

Increased progenitor cell proliferation in the peripheral blood of patients with bronchial asthma: The role of nitric oxide

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Background: Asthma exacerbation is associated with increased numbers of circulating CD34⁺ progenitor cells, which may migrate to airways and develop into mature cells under the effects of cytokines and hematopoietic factors. Nitric oxide (NO) generation is enhanced in asthma and is known to suppress human hematopoiesis.

Objectives: We studied circulating progenitor cells in the blood of patients with varying severity of asthma and examined the contribution of NO to their proliferation into eosinophil-forming colonies *ex vivo*.

Methods: With use of multiparameter flow cytometric analyses, the cell numbers and intracellular inducible NO synthase (iNOS) immunoreactivity of circulating CD34⁺ cells in peripheral blood was measured. The serum level of GM-CSF or IL-5 was also determined. The colonies grown from progenitor cells were cultured in methylcellulose either in the presence or absence of growth factors, including GM-CSF, stem cell factor, and IL-3.

Results: A significantly greater number of circulating CD34⁺ cells increased together with higher intracellular iNOS immunoreactivity in moderate asthmatics compared with mild intermittent asthmatics and healthy subjects. There was no significant difference in iNOS immunoreactivities or CD34⁺ progenitor cell numbers between healthy subjects and those with mild intermittent asthma. Serum levels of GM-CSF or IL-5 were significantly higher in all asthmatics compared with healthy subjects and correlated with circulating CD34⁺ cells. A greater number of colonies was grown either in the presence or absence of growth factors with a higher percentage of cells of eosinophil lineage in asthmatics than in health subjects. *N*^G-nitro-L-arginine methyl ester potentiated and sodium nitroprusside inhibited the colony growth in both asthmatic and healthy subjects without a significant change in the percentage of eosinophil lineage.

Conclusions: The production of NO from progenitor cells or other circulating cells may act in an autocrine or paracrine fashion to regulate progenitor cell growth and colony forma-

tion. However, this is not sufficient to control the increased proliferation of progenitor cells observed in asthma. (*J Allergy Clin Immunol* 1999;104:803-10.)

Key words: Asthma, progenitor cells, nitric oxide, cytokines, nitric oxide synthase

Chronic airway inflammation is an important characteristic feature of asthma involving the infiltration of eosinophils, neutrophils, monocytes-macrophages, mast cells, and T cells¹⁻³ and the expression of several cytokines including Th2-derived cytokines such as IL-3, IL-4, and IL-5, colony-stimulating factors such as GM-CSF and chemokines such as RANTES.⁴⁻⁶ Cells such as eosinophils, neutrophils, and monocytes are produced from progenitor cells in the bone marrow. The numbers of circulating eosinophil-basophil progenitors increase in atopic subjects,⁷ during asthma exacerbation by controlled withdrawal of inhaled steroid therapy,⁸ and by experimental exposure to allergen.⁹ These primitive hematopoietic cells express the CD34⁺ cell surface marker and have the capacity to develop into mature cells that could in turn contribute to the chronic inflammation of asthma.

Nitric oxide (NO), a radical produced from arginine catalyzed by nitric oxide synthase,¹⁰ is produced during inflammation and has been implicated as a signaling¹¹ as well as a toxic effector molecule.¹² NO mediates the activation or inhibition of various enzyme systems, DNA damage,¹³⁻¹⁵ and oxidative reactions, leading to a variety of biologic effects, including killing of micro-organisms, and stasis and cell death.¹⁶ Patients with bronchial asthma and allergic rhinitis demonstrate increased levels of NO in exhaled air.^{17,18} The inducible form of nitric oxide synthase (iNOS) is expressed in the bronchial mucosa¹⁹ of patients with bronchial asthma, but not of healthy subjects, and can also be detected in blood eosinophils.²⁰ Recently, iNOS has been demonstrated to be expressed in bone marrow CD34⁺ cells, and the generation of NO is known to suppress human hematopoiesis.²¹

We hypothesized that iNOS activities may be altered in the progenitor cells of patients with asthma, which could ultimately influence progenitor cell growth and development. We therefore studied progenitor cells in circulating blood of patients with varying severities of asthma and examined the contribution of NO to their proliferation into eosinophil-forming colonies *ex vivo*.

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TABLE I. Demographic features of patients

	Healthy subjects	Mild intermittent asthma	Moderately severe asthma
Age (y)	31.6 ± 3.4	36.9 ± 5.8	35.2 ± 6.2
Sex (male/female)	5:3	7:5	6:3
Atopy	0	7	6
FEV ₁ (% predicted)	87.9 ± 4.6	83.7 ± 3.2	71.1 ± 6.5
FEV ₁ /FVC (%)	85.3 ± 3.4	82.0 ± 1.7	68.5 ± 6.4
IgE (U/mL)	90.5 ± 34.2	251.4 ± 89.2	262.6 ± 89.6
PC ₂₀ (mg/mL)	>25	1.5 ± 0.7	0.5 ± 0.3

Data are mean symbol ± SEM. FVC, Forced vital capacity; PC₂₀, concentration of methacholine to induce 20% decrease in FEV₁.

