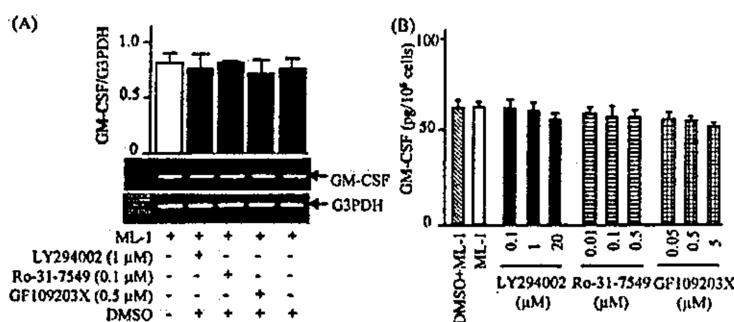

FIG 5. Effect of PD98059, U0126, Raf1 kinase inhibitor I, SB202190, and SP600125 on GM-CSF gene (A) and protein (B) expression in NHBEs.

PD98059 and 1 nmol/L Raf1 kinase inhibitor I inhibited, to a greater degree, the production of GM-CSF in NHBEs (Fig 3, *B* and *C*). The total number of cells and cell viability at the end of the culture period for each experiment did not differ among all culture conditions as determined by trypan blue exclusion assay, suggesting that the inhibition of ML-1–induced GM-CSF expression did not result from cytotoxicity.

The involvement of Raf1 kinase in ML-1–induced GM-CSF expression was further confirmed by the use of a Raf1 dominant-negative mutant with serine to alanine substitution at position 621. The results showed that overexpression of Raf1 dominant-negative mutants in BEAS-2B cells significantly inhibited ML-1–induced GM-CSF gene expression (Fig 4, *A*) and protein release (Fig 4, *B*), whereas the cells transfected with a control vector showed no significant change in the level of GM-CSF expression (Fig 4). These results suggest, therefore, the importance of the Raf1-MEK-ERK signaling pathway in ML-1–induced GM-CSF expression. It has been demonstrated that both PKC and PI3K are linked to the MAP kinase pathway, and that signaling from receptor tyrosine kinases to ERK1/2 is dependent on Ras and Raf1. Further, PKC has been shown to be a key activator of the Raf1/mitogen-activated protein kinase cascade at multiple steps that result in upregulation of ERK1/2. In our experiment, however, selective PKC inhibitors, Ro-31-7549 (0.1 μmol/L) and GF109203X (0.5 μmol/L), did not decrease the level of ML-1–induced GM-CSF gene expression in NHBEs (Fig 5, *A*). Similarly, no significant inhibitory effect was seen on ML-1–induced GM-CSF protein production when the inhibitors were used (Fig 5, *B*). Moreover, a PI3K inhibitor, LY294002, showed no significant inhibition of ML-1–induced GM-CSF gene expression in NHBEs (Fig 5, *A*) and protein production in NHBEs (Fig 5, *B*) when LY294002 (0.1-20 μmol/L) was used.

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DISCUSSION

In this report, we provide evidence that ML-1 (IL-17F) is able to induce GM-CSF expression in primary bronchial epithelial cells through the activation of the Raf1-MEK-ERK1/2 pathway. ML-1 is derived from activated CD4$^+$ T cells, basophils, and mast cells, which are important regulatory cells for allergic airway inflammation.[14] The ability of ML-1 to induce epithelium-derived GM-CSF raises a strong possibility that ML-1–induced GM-CSF is involved in allergic inflammation. In our previous report, the ML-1 gene is upregulated in the bronchoalveolar lavage cells from asthmatic patients after segmental allergen challenge.[14] The current study lends support for a role of ML-1 in the airway inflammatory responses via the induction of GM-CSF.

