Effects of Tachykinins and Histamine on the Expression of Thymus- and Activation-Regulated Chemokine mRNA in Peripheral Blood Mononuclear Cells and Human Bronchial Epithelial Cells

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We hypothesized that participation of sensory neuropeptides, substance P and neurokinin A in the airway inflammation caused to resistance against the glucocorticoids (GCs) treatment. We have measured the expression level of cytokines and chemokines mRNAs in bronchoalveolar lavage cells (BAL), transbronchial lung biopsies (TBLB) and peripheral blood mononuclear cells (PBMC) by reverse transcriptase polymerase chain reaction (RT-PCR). The mRNAs of interleukin-4 (IL-4) and -5, which are typical Thelper 2 (Th2)-type cytokines, were scarcely detected in all of bronchoalvelar lavage fluid (BALF) and TBLB samples. But the mRNA of Th1-type cytokines, such as IL-12, IL-18, and interferon-γ (IFN-γ), tended to be expressed higher than that of Th2-type cytokines at the early stage of asthma before intervention with GCs. The mRNAs of IL-18, IFN-γ, macrophage-derived chemokine (MDC) and thymus- and activation-regulated chemokine (TARC) tended to be expressed higher than that of non-asthmatic subjects. *In vitro* study demonstrated that the expression of TARC mRNA induced by the combination of IL-4, IFN-γ, and neurokinin A in human bronchial epithelial cells, BEAS-2B was not inhibited by the treatment of the cells with dexamethasone even at 10^{-6} M. We proposed that the part of resistance to GCs in the asthmatic patient without GC receptor β expression might be associated with the induction of TARC expression in airway epithelial cells by tachykinines and histamine.

Key words —— neurokinin A, histamine, thymus- and activation-regulated chemokine, asthma

INTRODUCTION

Resting $CD4^+$ T cells are polarized to either a Thelper 1 (Th1) or a Th2 phenotype by the immune response. Romagnani has reported the Th1/Th2 paradigm in which inflammation associated with allergic disorders is regarded as a Th2-dominant immune response.[1)] The cytokines released from allergic reactive Th2 cells control the processes leading to allergic inflammation.[2)] In a general way, Th1 and Th2 subsets are functionally separated based on their profiles of cytokine production. However, Calhoun *et al*. and other groups have demonstrated increased production of Th1-type cytokines and interferon-γ (IFN-γ) by bronchoalveolar lavage (BAL) cells in asthmatic compared with control subjects.[3–5)] Furthermore, increased levels of serum IFN-γ have been shown to be associated with disease severity in severe asthma.[6)] These findings suggest that asthma is not a pure Th2 disease.[7–9)] In addition, chemokine receptors are selectively expressed in either Th1 or Th2 cells. Th1 cells express C–C chemokine receptor 5 (CCR5) and CXC chemokine receptor 3 (CXC), whereas Th2 cells express CCR3, CCR4, and CCR8.[10–12)] The high affinity ligands for CCR4, macrophage-derived chemokine (MDC) and thymus- and activation-regulated chemokine (TARC), induce selective migration of Th2 cells.[13,14)] Although there are arguments about cells expressing CCR4, accumulated evidence to date strongly supports that both MDC and TARC play dominant roles in Th2-

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type disease conditions, such as atopic dermatitis and bronchial asthma.[15–18] Sekiya *et al*. have reported that the expression of TARC and MDC in human bronchial epithelial cells are induced by cytokines.[19]

Additionally, neural mechanisms have been suggested to contribute to the pathogenesis of chronic asthma. Excessive activity of cholinergic nerves may be important in asthma. Dysfunction of cholinergic receptor, muscarinic, 2 (M2) in asthma may lead to excessive bronchoconstriction and mucus secretion.[20] The sensory neuropeptides substance P (S-P) and neurokinin A (NKA) are localized to sensory airway nerves, from which they can be released by a variety of stimuli, including allergen, ozone, or inflammatory mediators. However, it is becoming increasingly evident that inflammatory cells such as eosinophils, macrophages, lymphocytes, and dendritic cells can produce the tachykinins S-P and NKA.[21] Moreover, immune stimuli can boost the production and secretion of S-P and NKA. S-P and NKA have potent effects on bronchomotor tone, airway secretions, and bronchial circulation and on inflammatory and immune cells. Tachykinins contract smooth muscle mainly by interaction with NK2 receptors, while the vascular and proinflammatory effects are mediated by the NK1 receptor.[21] In addition, tachykinins induce the release of histamine from human bronchoalveolar lavage mast cells as an indirect effect by degranulation.[22] The development of some allergic reactions, infections, and tumors are associated with excessive histamine production and a shift toward Th2 response.[23] However, the effect of tachykinin and histamine on neuroimmune functions has not been completely elucidated, and recent evidence suggests an important role for these molecules linking the neuroimmune system and inflammatory events.

MATERIALS AND METHODS

Reagents —— Recombinant human interleukin-2 (IL-2), IL-4, IL-18, IFN-γ and tumor necrosis factor-α (TNF-α) were purchased from Genzyme Corporation (Cambridge, MA, U.S.A.). Histamine dihydrochloride was obtained from NACALAI TESQUE (Kyoto, Japan). S-P and α-NKA were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). Bovine serum albumin (BSA) RIA and ELISA grade (fatty acid free) was obtained from United Biotechnological (Tokyo, Japan).