Abbreviations used

- iNOS: Inducible nitric oxide synthase
- L-NAME: *N*^G-nitro-L-arginine methyl ester
- MFI: Mean fluorescence intensity
- NAMNC: Nonadherent mononuclear cell
- NANT: Nonadherent non-T cell
- NO: Nitric oxide
- PE: Phycoerythrin
- SCF: Stem cell factor
- SNP: Sodium nitroprusside
- SSC: Side scatter

METHODS

Subjects and study design

Twenty-one nonsmoking adults with clinically defined mild intermittent (*n* = 12, 7 men/5 women) and moderate persistent (*n* = 9, 6 men/3 women) asthma were recruited from outpatient clinics (Table I). Asthma severity was assessed by an increase in asthma symptoms and increased bronchodilator requirements.²² Patients with intermittent daytime asthma symptoms less than one time a week, night time symptoms fewer than 2 times a month, and normal lung function between exacerbation are defined as mild intermittent asthma. Moderate persistent asthmatic patients are symptomatic daily, with asthma exacerbation after activity and sleep, and require daily use of inhaled short-acting β_2 -agonists. Those patients may have night time asthma symptoms more than 2 times a week.²² Seven patients with mild intermittent asthma and 6 with moderate persistent asthma were atopic, as determined by history and positive Phadiatop IgE serum testing (Pharmacia) (Table I). Airflow limitation was present at the time of the laboratory visit in all patients with asthma and had a >15% improvement in FEV₁ with inhaled salbutamol. None of the patients had received corticosteroids, cromoglycate, theophylline, or other medications 6 weeks before, except for inhaled β_2 -agonists as a rescue medication. Inhaled β_2 -agonists were withheld for 12 hours before venipuncture and methacholine testing, and subjects rested for 30 minutes before testing.

Eight normal nonsmoking subjects (5 men, 3 women) were recruited from our hospital staff without evidence of allergic rhinitis or asthma as identified by history, negative serum testing for Phadiatop and normal total IgE level, and eosinophil counts (Table I). Spirometry and methacholine airway hyperresponsiveness were within normal limits in those subjects.

Preparation of progenitor cells

PBMCs were separated from whole blood with use of dextran sedimentation and Ficoll-Hypaque density gradient centrifugation at 400g for 30 minutes. The mononuclear cells at the interface were harvested, washed twice, and resuspended in Iscove's modified

Dulbecco's medium. The nonadherent mononuclear cell (NAMNC) fraction was separated by adherence technique.

Quantitation of progenitor CD34⁺ cells in blood

The peripheral blood NAMNC at 1×10^6 cells per milliliter were fixed with 100 μ L of 4% paraformaldehyde at room temperature for 10 minutes and then washed twice with PBS, pH 7.0. The cell pellet was resuspended and incubated with anti-CD34-phycoerythrin (PE), anti-CD45-FITC mAbs (Dakopatts, Glostrup, Denmark) diluted in 1:25 in PBS in the dark for 1 hour at 4°C. After being washed twice with PBS, the cell pellet was immersed in 500 μ L of fluorescence-activated cell sorter permeabilizing solution (Becton-Dickinson, San Jose, Calif) and then incubated with anti-iNOS-PerCp rabbit polyclonal antibodies²³ (Transduction Laboratories, Lexington, Ky) diluted 1:25 in PBS in the dark for 1 hour at 4°C. After being washed and resuspended with PBS, the cell suspension was quantitated with a FACScan flow cytometer (Becton-Dickinson, Mountain View, Calif). Compensation settings were established with use of CalBrite beads (Becton-Dickinson Instrument Systems) and confirmed with use of NAMNC stained with anti-CD34-PE, anti-CD45-FITC, or anti-iNOS-PerCp. Off-line analysis was performed with QUEST software, as supplied by Becton-Dickinson.

A multiparameter sequential gating strategy as previously described^{24,25} was used to enumerate CD34⁺ progenitor cell numbers. Briefly, a region or gate (R1) representing CD45⁺ events (Fig 1, A, region R1) was set up to quantitate total nucleated white blood cells and to exclude contaminating events such as red blood cells, platelet aggregates, and other debris that can drastically inflate the number of CD34⁺ events. Simultaneous staining by CD34⁺ antibody then defined an approximate number for the CD34⁺ stem/progenitor subfraction. CD45⁺ events in region R1 were then analyzed for CD34 staining, and positive events corresponding to the latter were gated into a region R2 (Fig 1, B, region R2). The gating strategies of CD45⁺ and CD34⁺ events were set up with isotype-matched fluorochrome-conjugated control antibodies into less than 1% of cells that expressed positive events (dashed line in Fig 1, A and B). By the virtue of observation that true CD34⁺ cells express low levels of CD45 and have generally low side scatter,^{26,27} we can separate true CD34⁺ blast cells from other cell types that display similar or partially overlapping light-scattering characteristics. Thus lymphocytes that exhibit characteristics of side and forward scatter similar to some true CD34⁺ blasts can be separated from CD34⁺ cells because they stain brightly for CD45. Similarly, monocytes, which can partially overlap blast/lymphocyte gates on light-scatter analysis, can be distinguished by their high CD45 expression and increased side scatter. Granulocyte-series cells and their precursors, which stain weakly but nonspecifically with some CD34 antibodies, can also be distinguished from true CD34⁺ cells by high side scatter. Sequential gates were then set up: CD34 events in region R1

