co. (Milwaukee, Wis.), 2,4 dinitrobenzene sulfonic acid sodium salt (DNBSO₃) was purchased from Eastman Kodak Co. (Rochester, N.Y.), and 4 ethoxymethylene 2 phenyl oxazolone (Ox) was purchased from BDH Chemicals Ltd. (Poole, England). 4-Bromo calcium ionophore A23187 (4BrA23187) was purchased from Sigma Chemical Co. (St. Louis, Mo.). NS was kindly provided by Fisons Pharmaceuticals (Loughborough, U.K.).

Induction and transfer of contact sensitivity

DNFB immune lymph node cells (I-LNCs) were obtained from donor mice sensitized on day 0 and 1 with 0.5% DNFB or 2% Ox in 4:1 acetone:olive oil. Donors were sensitized with 25 μ l of DNFB or 50 μ l of Ox applied to the shaved abdomen, and 5 μ l of these haptens were applied to the feet and ears. On day 4 the draining lymph nodes were removed,

and single cell suspensions were prepared and washed (2 times) in RPMI 1640 medium (Biological Industries, Beth Haemek, Israel). Recipients were given 5×10^7 /mouse I-LNCs intravenously and were ear challenged within 1/2 hour of transfer. Examination of the BALB/c I-LNC preparations after May-Grünwald Giemsa staining showed that they contained very few granulocytes (<0.2%).

Elicitation of CS

Mice were ear challenged with 20 μ l of 0.2% DNFB or 20 μ l of 0.5% Ox (10 μ l to each side of the ear) in the same vehicle as described above. A constant area on the ears was measured immediately before challenge and 24 hours later with a Mitutoyo (Mitutoyo, Paramus, N.J.) engineer's micrometer. The CS reaction was determined as the increment of swelling expressed as the mean in units of 10^{-4} inch $\pm$ SEM. Percentage inhibition was calculated as the following:

$$\% \text{ Inhibition} = \frac{\text{Positive control} - \text{experimental group}}{\text{Positive control}} \times 100$$

The background (irritation) response of recipients of LNCs from naive unsensitized donor mice was subtracted from all groups before calculation of inhibition was done.

Mitogenic proliferation of lymphocytes

Spleen cells from BALB/c mice were minced through a metal screen, treated with TRIS ammonium chloride buffer to lyse the red cells, and then washed three times with RPMI medium. Then, 5×10^5 viable cells were cultured in round-bottom, 96-well culture plates (Nunc Delta, Roskilde, Denmark) containing 0.2 ml RPMI 1640, supplemented with L-glutamine, nystatin, penicillin, streptomycin, and 5% fetal calf serum.

NS (dissolved in RPMI) was added to some of the above samples in various concentrations, ranging between 10^{-5} to 10^{-8} mol/L (molecular weight 442). Unless specified otherwise, NS was added at the initiation of the culture and was present for the entire culture period.

Thirty minutes after the addition of NS, 1 μ g concanavalin A (ConA) (Difco Laboratories, Detroit, Mich.) was added to each milliliter of the culture suspension.

These cell cultures (in quadruplicate) were incubated for 72 hours at 37° C in a humidified atmosphere of 5% CO₂, 95% air. Eighteen hours before the conclusion of culture 1.0 μ Ci (methyl-[³H] thymidine [³H] TdR) (Sp.ac.25 Ci/mmol, Nuclear Research Center, Negev, Israel) was added to each microtiter well. Cells were subsequently harvested onto fiberglass filters by use of a multiple channel semiautomated cell harvester, and the cell-associated radioactivity was counted in an automated beta counter (Rack-beta, LKB Wallace, Finland).

Control samples included cultures that were prepared as described above without the addition of NS. Results are presented as average counts per minute (CPM) $\pm$ SEM. Inhibition of proliferation was calculated as described for CS.