The results of the current study demonstrate the involvement of the Raf1-MEK-ERK signaling pathway in ML-1–induced GM-CSF expression. It is of interest to note that the induction of IL-6 and IL-8 by ML-1 is dependent on the activation of ERK1/2, but not p38 and JNK,[19] suggesting that ML-1 is able to induce multiple cytokines via the same signaling pathway. Also, ERK1/2 may serve as a pivotal signaling molecule for IL-17 family members, such as IL-17A and ML-1 (IL-17F), and would be a potential therapeutic target for IL-17 family-associated airway diseases. Further, our pharmacologic studies showed that neither PKC nor PI3K is involved in ML-1–induced GM-CSF expression. This is in contrast to the finding that TNF-α induces GM-CSF expression via activation of PKC and subsequent activation of ERK1/2 kinases in NHBEs.[20] It is noted also that a PKC inhibitor, Ro-31-7549, does not affect IL-17A–induced IL-6 and IL-8 expression in bronchial epithelial cells.[21] Another PKC inhibitor, GF-109203X, also does not block diesel exhaust particle–induced GM-CSF expression.[22] Furthermore, a selective PI3K inhibitor, LY294002, does not affect IL-17A–induced IL-6 and IL-8 in bronchial epithelial cells.[23] Our data also showed that LY294002 did not inhibit ML-1–induced GM-CSF expression. Activated Ras promotes cell survival in epithelial cells through activation of PI3K and Akt/PKB, and it is known that at high dose (20 μmol/L), LY294002 induces apoptosis.[24] In the current study, the cell number and viability were not altered in the presence of 20 μmol/L of LY294002 (data not shown). This is likely a result of different culture conditions and cell types used. However, we cannot fully exclude the involvement of other molecules, such as RalGDS and PLC-ε, because recent

reports have demonstrated that Ras is able to activate these signaling molecules besides Raf1.[25,26] These results demonstrate, therefore, that multiple functional pathways exist in the regulation of GM-CSF expression.

To date, the upstream signaling pathway of Ras-Raf1-MEK-ERK1/2 induced by ML-1 is unclear. There have been few reports concerning the upstream signaling pathway of GM-CSF expression. However, the results of the current study suggest that Raf1 is predominantly associated with the activation of the MEK-ERK1/2 pathway. Therefore, we concluded that the Raf1-MEK-ERK1/2 pathway is a central upstream pathway of ML-1–induced GM-CSF expression in NHBEs. Further study is needed to identify functionally the putative receptor for ML-1 and its proximal signaling pathways. Also, the downstream signaling pathway is currently unclear. GM-CSF is known to be regulated by a transcription factor, nuclear factor κB (NF-κB).[27] IL-17A is known to activate NF-κB in chondrocytes and intestinal epithelial cells.[28] Because of high homology between IL-17A and ML-1, it is possible that ML-1 is able to activate NF-κB in the downstream signaling pathway. However, we could not detect the activation of NF-κB by ML-1 in NHBEs (data not shown), suggesting an NF-κB–independent pathway, for which further investigation is needed.

Bronchial epithelial cells are able to release GM-CSF in response to physiologic stimuli relevant for asthma, including dust mite proteolytic allergens, human rhinovirus 16, respiratory syncytial virus, and histamine.[29,30] Furthermore, bronchial epithelial cells have been demonstrated to secrete GM-CSF in response to several cytokines, such as TNF-α, IL-1β, IL-4, and IL-13, which are strongly involved in allergic response.[31,32] A substantial amount of GM-CSF is produced in the airway epithelium after antigen challenge in asthmatic subjects, and GM-CSF appears to be the major cytokine responsible for eosinophil survival in the bronchoalveolar lavage fluid of symptomatic asthmatic subjects.[33] In addition, GM-CSF acts as a cofactor for superoxide production and degranulation[33,34] and elicits multiple effects on other components of the immune systems, including dendritic cells, which initiate and perpetuate allergic inflammation.[35] Furthermore, GM-CSF also has multiple effects on neutrophil function, including increasing 5-lipooxygenase level, tyrosine phosphorylation, aggregation, superoxide anion production, degranulation, cytotoxicity, and adhesion to vascular endothelial cells.[36] Finally, GM-CSF stimulates monocyte survival and differentiation into mature macrophages, which are the largest cell population in the airways of patients with allergic inflammatory diseases.[37,38] Hence, these data strongly suggest that ML-1 shows multiple biological activities via the induction of GM-CSF, adding to a growing list of cytokines involved in the regulation of allergic inflammation.

In conclusion, this study reports that ML-1 induces GM-CSF via the activation of the Raf1-MEK-ERK1/2 pathway. These results suggest that ML-1 is involved in allergic inflammation via the induction of GM-CSF. ML-1 may cause and perpetuate airway inflammation at multiple steps. Furthermore, the control of the Raf1-MEK-ERK1/2 pathway is an attractive pharmacotherapeutic strategy for inhibition of ML-1–induced airway immunologic and inflammatory responses.