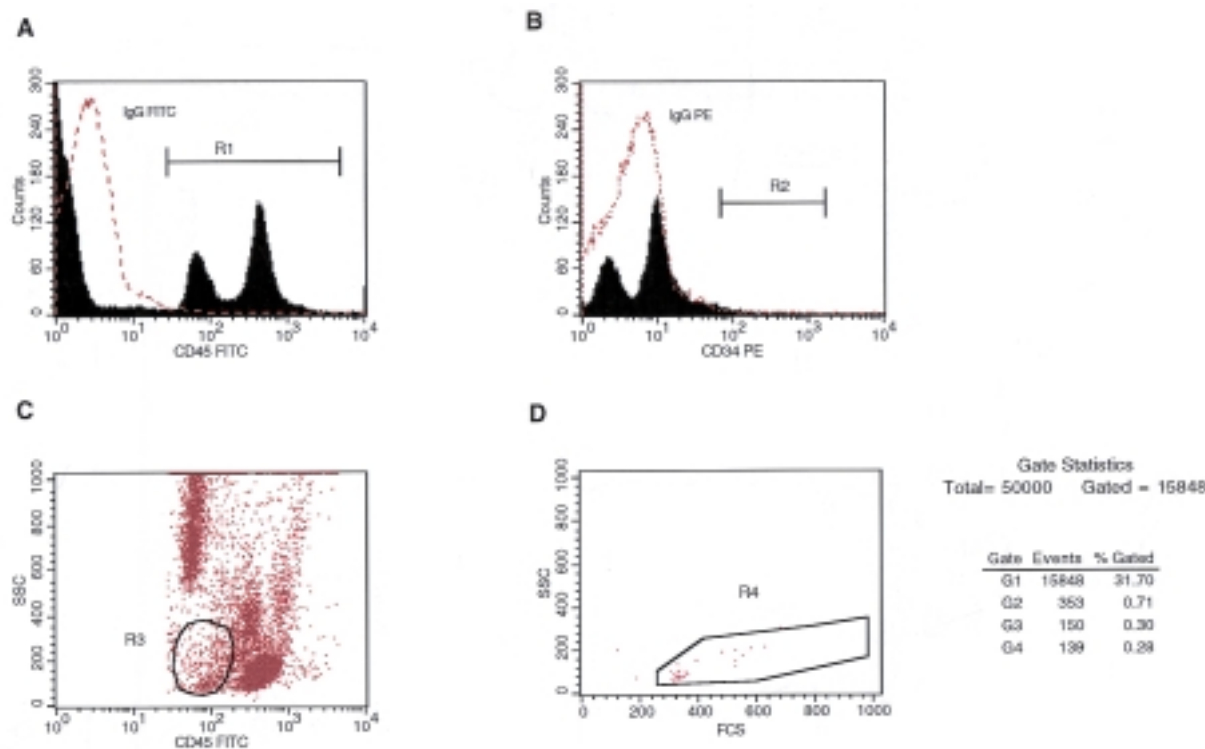


FIG 1. Multiparameter flow cytometric analysis for enumeration of CD34⁺ progenitor cells. Data show staining with anti-CD45-FITC and anti-CD34-PE of blood NAMNC from patient with asthma. **A**, Sample stained with anti-CD45-FITC; region R1 represents nucleated leukocytes. Dashed line, Events of IgG-FITC. **B**, Anti-CD34-PE staining of cells in region R1. Region R2 represents CD34⁺ cells. Dashed line, Events of PE-conjugated control IgG. **C**, CD45 versus side-scatter analysis of events gated in region R1; region R3 identifies true CD34⁺ blast cells with low CD45 and low SSC characteristics. **D**, Light-scatter properties of cells gated by R1, R2, and R3. Region R4 represents blast/lymphocyte region into CD34⁺ cells scatter. Gate statistics of cells gated in R1 (G1), R1 plus R2 (G2), R1 to R3 (G3), and R1 to R4 (G4).