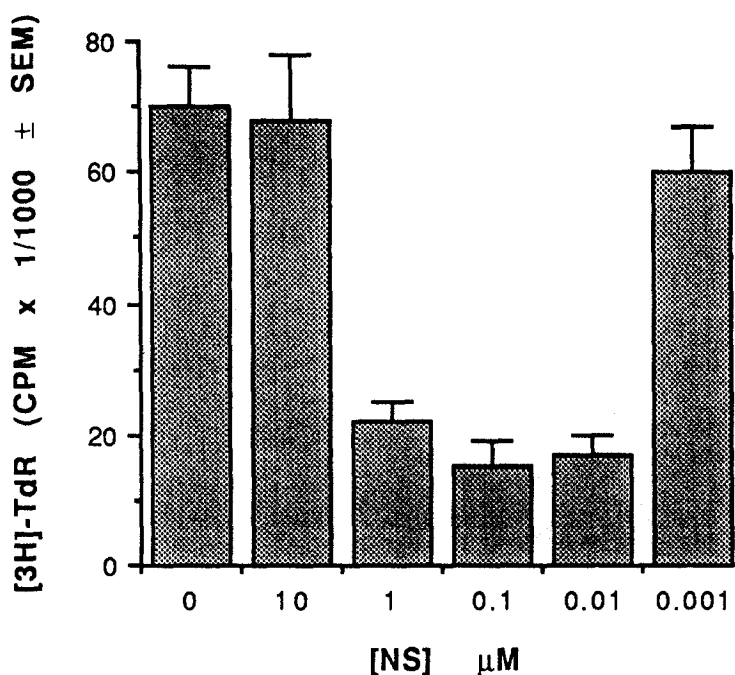


FIG. 1. Inhibition of ConA-stimulated spleen cells by NS. Several concentrations of the drug were added at culture initiation. Cells were activated with 1 $\mu\text{g}/\text{ml}$ of ConA, and [^3H] TdR incorporation was measured at 72 hours. Data, mean $\pm$ SEM of four different experiments performed in quadruplicate.

Enriched T-cell population was obtained by passage of plastic nonadherent spleen cells over nylon wool.¹⁵

IL-2 production assay

T cells were incubated for 24 to 48 hours under the same conditions as described above. After harvesting, supernatants were clarified by centrifugation and then extensively dialyzed against distilled H_2O , phosphate buffer, and RPMI 1640 for 36 hours in dialysis tubing retaining molecules in excess of 12,000 d. They were then sterile-filtered and stored at -20°C until used.

The IL-2 activity in supernatants was determined with use of a murine IL-2 dependent cytotoxic T-cell line (CTLL). Supernatants were added at different concentrations to 1×10^4 indicator cells. IL-2 activity was assessed by [^3H] TdR uptake by the CTLL after a 6-hour pulse with 1 $\mu\text{Ci}/\text{well}$ at the end of a 36-hour incubation. Recombinant IL-2 (Cetus Co., Emeryville, Calif.) in different concentrations was used for determination of the IL-2 content in the various samples. Quantities of IL-2 secreted by the different cultures were assessed by use of a computer program as described by Stadler et al.¹⁶

Measurement of TNF- α secretion

TNF- α bioactivity in spleen cell supernatants was detected with use of an MTT 18-h cytotoxicity assay with BALB/c Cl. 7 fibrosarcoma target cells.¹⁷ The specificity of the cytotoxic activity was determined with a monospecific

rabbit anti TNF- α antisera (donated by D. Aderka, MD, The Weizmann Institute, Rehovot, Israel), and recombinant murine TNF- α (Genzyme, Boston, Mass.) standards were included in each assay.

T-cell adhesion to fibronectin and laminin

This assay was recently described by us.¹⁸ In brief, ^{51}Cr -labeled T cells were pretreated (for 30 minutes) with NS and then activated with ConA (2 $\mu\text{g}/\text{ml}$) in fibronectin- or laminin-coated wells. After a 1-hour incubation period, the wells were washed three times with phosphate-buffered saline, and the remaining bound cells were lysed and transferred into testing tubes for the measurement of radioactivity. Percent of cell adhesion was calculated as follows:

$$\frac{\text{CPM of residual cells in the well}}{\text{Total CPM of cells seeded in the well} - \text{spontaneous release of } ^{51}\text{Cr}} \times 100$$