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Molecular mechanisms in allergy and clinical immunology

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IL-17 cytokine family

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A new family of cytokines, IL-17, has recently been defined that reveals a distinct ligand-receptor signaling system. Functional studies have provided evidence for its importance in the regulation of immune responses. Notably, 3 members, IL-17A, IL-17E (IL-25), and IL-17F, have been best characterized both *in vitro* and *in vivo*, and have been shown to be proinflammatory in nature. This proinflammatory activity is exemplified by their involvement in pulmonary inflammatory responses, in which both IL-17A and IL-17F are involved in the recruitment of neutrophils, and IL-17E is able to induce T_H2 cytokine production and eosinophilia. Although the elucidation of a detailed mechanism of action continues to be an active area of research, the potent inflammatory activity and its association with various human disease states suggest this new cytokine family as an important contributor to the pathophysiology of human disease conditions, in particular the pulmonary diseases. (J Allergy Clin Immunol 2004;114:1265-73.)

Key words: IL-17 family, IL-17A, IL-17E (IL-25), IL-17F

Abbreviations used

BALF:	Bronchoalveolar lavage fluid
COPD:	Chronic obstructive pulmonary disease
ENA-78:	Epithelial cell–derived neutrophil activating protein 78
ERK:	Extracellular signal–regulated kinase
G-CSF:	Granulocyte colony-stimulating factor
GROα:	Growth-related oncogene α
HUVEC:	Human umbilical vein endothelial cell
ICAM-1:	Intracellular adhesion molecule 1
IP-10:	IFN-γ inducible protein 10
MCP:	Monocyte chemoattractant protein
MIP-3:	Macrophage inflammatory protein 3
NFκB:	Nuclear factor κB

Cytokines are secreted proteins that regulate many biological activities, including hematopoiesis and immunity. The IL-17 cytokine family is a recently discovered group of cytokines. IL-17A, the original member of this family, was first identified in 1995[1] and was initially recognized for its similarity to a sequence belonging to open reading frame 13 of *Herpesvirus* saimiri. Subsequent functional analysis demonstrated its ability to induce the production of other cytokines and chemokines, such as IL-6, IL-8, and granulocyte colony-stimulating factor (G-CSF), from a variety of cell types, providing the basis for being a bona fide cytokine. Structurally, IL-17A has no sequence similarity to any other known cytokines or other mammalian proteins.[2] Also, its receptor, IL-17R, is not related to any of the other known cytokine receptors and does not possess any recognizable domains.[2] Thus, the IL-17 cytokine family appears to represent a distinct ligand-receptor signaling system.

In recent years, we have witnessed the explosion of novel gene discovery, a result in large part of the availability of human genome sequences and bioinformatics tools that have facilitated the gene discovery process. Indeed, 5 additional members of the *IL17* gene family were discovered primarily from the genome sequences within a short period between 2000 and 2002[3-9] and were sequentially named in the order of discovery. *IL17B* and *IL17C* were identified on the basis of a sequence similarity search of the expressed sequence tag database.[4] Similarly, 3 additional members, *IL17D*, *IL17E* (also called IL-25), and *IL17F* were discovered primarily on the basis of sequence homology search from the genomic DNA sequences. These molecules have a similar molecular weight of 20 to 30 kd and have overlapping, but not identical, biological activities. Although these new and exciting findings have been the basis for several recent reviews,[10-12] this review highlights the structural and functional features of the IL-17 cytokine family, particularly for the 3 best characterized members, IL-17A, IL-17E (IL-25), and IL-17F, and their involvement in pulmonary inflammatory responses.

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STRUCTURAL CHARACTERISTICS

Alignment of the predicted amino acid sequence of IL-17A and the other members reveals that the highest overall amino acid sequence identity (50%) is found between IL-17A and IL-17F, whereas only 10% to 30% sequence identity is seen between IL-17A and the other family members. They bear greatest similarity within the C-terminal 70 amino acids and have 4 well conserved cysteines. The crystal structure of one of the family members, IL-17F, has been determined recently.[9] The 4 conserved cysteines in the C-terminal half of the IL-17F sequence are shown to form a cystine knot in the crystal structure,[9] and interestingly, this cystine knot structure is similar to a common structural motif seen in several growth factors, such as bone morphogenic proteins, TGF-β, nerve growth factor, and platelet-derived growth factor. In addition, IL-17F is believed to form disulfide-bonded homodimers,[9] whereas other members of the IL-17 family are expressed as tightly associated dimers.