(Fig 1, B, region R2), CD45 versus side scatter (SSC) of the CD34⁺-gated events in R2 (Fig 1, C, region R3: to identify blast cells), and forward scatter versus SSC to confirm the lymphoblastoid characteristics of the gated CD34⁺ cells in region R3. Without changing any of the gates, analysis of the same cell sample stained with CD45-FITC and PE-linked isotype control antibody was performed. Enumeration data were derived from the gate statistics: events in gate G4 (Fig 1, D, = events in R1 to R4) after staining with CD45-FITC/CD34-PE minus events in G4 stained with CD45-FITC/PE-linked control antibody were used to calculate the absolute number of true CD34⁺ blast cells.

With use of 3-color analysis, events in region R4 were back-scattered onto a dot plot of CD34-PE versus staining by PerCp-linked iNOS mAbs or control antibody, and data were collected as percent positive cells at the 99% confidence limit. The data presented are the mean of duplicate assessments. The intra-assay variability was less than 6%.

Granulocyte-macrophage progenitor assay

The NAMNC cells obtained from 9 patients with mild intermittent asthma and from 8 healthy subjects were depleted of E-rosette-forming cells by a second Ficoll-Hypaque centrifugation of a mixture of NAMNC cells and sheep red blood cells. T-cell-depleted (NANT) cells were obtained at the interface and cultured at a concentration of 2×10^5 cells in 1 mL of 0.9% methylcellulose supplemented with Iscove's modified Dulbecco's medium, 5×10^{-5} mol/L 2-mercaptoethanol, 1% BSA, 30% FCS, with or without growth factors, GM-

CSF (500 U/mL), stem cell factor (SCF) (50 ng/mL), and IL-3 (100 U/mL) in 35-mm dishes.²⁸ N^G-nitro-L-arginine methyl ester (L-NAME, 1 mmol/L) was added in the culture medium to inhibit endogenous NO generation. Sodium nitroprusside (SNP) was used as the NO donor. SNP (10^{-4} mol/L) solution was prepared immediately before use and added directly to the cell cultures. The cultures that were treated with SNP were exposed to light for 30 seconds to enhance NO release by a photolytic effect. Duplicate cultures were prepared for each sample and incubated for 14 days in a humidified atmosphere of 5% carbon dioxide in air. Some dishes of cultured cells from one healthy subject were contaminated at 14-day culture. Therefore only data on 7 healthy subjects were included in the analysis. Fourteen days after plating colonies were scored with use of an inverted light microscope. A colony was defined as a group of 40 or more cells. Cytochrome preparation smears of the cultured colonies and discrete cells were made for differential cell count determination.

To determine the colony forming unit-granulocyte-macrophage assay, some NANT cells were cultured on a single-layer agar culture system. On day 14 of culture, the agar disks containing colonies from each well were fixed with citrate buffer acetone-methanol fixative (Sigma), dried overnight, and stained for eosinophil peroxidase,²⁹ which is present in both mature and immature forms even when eosinophilic granules are not prominent.³⁰ Neutrophils were not stained by eosinophil peroxidase staining.³⁰ All experimental procedures were performed in endotoxin-free plasticware; according to the manufacturers' information, the levels of endotoxin contamination in the cytokine preparations were 3 endotoxin units per milligram by

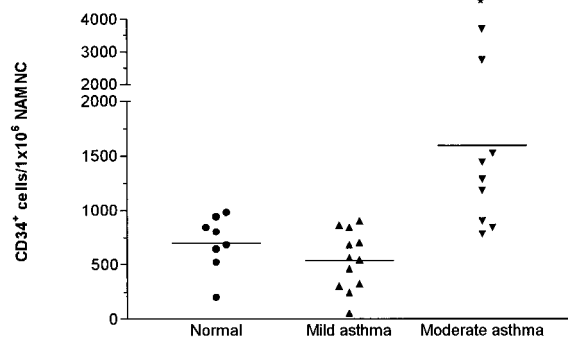


FIG 2. Numbers of CD34⁺ progenitor cells in peripheral blood of healthy subjects (normal, $n = 8$) or patients with mild intermittent (mild, $n = 12$) or moderate persistent asthma (moderate, $n = 8$). Asterisk, $P < .01$ compared with healthy subjects; horizontal line, mean value.

TABLE II. Serum levels of GM-CSF, IL-5, and IL-3 in healthy subjects and asthmatics

Cytokines (pg/mL)	Healthy subjects (n = 8)	Mild intermittent asthma (n = 12)	Moderately severe asthma (n = 8)
GM-CSF	2.1 ± 0.1	2.6 ± 0.1	3.2 ± 0.3*
IL-5	0.04 ± 0.02	0.6 ± 0.2†	1.2 ± 0.4†
IL-3	ND	ND	ND

Data are means ± SEM. ND, Not detectable.

* $P < .01$ compared with healthy subjects or mild intermittent asthmatics.

† $P < .01$ compared with healthy subjects.

the limulus assay, and antibodies used in all experiments contained <1 ng/mL endotoxin.