Determination of cytoplasmic free calcium content [Ca^{2+}]_i

T cells (3 to $4 \times 10^7/\text{ml}$) were loaded with the fluorescent indicator quin-2 by incubation with 50 $\mu\text{mol}/\text{L}$ of the acetoxymethyl parent compound (Sigma Chemical Co., St. Louis, Mo.) for 20 minutes at 37°C in Hanks' balanced salt solution (HBSS), and then they were diluted 10-fold and incubated for an additional 30 minutes. After they were loaded, the cells were centrifuged at 1000 g for 5 minutes

TABLE I. NS treatment of I-LNCs inhibits their ability to transfer CS

Donor cells	NS treatment	Ear swelling ($\times 10^{-4}$ inches) $\pm$ SEM	<i>p</i> value
(A) I-LNC (DNFB)	—	38 $\pm$ 4	<0.001 vs A
(B) I-LNC (DNFB)	10^{-7} M	21 $\pm$ 3	
(C) Naive-LNC	—	9 $\pm$ 2	

Four to five mice were included in each group.

Data represent mean $\pm$ SEM of three different experiments.

and resuspended in fresh HBSS at 2×10^6 cells/ml. The quin-2-loaded cells were incubated for 30 minutes at 37° C with different concentrations of NS or HBSS alone and then centrifuged (1000 g, 5 minutes) and resuspended with HBSS at 2×10^6 /ml.

[Ca²⁺]_i was determined before and at 5 minutes after the addition of 4 μ g/ml of ConA at 37° C.

Extracellular quin-2 fluorescence was quenched with use of 100 μ mol/L MnCl₂, and then chelated with an equimolar concentration of Ca-DTPA (calcium-diethylenetriaminepentaacetic acid).

Calculation of [Ca²⁺]_i was made as previously described,¹⁹ after correction for autofluorescence and extracellular quin-2.

Statistical analysis

The results of the experimental and control groups were compared for statistical significance ($p < 0.05$) by use of Student's *t* test (two-tailed).

RESULTS

NS inhibits proliferative responses of lymphocytes

To investigate the effect of NS on T-cell function we tested its effect on [³H] TdR incorporation by murine spleen cells in vitro. The drug was administered to the cells 30 minutes before introduction of the mitogen (ConA). Results are shown in Fig. 1.

In these experiments spleen cells were incubated with 10^{-5} to 10^{-9} mol/L solutions of the drug together with 1 μ g/ml of ConA for 72 hours. As can be seen in this figure, there was a small insignificant inhibition of proliferation when cells were incubated with either 10^{-5} mol/L (10 μ mol/L) or 10^{-9} mol/L (0.001 μ mol/L) of NS. However, inhibitions of [³H] TdR uptake of 78% and 76% were found at 10^{-7} mol/L and at 10^{-8} mol/L NS, respectively. At the concentrations tested, NS had no effect on lymphocyte viability (viability of cells after a 72-hour incubation was always >90% by trypan blue exclusion and by cell counts). The fact that the highest concentration of NS had minimal inhibitory effect on proliferation argues against a cytotoxic effect of the drug. Similar results were obtained when I-LNC's obtained from

mice that had been sensitized with DNFB were stimulated with the specific antigen DNBSO₃ (2.5 μ g/ml) (not shown).

NS treatment of I-LNC in vitro blocks their ability to transfer CS

The T cells that proliferate in vitro when restimulated with specific antigen represent a different population than the T cells capable of passively transferring CS.²⁰ We therefore determined whether NS could interfere with the function of effector cells that transfer CS.²¹

I-LNCs from mice sensitized to DNFB (I-LNC [DNFB]) were incubated for 90 minutes at 37° C with 10^{-7} mol/L of NS. The cells were then centrifuged, resuspended in fresh RPMI, and immediately injected intravenously into each of five syngeneic recipients (5×10^7 cells per recipient). Control cells included I-LNCs (DNFB) not treated with NS and LNCs recovered from mice that were not sensitized to DNFB (naive-LNCs). These populations were incubated, resuspended, and injected in the same manner as NS-treated I-LNCs (DNFB). Recipient animals were ear challenged within 30 minutes of cell transfer, and the contact response was measured 24 hours later (Table I).