EXPRESSION OF THE IL-17 CYTOKINE FAMILY

Members of the IL-17 cytokine family appear to have a very distinct expression pattern and are likely to have a distinct biological role. The original member of this family, IL-17A, is mainly expressed in $CD4^+$ T cells, but only on activation, with no expression seen in the resting state.[2] IL-17A–expressing T cells are not classified into either the T_H1 or the T_H2 subset.[13-15] Analysis of individual T-cell clones showed, however, that many IL-17–producing clones appear to represent a distinct cell population[15]; that is, they do not coexpress either T_H1 (IFN-γ) or T_H2 (IL-4 and IL-13) cytokines. Recent reports have indicated that in addition to the T cells, neutrophils, eosinophils, and $CD8^+$ T cells are sources of IL-17A.[16-18]

Two members of this cytokine family, IL-17B and IL-17C, are expressed in a wide range of tissues,[3,4] but the cell sources of IL-17B and IL-17C have not been identified. The *IL17D* gene appears to be most homologous to *IL17B* and is expressed widely in skeletal and heart muscle, brain, adipose tissue, lung, and pancreas.[5] The cellular source of IL-17D is resting $CD4^+$ T cells and $CD19^+$ B cells.[5] The expression of the *IL17E* gene is detected at very low levels in several tissues, including kidney, testis, lung, adrenal gland, brain, spinal cord, prostate, and trachea.[6] Unlike IL-17A, IL-17E (IL-25) expression is restricted to the T_H2 cells,[19] and interestingly, mouse bone marrow–derived mast cells are also able to produce IL-17E after the cross-linkage of the receptor for IgE.[20]

The expression pattern of the last member of this family, IL-17F, is more complex than those seen for other members. Two different isoforms (the longer and the shorter forms) of human *IL17F* have been found. The mechanism in the regulation of the expression for the longer versus the shorter form of the *IL17F* gene remains unclear but appears to be a result of differential splicing or the alternative usage of the translation start site.[7] On the basis of currently available data, no apparent functional difference has been noted between these 2 isoforms. Although both the longer and the shorter forms of the *IL17F* gene are expressed in activated $CD4^+$ T cells, increased gene expression of the shorter form is evident in additional cell types after activation, including peripheral blood basophils and cord blood–derived mast cells.[7,9] Also, the shorter form is expressed in allergen-specific $CD4^+$ T-cell clones with different cytokine phenotypes (T_H0, T_H1, and T_H2 subsets). Although the T cells appear to be the major cell source for IL-17A, unresolved issues are whether the same T-cell population is also able to coexpress IL-17F and/or IL-17E, 2 additional members produced from activated $CD4^+$ T cells, and whether there is a differential expression of these cytokines in response to different stimuli. This is a piece of the puzzle awaiting further investigation. It is of interest to note that although there is a significant degree of sequence homology and functional similarity between IL-17A and IL-17F, several types of tissues, including liver, lung, ovary, and fetal liver, show only the expression of *IL17F*,[7] suggesting a wider tissue distribution and a more diverse biological function of IL-17F compared with IL-17A.

Several attempts have been made to investigate the regulatory pathways leading to the induction of these 2 cytokines, and the results have been informative. Currently, in addition to the antigen as an inducer for the expression of IL-17A, 2 monocyte/dendritic cell lineage–derived cytokines, IL-15 and IL-23, are important stimuli for the induction of IL-17A. IL-15 has been shown to be able to induce IL-17A from $CD4^+$ T cells and neutrophils,[17] and IL-23 is also critical for IL-17A production from both $CD4^+$ and $CD8^+$ T cells.[21-23] Similar to IL-17A, IL-17F is inducible in IL-23–stimulated mononuclear cells isolated from mouse spleens.[21] Both IL-15 and IL-23 have been shown to be important in memory development of the T cells,[24] and IL-23 is involved in innate immunity and mucosal T-cell response.[25] The ability of these 2 cytokines to induce IL-17A (and perhaps IL-17F) may thus serve as a bridge between innate and adaptive immunity, wherein the IL-17 cytokine members, IL-17A and IL-17F, are the principal effector cytokines.