BAL —— BAL was performed under local anesthesia with 2% lidocaine. A fiberoptic bronchoscope was gently wedged into the segmental bronchus of the middle lobe of the right lung. Sterile 0.9% saline totaling 150 ml was instilled in aliquots and recovered by gentle hand suction. Mucus was removed from the fluid by filtration with two sheets of gauze. Lavage fluid was centrifuged at $400 \times g$ for 10 min at 4°C to separate cells from cell-free fluid. The cell-free lavage fluid was stored at –80°C until further analysis. The number of cells in the bronchoalvelar lavage fluid (BALF) was then determined using a hemocytometer. Cell differentials were determined by Giemsa staining for 500 cells prepared by cytocentrifugation.

Transbronchial Lung Biopsies (TBLB) —— Five transbronchial lung biopsies (TBLB) were taken using alligator forceps (Olympus, FB 15C, Tokyo, Japan) and stained with hematoxylin and eosin for routine clinical assessment of acute or chronic rejection according to standard criteria. The assessment, performed by an expert specialist transplant pathologist, was blinded with respect to the other investigations and yielded a standard grade of inflammation.

Patients Subjected to BAL or TBLB —— We studied 15 asthmatic subjects without inhaled steroid and 3 asthmatic subjects with inhaled steroid ranging in age from 23 to 66 years old. Interstitial pneumonia and sarcoidosis patients were used as 4 non-asthmatic subjects. Eighteen BAL samples, containing four non-asthmatic subjects, were obtained and 5 TBLB samples from asthmatic subjects. Their clinical characteristics are summarized in Table 1.

Separation and Cultivation of Peripheral Blood Mononuclear Cells (PBMC) and BEAS-2B Cells —— Subjects collected peripheral blood mononuclear cells (PBMC) were different from the patients obtained BALF and TBLB samples. In detail, there were 3 normal, 2 moderate asthmatic and 3 severe asthmatic subjects. PBMC were separated from heparinized venous blood samples by density gradient centrifugation on Lymphoprep (NYCOMED PHARMA AS, Oslo, Norway). The cells aspirated from the interface of the density gradient were washed three times with phosphate buffured saline (PBS). The cells were suspended in cell culture media RPMI 1640, containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin. The cells (1×10^6 /ml) were cultured in a 96 well flat-bottomed plate (SUMILON MS-8096F) at 5% CO_2, 37°C, and 95% humidity.

Table 1. Clinical Characteristics of the Study Subjects

	No.	Age (Y)	Sex	PC_{20} (mg/ml)	FEV_1 (%)	%FVC	Medication (μg/d)	RAST
Non-asthmatic subjects								
BAL	1	40	M	—	78.9	106.2		
	2	40	F	—	86.1	86.8	—	House dust mites
	3	44	M	—	80.2	67.7	—	Cat, Dog, House dust mites
	4	76	M	—	80.2	80.3	—	—
Asthmatic subjects								
BAL	5	23	F	250	64.3	113.4	BDP1200	Cat, Dog, House dust mites
	6	66	F	156	79.4	110.2	—	—
	7	30	F	180	76	67.5	—	—
	8	36	F	370	90	74.6	BDP800	—
	9	36	M	500	85.3	73.6	—	Cat, Dog, House dust mites
	10	57	F	8000	83.9	117.2	—	—
	11	33	F	470	94.1	115.8	—	—
	12	23*	F	10000	73.6	115.3	—	Cat, House dust mites
	13	33	M	65	71.9	105.9	—	—
	14	41	M	113	75.7	108.4	—	House dust mites
	15	23	M	—	88.1	80.4	—	House dust mites
	16	49	F	—	79.3	94.1	FP400	House dust mites
	17	40	M	1150	67.8	79.6	—	House dust mites
	18	43	M	—	84.6	103	—	—
TBLB	19	32	M	180	82.1	122.8	—	House dust mites
	20	23*	F	10000	73.6	115.3	—	Cat, House dust mites
	21	23	M	1100	75.5	115.9	—	Cat, House dust mites
	22	23	M	1570	75.5	103.7	—	Cat, Dog, House dust mites
	23	49	F	50	80	101.4	—	House dust mites

*: BALF (No. 12) and TBLB (No. 20) is the same asthmatic subject. BDP: beclometasone dipropionate. FP: fluticasone propionate.

BEAS-2B cells, human bronchial epithelial cell line, were cultured in LHC-8 medium (Biofluids, Rockville, MD, U.S.A.). The BEAS-2B cells were plated on 24-well culture plates (Coastar 3524) in DMEM/Ham's F12 medium containing 10% FCS and antibiotics. Cells were used between passages 39 and 46, and they were grown to 80% confluence in the medium containing 5% FCS for one day before usage. On the stimulation, all of the culture medium in each well was replaced by an identical formulation containing 0.1% BSA in place of FCS.

Treatment of Cultured Cells with Stimulators —— To induce cytokines and chemokines, the cultured cells were treated with histamine in PBS, S-P, or NKA in PBS containing 0.1% BSA at a final concentration of 10^{-4} M, 10^{-8} M and 10^{-8} M, respectively. Phytohemagglutinin (PHA; Invitrogen Co., Carlsbad, CA, U.S.A.) was used as a positive control. After the treatment for 16–18 hr, mRNA was extracted from the treated cells, and the expression of cytokine and chemokine mRNA was examined by reverse transcriptase polymerase chain reaction (RT-PCR).