At a concentration of 10^{-7} mol/L, NS produced 59% inhibition of the contact response. Once again it should be pointed out that there were no differences in cell numbers or viability (>90%) between NS-treated groups and the control groups.

One can argue that the treated I-LNCs simply transferred sufficient drug to block CS by a systemic action on other host cells. To test this possibility, we performed an experiment in which 5×10^7 I-LNCs from mice sensitized to Ox (I-LNC [Ox]) were cotransferred with 5×10^7 NS-treated or untreated I-LNCs (DNFB) (Table II). The left ears of recipient mice were then challenged with DNFB, and the right ears were challenged with Ox. Mice that received both NS-treated I-LNCs (DNFB) (10^{-7} mol/L NS) and untreated I-LNC (Ox) (group B) exhibited significant suppression of the response to DNFB (50% suppres-

TABLE II. The inhibition of transfer of CS by NS is specific for the treated cells

Donor cells	Ear	Challenge	Ear swelling ($\times 10^{-4}$ inch) $\pm$ SEM	p value
(A) I-LNC (DNFB)	Left	DNFB	28 $\pm$ 4	
+ I-LNC (Ox)	Right	Ox	36 $\pm$ 3	
(B) I-LNC (DNFB)-NS*	Left	DNFB	19 $\pm$ 2	<0.01 vs A NS vs A
+ I-LNC (Ox)	Right	Ox	34 $\pm$ 4	
(C) Naive-LNC	Left	DNFB	10 $\pm$ 2	
	Right	Ox	15 $\pm$ 2	

sion). By contrast, there was no detectable inhibition of the response to the unrelated antigen Ox (compare groups A and B for right ears). This experiment shows that NS blocks CS by an effect on the treated T cells and not on other target tissues.

Incubation with NS prevents ConA-induced production of lymphokines

We next evaluated the effect of NS on the production of IL-2 by mitogen-activated T cells. This aspect of the cellular immune response was tested directly by assaying the dialyzed supernatants of ConA-stimulated cells for IL-2 activity (Table III). This is the mean $\pm$ SEM of three different experiments. Incubation of murine spleen cells with 1 μ g/ml ConA induced the secretion of 6.0 μ g/ml IL-2 at 24 hours. The addition of 10^{-7} mol/L NS to this system blocked the IL-2 production. There was a mild inhibition of IL-2 production when cells were incubated with 10^{-8} mol/L of NS, and virtually no effect with the highest concentration of the drug (10^{-6} mol/L).

Once again, these results with the specific "therapeutic window" argue against a cytotoxic effect of NS on either the effector splenocytes or the target IL-2 dependent CTLL cell line. Furthermore, the dialyzates, which were tested for the presence of IL-2, contained substances with molecular weight (MW) greater than 12,000 d, whereas the MW of NS (which is water soluble) is 442 d.

NS at 10^{-8} and 10^{-7} mol/L (Table IV) abrogated the production of TNF- α by ConA-activated spleen cells. However, it did not affect TNF- α production by spleen cells when these were stimulated with lipopolysaccharide, indicating a specific effect of the drug on T cells (and not macrophages or B cells) in these cell populations.

NS inhibits T-cell adhesion to ECM components

To mediate an immune response lymphocytes must be able to interact with and respond to the surrounding extracellular environment. Besides interacting with

TABLE III. ConA-induced IL-2 secretion is prevented by NS

Incubation conditions			
Cells +	NS treatment	IL-2 (U/ml)	% Suppression
Medium	—	0.24 $\pm$ 0.02	
ConA	—	6.0 $\pm$ 0.50	0
ConA	10^{-8} mol/L	4.5 $\pm$ 0.62	25
ConA	10^{-7} mol/L	0.9 $\pm$ 0.01	85
ConA	10^{-6} mol/L	5.6 $\pm$ 0.25	6

TABLE IV. ConA-induced TNF- α production is prevented by NS

Incubation conditions			
Cells +	NS treatment	TNF- α (pg/ml) $\pm$ SEM	% Suppression
Medium	—	24 $\pm$ 5	
ConA	—	531 $\pm$ 28	
ConA	10^{-8} mol/L	228 $\pm$ 15	60
ConA	10^{-7} mol/L	75 $\pm$ 7	90
LPS	—	213 $\pm$ 30	0
LPS	10^{-8} mol/L	247 $\pm$ 19	0
LPS	10^{-7} mol/L	231 $\pm$ 20	0

other cells, lymphocytes in vivo interact with macromolecules found within the ECM as they move within various microenvironments.²² We therefore analyzed the effect of NS on T-cell adhesion to surfaces coated with the ECM components fibronectin and laminin.