IL-5, GM-CSF, and IL-3 assay

Sera from 16 asthmatic patients and 7 healthy subjects were freeze-dried at -76°C under -3.0 torr for 24 hours. The samples were then resuspended in one tenth of original volume of PBS and stored at -80°C for later measurement of IL-5, GM-CSF, and IL-3 concentrations with a specific ELISA kit (R&D Systems, Minneapolis, Minn) with use of the quantitative immunometric sandwich enzyme immunoassay technique. The limit of detection for IL-3, IL-5, and GM-CSF was 3 pg/mL for the 3 cytokines.

Statistical analysis

Standard formulas were used for the analysis. Data did not approximate a gaussian distribution, nonparametric statistical analyses were used, and the probability of differences among groups was initially assessed by Kruskal-Wallis analysis. The number of subjects in each group was too small to allow for a strict median test between groups, and subsequent analysis was performed with the Mann-Whitney U test (2-tailed) to assess the significance of differences among groups. The relationship between serum levels of GM-CSF or IL-5 and the numbers of peripheral blood CD34⁺ progenitor cells in patients with asthma was sorted by the Spearman rank correlation test. Data are represented as mean ± SEM. The null hypothesis was rejected at $P < .05$.

RESULTS

CD34⁺ progenitor cells in peripheral blood

With use of multiparameter flow cytometric analyses, the number of bone marrow-derived CD34⁺ cells in the

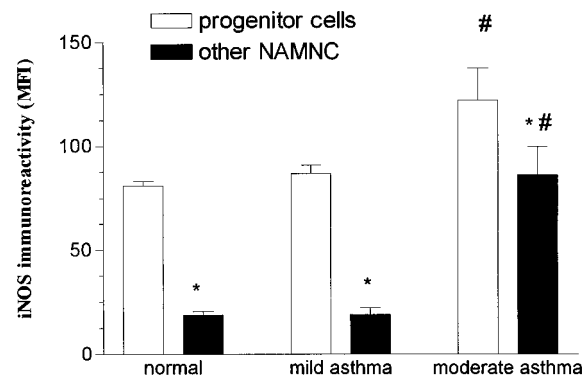


FIG 3. Mean fluorescence intensity (MFI) of iNOS activity of peripheral blood progenitor cells (open bars) or other NAMNCs (solid bars) in healthy subjects (normal, $n = 8$) or patients with mild intermittent (mild, $n = 12$) or moderate persistent (moderate, $n = 8$) asthma. Asterisk, $P < .01$ compared with progenitor cells in same group; pound sign, $P < .01$ compared with normal subjects. Data are means ± SEM.

peripheral blood was significantly increased in patients with moderate persistent asthma (1591.0 ± 323.8 cells/ 1×10^6 NAMNC, $n = 8$, $P < .001$) compared with those with mild intermittent asthma (537.7 ± 78.2 cells/ 1×10^6 NAMNC, $n = 12$) and healthy subjects (700.0 ± 89.8 cells/ 1×10^6 NAMNC, $n = 8$) (Fig 2). There was no significant difference in progenitor cell numbers between mild asthmatics and healthy subjects.

iNOS immunoreactivity of progenitor cells

The intracellular iNOS immunoreactivity was significantly higher in progenitor cells than that of other NAMNCs either in asthmatic or healthy subjects (Fig 3). Compared with healthy subjects, the iNOS immunoreactivities of progenitor cells or other NAMNCs were significantly higher in patients with moderate persistent asthma (121.7 ± 15.6 and 85.8 ± 13.8 mean fluorescence intensity [MFI] respectively, $n = 8$) than those of healthy subjects (80.8 ± 2.2 and 18.9 ± 1.5 MFI, respectively, $n = 8$, $P < .01$) (Fig 3). There was no significant difference in iNOS immunoreactivities in either CD34⁺ progenitor cells or other NAMNCs between healthy subjects and those with mild intermittent asthma (Fig 3).

Serum levels of GM-CSF, IL-3, and IL-5

There was no detectable level of IL-3 either in asthmatic or healthy subjects. Serum levels of GM-CSF were significantly higher in patients with moderate persistent asthma (3.2 ± 0.3 pg/mL, $n = 8$, $P < .01$) and in those with mild intermittent asthma (2.6 ± 0.1 pg/mL, $n = 12$, $P < .01$) than those of healthy subjects (2.1 ± 0.1 pg/mL, $n = 8$) (Table II). There were also significantly higher levels of IL-5 in both mild intermittent (0.6 ± 0.2 pg/mL, $n = 12$, $P < .01$) and moderate persistent asthma (1.2 ± 0.4 pg/mL, $n = 8$, $P < .01$) than in healthy subjects (0.04 ± 0.02 pg/mL, $n = 8$) (Table II). There was a significant correlation between the numbers of peripheral blood progenitor cells in asthmatics and the serum IL-5