RT-PCR and PCR —— The presence of cytokine-specific transcripts was detected using RT-PCR. Total RNA from PBMC, BEAS-2B cells, BAL cells, and TBLB samples was extracted by the ISOGEN (Nippon Gene, Toyama, Japan) and reverse transcribed using the Thermoscript RT-PCR kit (Invitrogen Co., Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. The RNA (PBMC: 200 ng, BALF and TBLB: 1 μg, BEAS-2B: 3 μg) was washed, denatured (at 65°C for 5 min), and exposed to avian myebblastosis virus (AMV) reverse transcriptase (15 U) in the additional presence of RNAaseOUT (40 U), deoxyribonucleoside triphosphate mix (10 mmol/l) and the supplied reverse transcription (RT) buffer. The reaction was allowed to continue for 1 hr at 50°C, after which the RT product was denatured (at 95°C for 2 min). PCR

Table 1. Continued

	No.	Infant asthma	Smoke	Number of cells (cells/mlj	% of cells population				
Non-asthmatic subjects					Macrophage	Lymphocyte	Eosinophil	Neutrophil	Basophil
BAL	1	—	—	2.0×10^5					
	2	—	+	2.0×10^5	81	17	1	1	0
	3	—	+	4.9×10^5	87	5	8	0	0
	4	—	+	2.2×10^5	100	0	0	0	0
Asthmatic subjects									
BAL	5	—	—	4.9×10^5	52	40	7	0	1
	6	—	+	2.8×10^5					
	7	—	—	4.4×10^5	57	28	14	0	1
	8	—	+	5.6×10^5	97	3	0	0	0
	9	+	—	4.5×10^5	67	0	33	0	0
	10	—	—	7.4×10^5	77	21	2	0	0
	11	—	+	6.6×10^5	97	1	2	0	0
	12	+	+	2.1×10^5	94	5	1	0	0
	13	+	+	3.7×10^5	93	4	1	1	1
	14	—	+	4.3×10^5	97	3	0	0	0
	15	+	+	2.7×10^5	73	20	7	0	0
	16	—	—	2.9×10^5	99	1	0	0	0
	17	—	+	3.9×10^5	61	1	38	0	0
	18	—	+	7.1×10^5	50	27	23	0	0
TBLB	19	+	+						
	20	+	+						
	21	+	+						
	22	—	—						
	23	—	—						

amplification was carried out on 2–5 μl aliquots of reverse transcribed cDNA in 50 μl reaction using 0.5 U of EX Taq polymerase (TAKARA SHUZO Co., Shiga, Japan) as suggested by the supplier. The specific primers for cytokines or chemokines were employed to generate the cDNAs. The sequences of specific primers used are given in Table 2. The PCR products were visualized on 2% agarose gels and were analyzed comparatively to the amount of β-actin (internal standard) detected in the same mRNA sample.

RESULTS

Expression of Cytokine and Chemokine mRNAs in BALF and TBLB

To investigate the inflammatory process of bronchial asthma, we isolated mRNA from BALF and TBLB obtained from asthmatic subjects, and semiquantitative RT-PCR was performed to detect the expression of cytokines and chemokines. The clinical characteristics of the tested subjects are summarized in Table 1. As shown in Table 3, the mRNAs of IL-4 and 5, which are typical Th2-type cytokines, were not detected in all BALF and TBLB samples tested, except for the TBLB sample from patient 19. IL-13 mRNA was detected only in the BALF samples from Pts. 6, 10, and 15, and in the TBLB sample from Pt. 19, but the expression level was very low. IL-10 mRNA was detected in almost all of the tested BALF samples from asthmatic subjects, but not in Pt. 13. The mRNA of the Th1-type cytokines, such as IL-12, IL-18, and IFN-γ tended to be expressed higher than that of the Th2-type cytokines. In addition, the MDC and TARC mRNAs in BALF and TBLB samples from asthmatic subjects were relatively higher than that of normal subjects (Fig. 1).

The mRNA of granulocyte macrophage-colony stimulating factor (GM-CSF) involved in eosinophil survival and proliferation was detected in BALF samples from asthmatic patients, but not in TBLB samples (Table 3).