As can be seen in Table V, 38% and 40% of ConA-activated T cells adhered to fibronectin and laminin, respectively. There was no detectable adherence to uncoated plates, whereas the adhesion to bovine serum albumin-coated wells of activated T cells was always 5% or less. Pretreatment (30 minutes) of these cells

TABLE V. NS inhibits T-cell adherence to ECM components

NS pretreatment (mol/L)	% Adhesion $\pm$ SEM	
	Fibronectin (% Suppression)	Laminin (% Suppression)
—	38 $\pm$ 2	40 $\pm$ 5
10 ⁻¹⁰	29 $\pm$ 4 (24)	30 $\pm$ 3 (25)
10 ⁻⁸	20 $\pm$ 3 (47)	23 $\pm$ 1 (43)
10 ⁻⁶	19 $\pm$ 7 (50)	21 $\pm$ 4 (47)

Adherence of nonactivated T cells was 2% to 5%.

Data, mean $\pm$ SEM of three different experiments.

within 10⁻¹⁰ mol/L NS before activation caused a mild inhibition of adhesion, whereas 10⁻⁸ mol/L and 10⁻⁶ mol/L of NS induced a significant (>40%) inhibition of adhesion. Washing the drug out before cell activation reversed the inhibitory effect (not shown).

The effect of NS on lectin-induced uptake of calcium

Proliferative responses of lymphocytes to mitogens and antigens are associated with early changes in cytosolic calcium.^{23, 24} It has also been shown that changes in cytosolic calcium are required for lectin-induced secretion of IL-2.²⁵ We therefore evaluated the effect of NS on ConA-induced changes in [Ca²⁺]_i (Table VI; mean $\pm$ SEM of four different experiments).

Within the first 5 minutes of the addition of ConA, the resting [Ca²⁺]_i of enriched T-cell population increased from 115 $\pm$ 5 to 271 $\pm$ 11 nmol/L (135% increase). A 30-minute incubation of quin-2-loaded cells with either 10⁻⁸ mol/L or 10⁻⁷ mol/L NS did not significantly alter the resting [Ca²⁺]_i, but the presence of the drug abrogated the increase in [Ca²⁺]_i that occurred after the addition of the mitogen (59% increase and 43% increase, respectively). The effects of NS were reversible because washing the drug out restored the ConA-induced increase in the cytosolic calcium (Table V, groups D and E). These findings were further explored by attempting to bypass the effect of NS on [Ca²⁺]_i by use of the calcium ionophore 4-BrA23187. The addition of 10⁻⁷ mol/L of the ionophore to the NS-pretreated (10⁻⁷ mol/L) groups resulted in recovery of the suppression that had been induced by the drug [Ca²⁺]_i = 405 $\pm$ 104).

DISCUSSION

The pharmacologic actions of NS in asthma are many and include suppression of mediator release,²⁶

TABLE VI. Effect of NS on ConA-induced changes in cytosolic calcium

	Preincubation conditions NS treatment	[Ca ²⁺] _i (nmol/L)	
		Pre-ConA	Post ConA
A	—	115 $\pm$ 5	271 $\pm$ 11
B	10 ⁻⁸ mol/L	130 $\pm$ 15	207 $\pm$ 14
C	10 ⁻⁷ mol/L	100 $\pm$ 9	143 $\pm$ 23
D	10 ⁻⁸ mol/L + washing	92 $\pm$ 6	277 $\pm$ 18
E	10 ⁻⁷ mol/L + washing	109 $\pm$ 13	237 $\pm$ 12

antiinflammatory effects on eosinophils, macrophages, and neutrophils,^{27, 28} and an ability to reduce bronchial hyperresponsiveness.^{1, 3} Although it is effective in the clinical treatment of asthma, the precise mechanisms by which NS accomplishes this are not known.