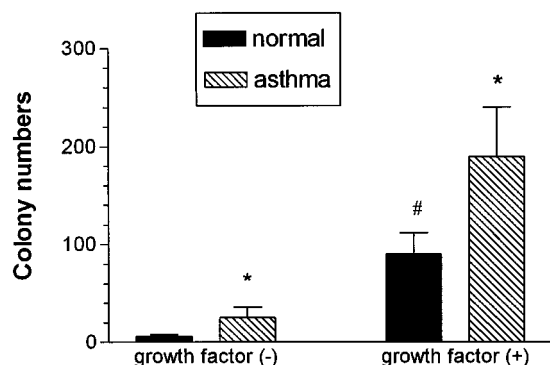


FIG 4. Colony numbers of progenitor cells cultured for 14 days in methylcellulose in presence (growth factor [+]) or absence of growth factors (growth factor [-]) from healthy subjects (normal, $n = 7$) or patients with mild intermittent asthma (asthma, $n = 9$). Asterisk, $P < .01$ compared with corresponding healthy subjects; pound sign, $P < .01$ compared with corresponding groups in absence of growth factors.

levels ($r_s = 0.41$, $P < .05$, $n = 20$) or GM-CSF ($r_s = 0.68$, $P < .001$, $n = 20$).

Colony formation of peripheral blood CD34⁺ cells

Colony formation from progenitor cells was performed by culturing NANT cells from healthy subjects ($n = 7$) and patients with mild intermittent ($n = 9$) because both had a similar percentage of CD34⁺ progenitor cells. There was a significantly greater number of colonies grown from NANT cells cultured in methylcellulose in the presence of growth factors, GM-CSF (500 U/mL), SCF (50 ng/mL), and IL-3 (100 U/mL) in asthmatic patients (189.9 ± 50.3 colonies, $n = 9$, $P < .01$) than in healthy subjects (90.0 ± 21.8 colonies, $n = 7$) (Fig 4). Even in the absence of growth factors, there was still a significant number of colonies grown from NANT cells from asthmatic patients (25.5 ± 10.4 colonies, $n = 7$, $P < .01$) compared with healthy subjects (5.8 ± 2.0 colonies, $n = 6$) (Fig 4).

No pure eosinophil colonies could be detected in agar cultures of NANT cells without growth factors. With growth factors (SCF, GM-CSF, and IL-3), a higher percentage of pure eosinophil colonies were grown from asthmatic ($61.0\% \pm 1.6\%$, $n = 3$) than from healthy subjects ($10.3\% \pm 2.4\%$, $n = 3$) (Table III). Cytochemical staining of cytopspin preparations of the cultured progenitor cells revealed a significantly higher percentage of cells of eosinophil lineage in asthmatic subjects ($12.6\% \pm 4.9\%$, $n = 9$, $P < .01$) than that in healthy subjects ($0.04\% \pm 0.04\%$, $n = 7$) in the presence of growth factors (Table IV).

Effect of NO on colony formation of CD34⁺ cells

There was a significantly greater number of colonies grown from NANT cells treated with L-NAME (1 mmol/L) than those from cells with growth factors alone either in asthmatic (273.8 ± 68.4 colonies, $n = 9$) or healthy subjects (163.9 ± 14.3 colonies, $n = 7$) (Fig 5).

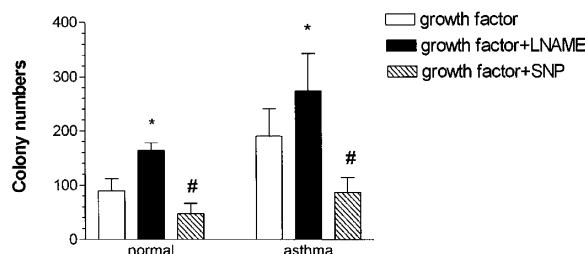


FIG 5. Effect of L-NAME (1 mmol/L) or SNP on colony numbers of progenitor cells cultured for 14 days in methylcellulose in presence of growth factors, GM-CSF, SCF, and IL-3 from healthy subjects (normal, $n = 7$) or patients with mild intermittent asthma (asthma, $n = 9$). Asterisk, $P < .01$; pound sign, $P < .05$ compared with corresponding groups with growth factors alone.

TABLE III. Percentage of pure eosinophil colonies grown on single-layer agar culture for 14 days in healthy ($n = 3$) and asthmatic subjects ($n = 3$)

	G(-)	G(+)	G(+)+LN	G(+)+SNP
Normal	0.0 $\pm$ 0.0	10.3 $\pm$ 2.4	10.7 $\pm$ 2.3	7.5 $\pm$ 1.9
Asthma	0.0 $\pm$ 0.0	61.0 $\pm$ 1.6*	55.4 $\pm$ 4.4*	49.6 $\pm$ 1.0

Data are means $\pm$ SEM. G(-), Without growth factors; G(+), with growth factors; LN, L-NAME.

* $P < .01$ compared with corresponding group of healthy subjects.