Table 2. Primers for RT-PCR

Gene	Sense primers	Anti-sense primers
β-actin	5′-GAC CCA GAT CAT GTT TGA GAC-3′	5-CTT C′AT GAG GTA AGT CAG G-3′
GM-CSF	5′-AGC ATG TGA ATG CCA TCC-3′	5′-ATC TGG GTT GCA CAG GAA G-3′
IL-2	5′-CAA CTC CTG TCT TGC ATT GC-3′	5′-ATG GTT GCT GTC TCA TCA TCA GC-3′
IL-4	5′-GAC ACA AGT GCG ATA TCA CC-3′	5′-CCA ACG TAC TCT GGT TGG CT-3′
IL-5	5′-CGA ACT CTG CTG ATA GCC AA-3′	5′-CCA CTC GGT GTT CAT TAC AC-3′
IL-6	5′-ACA GAC AGC CAC TCA CCT C-3′	5′-CAG GAA CTC CTT AAA GCT GC-3′
IL-8	5′-CTG ATT TCT GCA GCT CTG TG-3′	5′-TTC ACT GGC ATC TTC ACT G-3′
IL-10	5′-ATG CAC AGC TCA GCA CTG CT-3′	5′-CCT TGA TGT CTG GGT CTT GG-3′
IL-12 p40	5′-GTC ACC AGC AGT TGG TCA TC-3′	5′-CAA ATC AGT ACT GAT TGT CGT CAG-3′
IL-13	5′-TGA CCA CGG TCA TTG CTC-3′	5′-TCT CGG ACA TGC AAG CTG-3′
IL-18	5′-ATG GCT GCT GAA CCA GTA GA-3′	5′-AGT CTT CGC TTT GAA CAG TG-3′
MDC	5′-ACA TAC AGG ACA GAG CAT GG-3′	5′-AGT AGG CTC TTC ATT GGC TC-3′
TARC	5′-TCT CTG CAG CAC ATC CAC G-3′	5′-GAC CTC TCA AGG CTT TGC AG-3′
IFN-γ	5′-GTT CTC TTG GCT GTT ACT GC-3′	5′-CGA AAC AGC ATC TGA CTC C-3′

Table 3. Cytokine mRNA Expression in BALF and TBLB Samples Obtained from the Non-Asthmatic and Asthmatic Subjects

BALF

	No. 1*	No. 2*	No. 3*	No. 4*	No. 5	No. 6	No. 7	No. 8	No. 9	No. 10	No. 11	No. 12
IL-2	—	—	—	—	—	0	0	0	0	—	—	0
IL-4	—	—	—	—	—	0	0	0	0	—	—	0
IL-5	—	—	—	—	—	0	0	0	0	—	—	0
IL-6	—	—	—	—	—	0.136	0.108	0.121	0	—	—	0.684
IL-8	—	—	—	—	0.752	0.617	0.689	0.81	0.859	0.899	0.795	1.055
IL-10	—	—	—	—	0.696	0.013	0.14	0.11	0.067	0.786	—	0.248
IL-12	0	—	—	—	0.453	—	0.141	0.054	—	0.453	—	—
IL-13	—	—	—	—	—	0.013	0	0	0	0.145	—	0
IL-18	0.071	—	—	0.11	0.236	0.809	0.518	0.691	0.448	0.83	0.583	1.12
IFN-γ	0.509	—	—	—	0.661	0.098	0.068	0.507	0.274	1.006	0	0.863
MDC	0.29	—	—	0.182	0.193	0.832	0.824	0.868	0.275	0.774	0.644	0.709
TARC	0.201	—	—	0.050	0	0.805	0.720	0.801	0.198	0.711	0.805	0.564
GM-CSF	—	—	—	—	0.438	0.064	0	0.074	0.041	0.686	—	0.316

BALF TBLB

	No. 13	No. 14	No. 15	No. 16	No. 17	No. 18	No. 19	No. 20	No. 21	No. 22	No. 23
IL-2	—	—	0	—	—	—	0.013	0	0	0	0
IL-4	—	—	—	—	—	—	0	0			
IL-5	—	—	—	—	—	—	0.067	0	0	0	0
IL-6	—	—	0.188	—	—	—	—	0.133	0	0.234	—
IL-8	0.378	0.885	—	—	—	—	—	0.608	0.593	0.099	—
IL-10	0	0.255	0.792	—	—	—	0.047	0.108	0	0	—
IL-12	0.041	0.061	0.396	—	—	—	0	0.114	0	0	—
IL-13	—	—	0.063	—	—	—	0.06	0	0	0	0
IL-18	0	1.01	0.672	0.219	0.254	—	0.407	0.228	0	0	0.218
IFN-γ	1.014	1.051	0.995	—	—	—	0	0.11	0.134	—	—
MDC	0.712	0.782	0.775	0.377	0.476	0.504	0.647	0.392	0.475	0.207	0
TARC	0.250	1.021	0.385	0.397	0.065	0.185	0.147	0.095	0.373	0.159	0.995
GM-CSF	0.055	—	0.245	—	—	—	0	0	0	0	—

—: did not measured. *: The number indicated a non-asthmatic subject. BALF (No. 12) and TBLB (No. 20) is the same asthmatic subject. The data showed the ratio of cytokines to β-actin.