In the present study we examined the effect of NS on various aspects of T-cell activity. First we showed that this drug inhibited the mitogenic response of spleen cells and the antigen-induced proliferative response of I-LNCs.

In addition, a 90-minute incubation of I-LNCs with 10⁻⁷ mol/L of NS significantly inhibited their ability to transfer CS to naive recipients (Tables I and II). These results indicate that NS influenced different populations of T cells, because distinct T-cell subpopulations were identified to mediate in vivo (transfer) and in vitro (proliferative response) manifestations of delayed hypersensitivity.²⁰ The I-LNC preparations used in our studies contained negligible numbers of either granulocytes or MCs according to May-Grünwald or toluidine blue staining, indicating that the drug influenced directly the T-cell compartment included in these populations. Moreover, NS was previously found to be ineffective against mouse peritoneal MCs.²⁹ We have also shown that the inhibition of CS was specific for the treated I-LNCs. Animals that received both NS-treated I-LNCs from mice sensitized to DNFB (I-LNC [DNFB]) and I-LNCs from mice sensitized with Ox expressed abrogated response to DNFB challenge but responded normally to challenge with Ox on the opposite ear. Thus there was no systemic effect of NS transferred with the treated cells.

In the light of the pivotal role of IL-2 in the immune response in general and in T-cell activation in particular, inhibition of its production may explain the biologic effect of NS. Indeed, NS inhibited IL-2 production. We were concerned that the drug may have an inhibitory effect on the CTLL assay, therefore all

supernatants of the cultures under investigation had been intensively dialyzed before they were assayed for lymphokine production. Once again, the same dose-response relationship was observed as in the proliferative responses, that is, the highest concentration of the drug was ineffective. We currently do not have any clear explanation for this observation; however, it should be mentioned that the very same dose-response relationship was found when the effect of NS on eosinophil activation was examined.²⁷ The optimal concentration of the drug in inhibiting eosinophil cytotoxic activity was 10^{-7} mol/L, whereas 10^{-5} mol/L was ineffective.

Preincubation with NS was also found to inhibit adhesion of activated T cells to ECM components (Table V). This effect of the drug may interfere with yet another essential function of T cells, because in vivo they interact with macromolecules found within the ECM as they move within various microenvironments by way of a number of lymphocyte ECM receptors termed β_1 -integrins.^{30, 31} These integrins facilitate the ability of these cells to interact with and respond to a wide variety of microenvironments.²² This ability of lymphocytes was also found to be functionally fundamental in facilitating their migration from the vascular lumen into the surrounding tissues through the basement membrane on one hand, and from tissues into lymphatic vessels on the other hand. For example, VLA-6 ($\alpha_6 \beta_1$ integrin)-mediated adhesion to laminin is likely to be critical to migration through the basement membrane where laminin is a major constituent.³² Adhesion of lymphocytes to ECM not only facilitates their migration from the intravascular compartment but also temporarily immobilizes them at the site of the ongoing immune response. Thus at least a part of the inhibitory effect of NS on the elicitation of CS may be attributed to the ability of the drug to inhibit adhesion to ECM. Indeed, in a recent study Ferguson et al.³³ have shown that inhibition of adhesion to ECM components abrogated T-cell mediated immune responses (such as CS) in vivo.

The above-mentioned effects of NS on T-cell function may be attributed at least in part to its ability to block the rise in cytosolic calcium, which accompanies lymphocyte activation (Table VI).^{24, 25} The production and secretion of IL-2 after lectin treatment appears to be dependent on this mitogen-induced rise in $[Ca^{2+}]_i$.²⁵ Preventing the increase of the cytosolic calcium is associated with inhibition of IL-2 production and subsequent proliferation.²⁵ This effect of NS on calcium ion flux was reversible, because washing the drug out restored the response (Table VI).

Furthermore, T-cell adhesion to protein components of the ECM was also shown to be an active process that requires the stimulation of the T cells by

a peptide antigen or mitogen,^{18, 30} therefore inhibition of calcium flux and cell activation resulted in interference with cell adhesion.