L-NAME treatment did not affect colony formation in the absence of growth factors. SNP significantly inhibited the growth of colonies either in asthmatic (86.7 ± 27.5 colonies, $n = 9$) or healthy subjects (47.6 ± 19.1 colonies, $n = 7$) (Fig 5). SNP did not affect the viability of cultured cells at 30 minutes and 14 days when tested by trypan blue exclusion. There was no significant change in the percentage of eosinophil lineage development determined by cytopspin preparations of methylcellulose cultured cells by L-NAME or SNP treatments either in asthmatic ($17.4\% \pm 5.6\%$ and $18.3\% \pm 2.1\%$, $n = 9$, respectively) or healthy subjects ($0.3\% \pm 0.2\%$ and $1.4\% \pm 0.8\%$, $n = 7$, respectively) (Table IV). Neither was there any significant change in the percentage of pure eosinophil colonies grown from agar culture or pure eosinophil colonies grown from agar culture by L-NAME or SNP (Table III).

Comparison between atopic and nonatopic asthmatics

There was no significant difference in the CD34⁺ progenitor cell number, serum IL-5 or GM-CSF levels in the peripheral blood, and iNOS activities of progenitor cells between atopic and nonatopic mild intermittent asthmatics. Neither was there any significant difference in progenitor cell growth in response to L-NAME or SNP treatments between atopic and nonatopic patients with mild intermittent asthma.

DISCUSSION

In the current study we have shown that there are increased numbers of CD34⁺ progenitor cells in the

TABLE IV. Differential cell count (percentage) of colonies after culture for 14 days in healthy subjects (n = 7) and mild asthmatics (n = 9)

	Blast	Pro	Myelo	Meta	Band	Seg	Eos	Baso	Mac
Normal									
G(-)	78.5 ± 4.4	4.0 ± 2.4	1.7 ± 0.7	0.4 ± 0.2	0.3 ± 0.1	1.1 ± 1.0	0.0 ± 0.0	0.2 ± 0.2	14.2 ± 2.2
G(+)	61.0 ± 8.0	14.1 ± 5.9	6.9 ± 2.6	1.7 ± 0.7	3.6 ± 0.8	5.0 ± 0.8	0.04 ± 0.04	0.3 ± 0.3	7.2 ± 3.7
G(+) + LN	47.4 ± 9.3	15.6 ± 5.6	8.9 ± 1.3	1.9 ± 0.8	7.4 ± 2.3	6.2 ± 1.2	0.3 ± 0.2	0.9 ± 0.4	11.0 ± 2.7
G(+) + SNP	48.5 ± 4.7	12.4 ± 4.0	19.6 ± 4.1	0.6 ± 0.3	5.6 ± 2.4	5.1 ± 2.2	1.4 ± 0.8	2.1 ± 1.0	6.2 ± 2.9
Asthma									
G(-)	74.3 ± 5.3	1.3 ± 0.5	5.9 ± 2.9	0.7 ± 0.7	3.1 ± 0.9	3.7 ± 1.1	2.1 ± 0.7	0.0 ± 0.0	8.9 ± 4.1
G(+)	51.2 ± 9.2	3.5 ± 2.1	14.0 ± 5.5	1.6 ± 0.8	4.7 ± 1.9	7.8 ± 3.1	12.6 ± 4.9*	0.4 ± 0.3	3.4 ± 1.2
G(+) + LN	47.8 ± 8.8	4.0 ± 1.2	10.6 ± 2.1	1.2 ± 0.5	5.7 ± 1.2	9.5 ± 2.7	17.4 ± 5.6*	0.1 ± 0.1	4.1 ± 1.8
G(+) + SNP	33.8 ± 4.3	2.4 ± 0.7	14.3 ± 2.8	4.2 ± 2.4	7.9 ± 1.5	12.9 ± 3.3	18.3 ± 2.1*	0.0 ± 0.0	4.2 ± 1.0

Data are means ± SEM. *Pro*, Promyelocytes; *Myelo*, myelocytes; *Meta*, meta-myelocytes; *Seg*, segment; *Eos*, eosinophils; *Baso*, basophils; *Mac*, macrophages; *G(-)*, without growth factors; *G(+)*, with growth factors; *LN*, L-NAME.

**P* < .01 compared with corresponding group of healthy subjects.

peripheral blood of patients with moderate persistent asthma but not in that of patients with mild intermittent asthma. When cultured on methylcellulose, CD34⁺ progenitor cells of mild intermittent asthmatics formed a greater number of colonies in culture with an increased proportion of eosinophil lineage compared with those of healthy subjects. Progenitor cells in the peripheral blood of asthmatic patients therefore possessed enhanced growth potential and were committed to the preferential development of the eosinophil lineage.

IL-5 is an important growth factor acting specifically on the eosinophil precursors. However, there is growing evidence suggesting that progenitor cells from asthmatic patients are hyperresponsive to the exogenous IL-5 treatment, probably as a result of an increase in IL-5 receptor expression.^{31,32} Thus, to avoid the enhancement effect of IL-5 to render the interpretation of the inhibitory effect of NO on progenitor cell growth in asthmatic patients, we chose not to use exogenous IL-5 as a growth factor in our culture system.