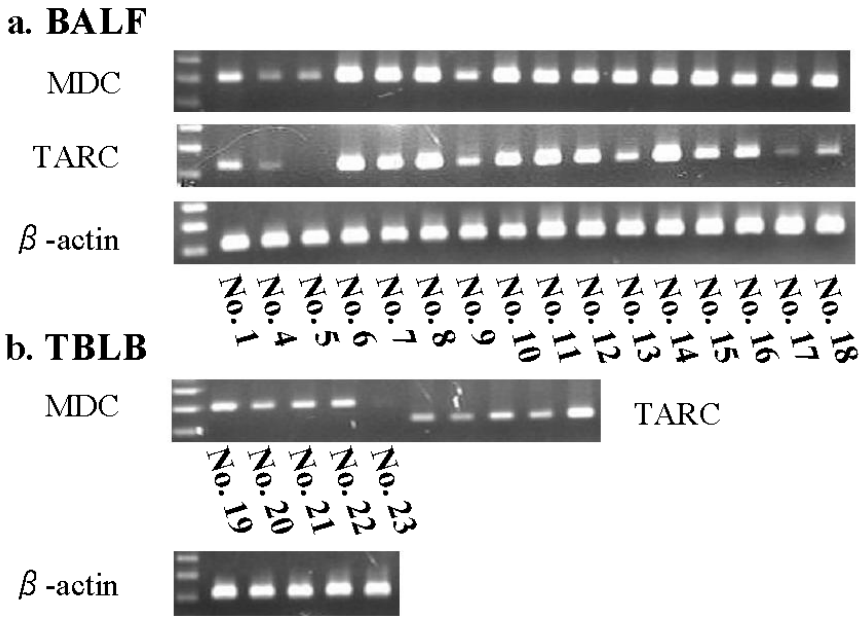

Fig. 1. Expression of TARC and MDC mRNA in BALF Cells (a) and TBLB (b)

The TARC and MDC transcripts in the samples from non-asthmatic and asthmatic patients were identified by RT-PCR and then agarose gel electrophoresis. The clinical characteristics of these are summarized in Table 1. BALF (No. 12) and TBLB (No. 20) were the samples from same asthmatic subject.

Effect of Tachykinins and Histamine on the Expression of TARC mRNA in PBMC

To investigate the effects of tachykinins and histamine on the expression of TARC mRNA in PBMC, the PBMCs from moderate asthmatic patients, steroid-resistant severely asthmatic patients, and normal subjects were treated with tachykinin or histamine. After the incubation with stimulators for 16–18 hr, total RNA was extracted from PBMC, reverse-transcribed, and subjected to TARC specific PCR. As a positive control, phytohemagglutinin (PHA) was used to activate the PBMC. In all of the experiments, the induction level was expressed as a ratio of TARC mRNA to β-actin mRNA. As shown in Fig. 2, although the expression of TARC mRNA was not induced in normal PBMC by NKA, S-P, or histamine, the different responses to these stimulators were detected in PBMC samples from patients. In the PBMC from moderate asthmatic subjects, the strongest induction of the TARC mRNA by PHA was detected as well as normal subject. The ratio of TARC/β-actin was 0.574, and 0.558 in the normal subjects and moderate asthmatic subjects, respectively. The induction of TARC mRNA by NKA, S-P, or histamine was very weak in both moderate and normal subjects. The same results were obtained from 2 or 3 independent experiments. In contrast, the TARC mRNA was not induced by PHA in PBMC from the glucocorticoid-resistant (GC-resistant) severe asthmatic patient, but was strongly induced by NKA or histamine. In this patient, the ratio of TARC/β-actin was 0.487 by NKA, and 0.370 by histamine. It showed a marked augmentation among 3 independent experiments of GC-resistant severe asthmatic patients. In the other 2 severe asthmatic patients, the TARC mRNA was completely inhibited under all stimulated conditions.

Effect of Dexamethasone (DEX) on the Expression of TARC and GM-CSF mRNA by IL-4 in PBMC

The TARC mRNA induced by PHA in normal PBMC was reduced to control levels by the treatment with 10^{-6}–10^{-7} M dexamethasone (DEX). While the inhibitory effect of DEX on the TARC mRNA expression by IL-4 was a little even at 10^{-6}–10^{-7} M DEX. When TARC expression was induced by the combination of IL-4 and NKA or Histamine (His), DEX inhibited the induction in a dose-dependent manner, but the inhibitory level was very low. (Fig. 3a) The same experiment was performed for GM-CSF mRNA. It was completely inhibited to the control level by DEX in all conditions (Fig. 3b). Similar results were obtained when normal PBMC were treated with the combination of IL-4, IFN-γ and TNF-α to induce the expression of TARC and

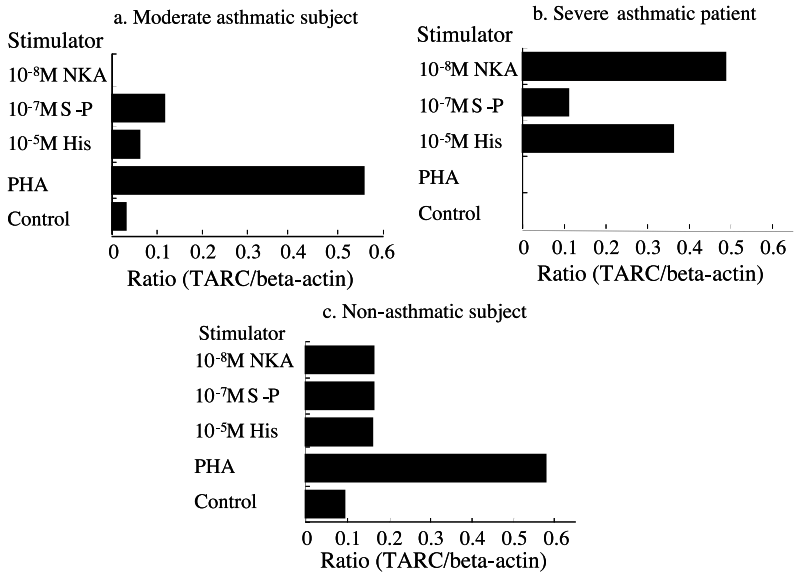

Fig. 2. Induction of TARC mRNA in PBMC from Normal, Moderate Asthmatic and GC-Resistant Severe Asthmatic Subject

PBMC (1×10^6 cells/ml) from each subject were cultured in a 96 well flat-bottomed plate at 100 μl/well and added the indicated stimulator. After 16–18 hr cultivation, the total RNA was isolated from the cultured cells, and the mRNA for TARC was detected by semiquantitative RT-PCR. The data indicated as typical for 3 experiments with normal subjects and 2 experiments with moderate asthmatic subjects, but the specific subject was presented on Fig. 2b among 3 experiments with severe asthmatic subjects. The data was presented as the ratio of TARC to β-actin (see Materials and Methods).