Do the effects of NS on the various aspects of T-cell activity explain its effectiveness in the clinical treatment of asthma? Although the presence of T cells has been demonstrated in asthma and in allergic LPR,⁷⁻¹³ their precise role in the pathogenesis of asthma has not been fully elucidated and remains so far largely indirect. Therefore the causal relationship between the effects of NS on T-cell function and its clinical effectiveness in asthma remains largely speculative. Moreover, the information that has been gathered so far points to the fact that T cells that are involved in allergic reactions belong to the T_{H2} subpopulation.^{11, 13} These cells are characterized by their ability to produce and release IL-4, IL-5, and IL-10 but no IL-2 or interferon gamma, whereas T_{H1} clones produce IL-2 and interferon gamma but no IL-4 or IL-5.³⁴ T_{H1} clones mediate delayed type of hypersensitivity reactions, whereas the T_{H2} promote preferential B-cell production of IgA, IgE, IgG1, and IgM and the growth of eosinophils.^{35, 36}

Our data suggest that NS affects a pattern of activity that characterizes the T_{H1} subpopulation, namely IL-2 production and elicitation of CS, which is a prototype of delayed hypersensitivity reaction. As mentioned above, this subpopulation has not been shown to participate in asthma. It is interesting to note that O'Hehir et al.⁵ could not find any effect of NS on cloned human allergen-specific CD4 T-cell function, which most probably belonged to the T_{H2} subpopulation.

It seems that our results may explain at least in part the effect of NS on inflammatory processes such as EAN.¹⁴ Whether this effect is specific for the T_{H1} population is currently examined in our laboratory.

REFERENCES

1. Dahl R, Pederson B. Influence of nedocromil sodium on the dual asthmatic reaction after allergen challenge. *Eur J Respir Dis* 1986;69(suppl 147):263-5.
2. Church MK, Huston PA, Holgate ST. Effect of nedocromil sodium on early and late phase responses to allergen challenge in the guinea-pig. *Drugs* 1989;(suppl 1):101-8.
3. Aalbers R, Kauffman HF, Goren H, Koeter GH, De Monchy JGR. The effect of nedocromil sodium on the early and late reaction and allergen-induced bronchial hyperresponsiveness. *J ALLERGY CLIN IMMUNOL* 1991;87:993-1001.
4. Busse WW, Orr TSC, Pauwels R eds. International symposium on nedocromil sodium. *Drugs* 1989;(suppl 1):1-77.
5. O'Hehir RE, Moqbel R. Action of nedocromil sodium and sodium cromoglycate on cloned human allergen specific CD4+ T lymphocytes. *Drugs* 1989;(suppl 1):23-5.
6. Metzger WJ, Hunninghake G, Richerson H. Late asthmatic responses: inquiry into mechanisms and significance. *Clin Rev Allergy* 1985;3:145-9.