The factors responsible for stimulating bone marrow progenitors in asthma are not yet completely elucidated. Evidence for the generation of a serum hematopoietic factor during airway allergen challenge that can prime the bone marrow for increased production of granulocyte progenitor cells has been demonstrated in a canine model of airway hyperresponsiveness.³³ Of the cytokines that can support eosinophilopoiesis (IL-5, IL-3, and GM-CSF), GM-CSF and IL-3 enhance pluripotential CD34⁺ progenitor cells to differentiate into eosinophils in response to IL-5,³⁴ with up-regulation of IL-5 receptor expression.³¹ IL-5 is crucial in the terminal differentiation of less-primitive progenitor cells into eosinophils.³⁵ Elevated serum IL-5 levels have been demonstrated in asthmatic patients.³⁶ In the current study we also showed that there were increased levels of GM-CSF and IL-5 in the serum of asthmatic compared with healthy subjects. The source of GM-CSF and IL-5 in the sera of asthmatic subjects is not known. The numbers of CD34⁺ progenitor cells in the peripheral blood were significantly correlated to GM-CSF and IL-5 levels, supporting the concept

that these cytokines can prime progenitor cells to proliferate and enhance their subsequent ability to differentiate into eosinophils.

There was an increased intracellular iNOS immunoreactivity in the progenitor cells of patients with moderate persistent asthma. Although we did not provide direct evidence to show that the immunoreactivity of iNOS reflects the enzyme activity and NO release in this study, our previous study²³ using the similar technique has demonstrated that there is a high correlation between iNOS immunoreactivity and NO production in alveolar macrophages. We also demonstrated that an inhibition of iNOS activity by L-NAME significantly increased the colony growth of CD34⁺ progenitor cells, whereas an NO donor decreased the colony growth from these cells. There were higher iNOS activities in CD34⁺ progenitor cells than in other NAMNCs in either asthmatic or healthy subjects. That may suggest that endogenous NO production in peripheral blood progenitor cells of asthmatic patients is enhanced and could play a regulatory role in inhibiting the expansion of the progenitor cells in an autocrine fashion. However, iNOS expression is not restricted to CD34⁺ progenitor cells but is also present in other NAMNCs. Thus the colony growth of CD34⁺ progenitor cells may be also affected by NO released from other cells in a paracrine fashion. This notion was supported by our results that exogenous administration of SNP, an NO donor, significantly inhibited the colony growth of progenitor cells. However, it is also possible the effect of SNP was through a nonspecific inhibitory or toxic effect on the cultured progenitor cells or inhibiting cytokine-mediated signaling by mechanisms other than through NO production.

There was no significant change in the percentage of eosinophil lineage after L-NAME or SNP treatment compared with growth factor treatment alone, suggesting that NO inhibited nonselectively all myeloid colony growth. Thus modulation of NO generation may influence not only the progenitor cells of eosinophil lineage but also the colony growth of other myeloid committed progenitor cells that were present in the mononuclear cell populations.

The mechanisms for NO-mediated suppression of myeloid colony growth were not elucidated in the current study. NO production has been demonstrated to be toxic to surrounding cells or autotoxic by binding to iron in the prosthetic groups of a variety of enzymes,^{10,12,37,38} by S-nitrosation of proteins,^{39,40} and by adenosine diphosphate ribosylation leading to posttranslational modification of proteins.⁴¹ DNA toxicity with single-strand DNA breaks and base deamination may be also responsible for NO-mediated cytotoxicity.^{15,42,43} NO-mediated DNA damage might be responsible for poly-adenosine diphosphate ribosylation and subsequent apoptosis.⁴⁴ These mechanisms have been proposed for NO-mediated inhibition of colony formation from bone marrow CD34⁺ cells.²⁵

Several proinflammatory cytokines in asthmatic inflammation, including TNF- α , IL-1 β , and IFN- γ ²⁰ have been demonstrated to induce iNOS expression of progenitor cells.²¹ The pathophysiologic consequences of increased NO production in asthma is not known. NO inhibits IFN- γ production by T-helper-1 cells, leading to proliferation of T-helper-2 cells, resulting in increased production of IL-4 and IL-5, which leads to IgE synthesis, mast cell sensitization, and eosinophil recruitment.⁴⁵ We now show that the increased NO production in asthma may limit bone marrow-derived proinflammatory cell growth and colony formation. This role for endogenous NO may also be important in progenitor cells from nonasthmatic patients and those with mild asthma.

In conclusion, we have demonstrated that progenitor cells in the peripheral blood of patients with asthma possess higher proliferation potential and are particularly committed into an eosinophil lineage. The production of NO from progenitor cells or other circulating cells may act in an autocrine or paracrine fashion to suppress progenitor cell growth and colony formation. This could represent a beneficial effect of endogenous NO.

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