GM-CSF mRNA (data not shown).

Effect of DEX on the Expression of TARC and GM-CSF mRNA Induced by the Combination of IL-4, IFN-γ, and TNF-α in Human Bronchial Epithelial Cells BEAS-2B

To investigate the expression of TARC and GM-CSF mRNAs in airway epithelium, human bronchial epithelial cells, BEAS-2B, were treated with IL-4, IFN-γ and TNF-α to induce the expression of TARC and GM-CSF mRNAs. As shown in Figs. 4a and 4b, the expression of GM-CSF mRNA induced by the combination of these cytokines was inhibited with DEX in a dose-dependent manner. Under the influence of NKA or His in addition to the above, the expression of GM-CSF was also inhibited by DEX. While the expression of TARC by the combination of cytokines was slightly inhibited by DEX at a highest concentration, the inhibitory effect of DEX was completely abolished by the addition of NKA or His to the combination of IL-4, IFN-γ and TNF-α (Figs. 4c and 4d).

DISCUSSION

In general, the inflammation and hypersensitivity of the airway have been established early in the onset of asthma. It is thought that this inflammation resembles Th2-type allergy. We investigated expression of cytokine mRNAs as an index of inflammation at the early stage of asthma before intervention with GCs (Table 3). IL-4 and IL-5 mRNA expression were not detected in the BALF and TBLB samples from asthmatic subjects, except for the TBLB sample from No. 19 patient. In contrast, the mRNAs of IL-18, IFN-γ, MDC and TARC tended to be expressed higher than that of non-asthmatic subjects. The pattern of cytokine expression at an early stage of asthma reflects a Th1-phenotype rather than a Th2-type allergy, because the all tested subjects were almost the first visit patients without invention with GCs.

Although the majority of cells in BALF from non-asthmatic and asthmatic subjects were macrophages, the number of eosinophils increased in proportion to the severity of disease. In addition to the direct effects of smoking, it is quite possible that smoking led the increase of eosinophils, resulting in aggravation of asthma.[24] As shown in Table 1, more than 60% of tested asthmatic patients have or do smoke. Smoking leads to wheezing, increased sensitization to certain allergens, chronic bronchitis, bronchial hypersensitivity, and thus exacerbates the symptoms of asthma.[25] Activated macrophages in the airways of asthmatic subjects are thought as the main producer of IL-18 and IFN-γ. IL-18 enhanced IFN-γ production from T cells or macrophages con-

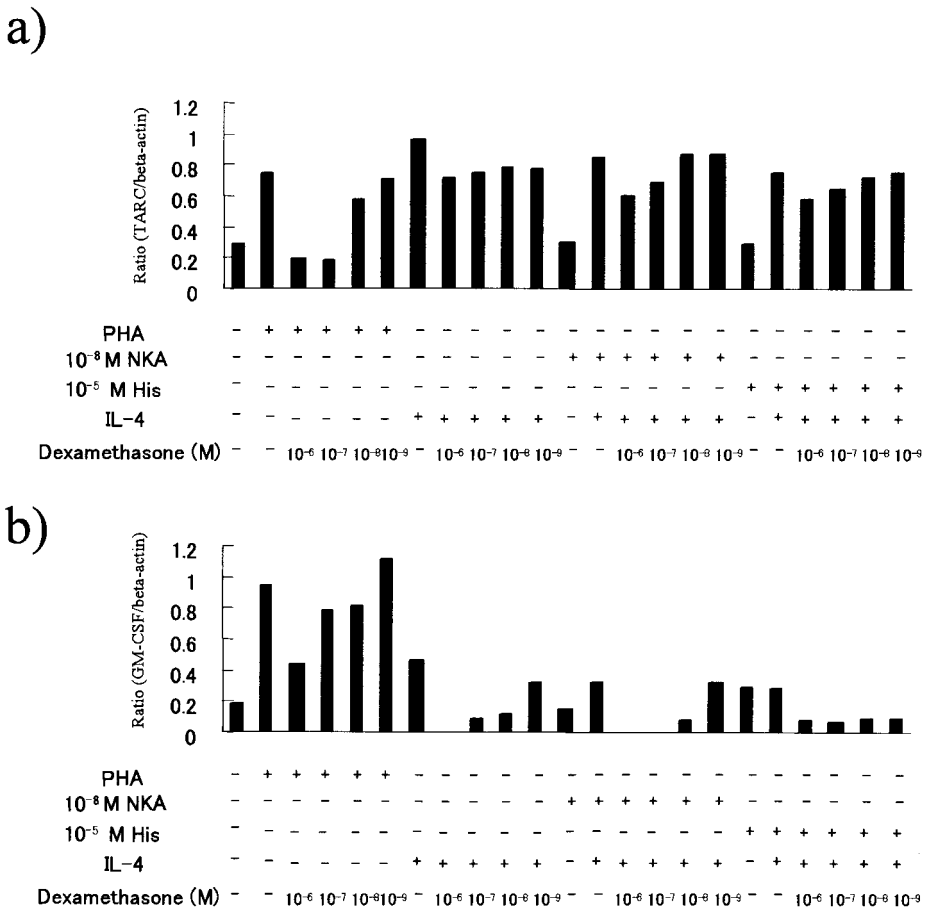

Fig. 3. Effect of DEX on the Induction of TARC and GM-CSF mRNA by PHA, IL-4, NKA or His in Normal PBMC