7. Kay AB. Asthma and inflammation. *J ALLERGY CLIN IMMUNOL* 1991;87:893-910.
8. Barnes PJ. A new approach to the treatment of asthma. *N Engl J Med* 1989;321:1517-27.
9. Frew AJ, Kay AB. The relationship between infiltrating CD4 + lymphocytes, activated eosinophils and the magnitude of the allergen-induced late phase cutaneous reaction in man. *J Immunol* 1988;141:4158-64.
10. Frew AJ, Moqbel R, Azzawi M, et al. T lymphocytes and eosinophils in allergen-induced late-phase asthmatic reactions in the guinea pig. *Amer Rev Respir Dis* 1990;141:407-13.
11. Kay AB, Ying S, Varney V, et al. Messenger RNA expression of the cytokine gene cluster, IL-3, IL-5 and GM-CSF in allergen induced late phase cutaneous reactions. *J Exp Med* 1991;173:775-9.
12. Azzawi M, Bradely B, Jeffery PK, et al. Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma. *Am Rev Respir Dis* 1990;142:1407-13.
13. Robinson DS, Hamid Q, Ying S, et al. Predominant T_{H2}-like bronchoalveolar T lymphocyte population in atopic asthma. *N Engl J Med* 1992;362:298-304.
14. Seeldrayers PA, Yasui D, Weiner HL, Johnson D. Treatment of experimental allergic neuritis with nedocromil sodium. *J Neuroimmunol* 1989;25:221-6.
15. Julius MH, Simpson E, Herzenberg LA. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur J Immunol* 1973;3:645.
16. Stadler BM, Dougherty SF, Farrar JJ, Oppenheim JJ. Relationship of cell cycle to recovery of IL-2 activity from human mononuclear cells, human and mouse T-cell lines. *J Immunol* 1981;127:1936-40.
17. Gallily R, Sher T, Ben Av P, Loewenstein J. Tumor necrosis factor as a mediator of mycoplasma orale induced tumor cell lysis by macrophages. *Cell Immunol* 1989;121:146-53.
18. Herschkoviz R, Miron S, Cohen IR, Miller A, Lider O. T-lymphocyte adhesion to fibronectin and laminin components of the ECM is regulated by the CD4 molecule. *Eur J Immunol* 1992;22:7-13.
19. Rink TJ, Pozzan T. Using quin 2 in cell suspensions. *Cell Calcium* 1985;6:133-44.
20. Moorhead JW. Tolerance and contact sensitivity to DNFB in mice. VIII. Identification of distinct T cell subpopulations that mediate in vivo and in vitro manifestations of delayed hypersensitivity. *J Immunol* 1978;120:137.
21. Mekori YA, Weitzman GL, Galli SJ. Reevaluation of reserpine-induced suppression of contact sensitivity. *J Exp Med* 1985;162:1935-53.
22. Shimizu Y, Shaw S. Lymphocyte interactions with extracellular matrix. *FASEB J* 1991;5:2292-9.
23. Tsien RY, Pozzan T, Rink TT. T cell mitogens cause early changes in cytoplasmic free Ca⁺⁺ and membrane potential in lymphocytes. *Nature (London)* 1982;295:68-71.
24. Shapiro DN, Adams BS, Niederhuber JE. Antigen specific T-cell activation results in an increase in cytoplasmic free calcium. *J Immunol* 1985;135:2256-61.
25. Mills GB, Cheung RK, Grinstein S, Gelfand EW. Increase in cytosolic free calcium concentration is an intracellular messenger for the production of interleukin 2 but not for the expression of the IL-2 receptor. *J Immunol* 1985;134:1640-3.
26. Leung KBP, Flint KC, Brostoff J, et al. A comparison of nedocromil sodium and sodium cromoglycate on human lung mast cells obtained by bronchoalveolar lavage. *Eur J Respir Dis* 1986;69(suppl 147):223-6.
27. Moqbel R, Cromwell O, Kay AB. The effect of nedocromil sodium on human eosinophil activation. *Drugs* 1989;(suppl 1):19-22.
28. White MV, Phillips RL, Kaliner MA. Neutrophils and mast cells: nedocromil sodium inhibits the generation of neutrophil-derived histamine releasing activity. *J ALLERGY CLIN IMMUNOL* 1991;87:812-20.
29. Pearce FL, Al-Laith M, Bosman L, et al. Effects of sodium cromoglycate and nedocromil sodium on histamine secretion from mast cells from various locations. *Drugs* 1989;(suppl 1):37-43.
30. Springer TA. Adhesion receptors of the immune systems. *Nature* 1990;346:425-33.
31. Ruoslahti E. Integrins. *J Clin Invest* 1991;87:1-5.
32. Yurchenco PD, Schittny JC. Molecular architecture of basement membranes. *FASEB J* 1990;4:1577-90.
33. Ferguson TA, Mizutani H, Kupper TS. Two integrin binding peptides abrogate T-cell mediated immune responses in vivo. *Proc Natl Acad Sci U S A* 1991;88:8072-6.
34. Mosmann TR, Cherwinski H, Bond WW, Giedlin MA, Coffman RL. Two types of murine helper T-cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 1986;136:2348-57.
35. Fong TA, Mosmann TR. The role of IFN- γ in delayed type hypersensitivity mediated by T_{H1} clones. *J Immunol* 1989;143:2887-93.
36. Mosmann TR, Coffman RL. T_{H1} and T_{H2} cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989;7:145-73.