PBMC (1×10^6 cells/ml) from normal subject were cultured in a 96 well flat-bottomed plate at 100 μl/well and added the indicated stimulator. After 16–18 hr cultivation, the total RNA was isolated from the cultured cells, and the mRNAs for TARC and GM-CSF were detected by semiquantitative RT-PCR. Results are from 1 of 2 similar experiments. The data was presented as the ratio of TARC or GM-CSF to β-actin (see Materials and Methods). Upper panel (a) shows the effect of 10^{-6}–10^{-9} M DEX on TARC mRNA expression induced by 10^{-8} M NKA or 10^{-5} M His in addition to 10 ng/ml of IL-4. Lower panel (b) shows the effect of 10^{-6}–10^{-9} M DEX on GM-CSF mRNA expression induced by 10^{-8} M NKA or 10^{-5} M His in addition to 10 ng/ml of IL-4.

comitant with IL-12. However, IL-18 aggravated the allergic reaction, such as bronchial asthma, by increasing IL-4 or IL-13 production, which is involved in T cell activation via IL-2 as well as basophil and mast cell activation via IL-3.[26,27] In addition, IL-18 might be able to change the course of an allergy reaction to either a Th1 or a Th2-type cytokine pattern.

MDC was thought to be mainly produced in antigen stimulated dendritic cells.[18, 19] Sekiya *et al.* have reported that combined treatment of TNF-α, IFN-γ, and IL-4 induced TARC mRNA expression in bronchial epithelial cells.[19] Treatment with IL-4 alone induced TARC expression in PBMC (Fig. 3a).[11,19] The essential cytokines for TARC expression in PBMC were different from those in bronchial epithelial cells. These reports suggested the possibility that TARC and MDC played important roles in asthma, since the importance of CCR4 ligands in the pathogenesis of Th2-type disease had been increasingly accepted.

Reinhardt *et al.* reported that memory-phenotype T cells were divided into two populations on the experimental model mouse. One produced IL-2 in lymph nodes and the other produced the anti-microbial lymphokine IFN-γ in non-lymphoid tissues.[28] Former cells produced more memory cells in the lymph nodes, and migrated to local sites after the activation by certain antigen stimulation. Our data showed that the cell population of lymphocytes in BALF from asthmatic subjects, patient numbers 5, 7, 10, 15 and 18 were relatively high compared with other patients, and the expression of IL-12 p40 mRNA in these BALF was higher than others (Table 1 and 3). As the result of infection, the IFN-γ producing cells with anti-microbial activity prolif-

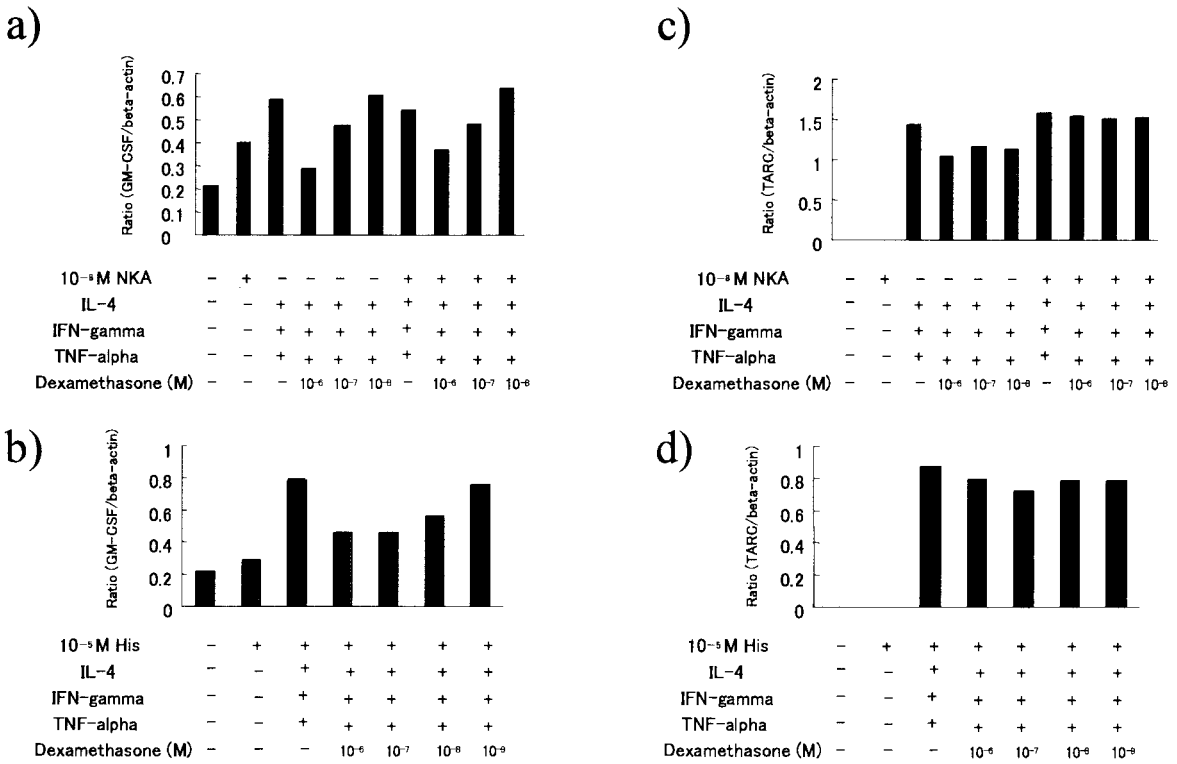

Fig. 4. Effect of DEX on the Expression of TARC and GM-CSF mRNA Induced by the Combination of IL-4, IFN-γ and TNF-α in BEAS-2B Cells

BEAS-2B cells were cultured at 1 ml/well in a 24 well flat-bottomed plate and grown to 80% confluence in DMEM/F12 medium containing 5% FCS. After the culture medium was replaced with DMEM/F2 containing 0.1% BSA, the cells were treated with the indicated stimulators for 16–18 hr. Total RNA was isolated from the treated cells, and the analysis of TARC and GM-CSF mRNA was carried out by semiquantitative RT-PCR. Results are from 1 of 2 similar experiments. The data was represented as the ratio of TARC or GM-CSF to β-actin (see Materials and Methods). Left-upper panel shows the effect of 10^{-6}–10^{-9} M DEX on GM-CSF mRNA expression induced by 10^{-8} M NKA in addition to 10 ng/ml of IL-4, 50 ng/ml of TNF-α and 300 U/ml of IFN-γ (a). Left-lower panel shows the effect of DEX on GM-CSF mRNA expression induced by His in addition to the above three combined cytokines (b). Right-upper panel shows the effect of DEX on TARC mRNA expression induced by NKA in addition to the three combined three cytokines (c). Right-lower panel shows the effect of DEX on TARC mRNA expression induced by His in addition to the three combined cytokines (d).

erated at local sites via synergy of IL-12 and IL-18.[29] These cells may act in the bronchoalveolar cavity such as Th0 or Th1-type cells. The latter, that are present in non-lymphoid tissues, produce IFN-γ for protective immunity at the site of infection. Thus, the population of memory T cells in the airways of asthmatic subjects seems to consist of the migrated cells from lymph nodes and the resident cells which were located in airways before inflammation. Since viral or bacterial infection can enhance IFN-γ production from these memory cells and alveolar macrophages, the inflammatory symptoms of asthmatic patients may worsen by the IFN-γ from these cells with TNF-α and IL-4.

NKA and histamine had no effect on expression of TARC mRNA in PBMC of the normal subjects and moderately asthmatic patients, but these stimulators strongly augmented TARC expression in PBMC of the GC-resistant severely asthmatic subject (Fig. 2). This GC-resistant severely asthmatic patient was medicated with 10 mg/day of oral GC predonisolone, but the asthmatic symptoms scarcely improved. TARC expression by PHA was completely inhibited by addition of DEX at concentration of 10^{-7} M in normal PBMC cultivation. The TARC expression in BEAS-2B cells with the combined treatment of IL-4, IFN-γ and TNF-α in addition to NKA was not inhibited by steroid even at 10^{-6} M (Fig. 4c). GC-resistance of TARC expression in bronchial epithelial cells caused by NKA or histamine may be involved in the chronic airway inflammation and hypersensitivity of asthma.

Leung *et al*. have proposed that the mechanism of GC-resistance may account for the increasing expression of glucocorticoid receptor β (GCRβ), resulting in significant reduction of GCRα DNA binding capacity.[30] It was reported that the combination of IL-2 and IL-4 induced GCRβ in PBMC and

blunted the T cell response to GCs.[31] GC-resistant bronchial asthma is thought to be associated with increased c-fos expression in monocytes and T lymphocytes, by which AP-1 is increased and GCR-DNA binding is decreased.[32] GCRβ seems to be expressed in airway epithelial cells, T cells, and macrophages from a GC-resistant severely asthmatic patient, because the expression of GCRβ was significantly higher in airway T cells than PBMC.[30,31]. However, all aspects of GC-resistant asthma could not be explained by the expression of GCRβ. Although the expression of GCRβ was detected in PBMC of the tested 2 GC-resistant severe asthma, in the patient showed Fig. 2, GCRβ expression was not detected (data not shown). Taking into account the high expression of TARC mRNA in BALF and TBLB from asthmatic patients, we propose that the part of resistance to GC in the asthmatic patient without GCRβ expression might be dependent on the induction of TARC expression in airway epithelial cells by tachykinines and histamine. Furthermore, the process which TARC led cells bearing CCR4 to inflammatory sites might be not inhibited by steroids.

The results described here will be useful to address one mechanism of GC-resistant asthma. We would like to investigate the cells producing each cytokine and the cell-signaling pathway of GC-resistant asthma by neuropeptides.

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