FUNCTIONAL ASPECTS

Investigation into the function of the IL-17 cytokine family has been extensive, but the majority of the effort has centered on the role of 3 members, IL-17A, IL-17E, and IL-17F, in immunoregulation. The fact that these 3 members of the IL-17 cytokine family are expressed in diverse cell types and tissues suggests a pleiotropic biological activity. Indeed, *in vitro* experiments using various target cell types and recombinant forms of cytokines have shown a wide variety of functions. Further, increased *in vivo* expression of these 3 members in various disease states

TABLE I. Summary of key functional features

IL-17 family	Model	Functional effect	Comment	Reference
IL-17A	Bronchial epithelial cells	Increased expression of IL-6, IL-8, G-CSF, GM-CSF, and granulocyte chemotactic protein 2	No effect on CC chemokine expression	27-29
	Bronchial epithelial cells	Increased expression of mucin genes		46
	Fibroblasts	Increased expression of IL-6, IL-8, IL-11, and GROα	Inhibited by dexamethasone	48
	IL-17A–deficient mice	Decreased AHR Decreased expression of IL-4, IL-5 and IgE		50
	Mice (intratracheal instillation of IL-17A)	Induction of neutrophilia	Via induction of IL-8	34
	Mice (adenovirus-mediated gene transfer)	Peripheral neutrophilia	Lung neutrophilia not tested	35
	Mice (treatment with anti–IL-17A mAb)	Reduction of neutrophilia		36
	Mice (IL-17R–deficient)	Reduction of *K pneumoniae*-induced neutrophilia		55
	Mice (adenovirus-mediated gene transfer)	Host defense and early survival after *K pneumoniae* infection; reduced bacterial growth		56
	Human (asthma)	Increased expression of IL-17A		18,48
	Human (asthma and COPD)	Increased expression of IL-17A correlates with AHR		51
	Human (asthma)	Increased expression of IL-17A correlates with severity		18
IL-17E	Transgenic mice	Induction of AHR; High level of T_H2 cytokines, eotaxin, IgE, eosinophilia, and mucus hypersecretion		59,60
	Mice (adenovirus-mediated gene transfer)	Increased expression of IL-4, IL-13, eotaxin, and CCR3	Induction of AHR by administration of IL-17E	38
IL-17F	Bronchial epithelial cells	Increased expression of IL-6, IL-8, GROα, ENA-78, GM-CSF, and ICAM-1	No effect on CC chemokine expression	7,31-33
	HUVECs	Increased expression of IL-6, IL-8, GROα, and ENA-78		31,32
	Human (asthma)	Increased *IL17F* gene expression in the BALFs		7
	Mice (adenovirus-mediated gene transfer)	Increased expression of IL-6, IFN-γ, IP-10, monokine induced by IFN-γ, MCP-1, and MCP-3; induction of neutrophilia		38
	Mice (intratracheal delivery of IL-17F)	Induction of neutrophilia and CXC chemokines		39
		Increased AHR and mucus hypersecretion		

AHR, Airway hyperreactivity.

supports this view. To date, the major function of these 3 members is chemoattractant activity through the induction of cytokines and chemokines. Notably, IL-17A and IL-17F share similar functions in terms of their ability to induce chemokines that are important in neutrophil recruitment and activation *in vitro* and *in vivo*, whereas IL-17E is involved in the induction of T_H2 cytokines and eosinophilic recruitment. Further, several studies have provided evidence for the role of IL-17A and IL-17F in the induction of airway hyperresponsiveness and other pathological changes, including goblet cell metaplasia and airway remodeling. The salient features of the current findings are summarized in Table I and illustrated in Fig 1.

IL-17A and IL-17F

Since the initial gene discovery of IL-17A and IL-17F, many *in vitro* studies using various target cell types have been pursued, and by far, the most extensively investigated function involves the ability of these 2 cytokines to induce other cytokines, such as IL-6 and GM-CSF, as well as a panel of chemokines from epithelial and vascular endothelial cells.[26-30] For example, in bronchial epithelial cells, IL-17A is able to induce 2 CXC chemokines: IL-8, growth-related oncogene α (GROα), and epithelial cell–derived neutrophil activating protein 78 (ENA-78). Two additional chemoattractant factors, granulocyte chemotactic protein 2 and monocyte chemoattractant protein (MCP)–1, are also induced in IL-17A–stimulated bronchial epithelial cells *in vitro*. IL-17F has been shown to be able to induce a similar panel of cytokines and chemokines in bronchial epithelial cells and human umbilical vein endothelial cells (HUVECs).[31-33] Besides epithelial and endothelial cells, fibroblasts are known to produce neutrophil-activating factors, IL-6 and IL-8, in response to both IL-17A and IL-17F.[9,29]

Recent efforts with mouse models have corroborated these *in vitro* findings and provided further insight into the functional role of these 2 cytokines *in vivo*. These studies have primarily focused on the regulation of pulmonary responses, involving local (mucosal) administration of

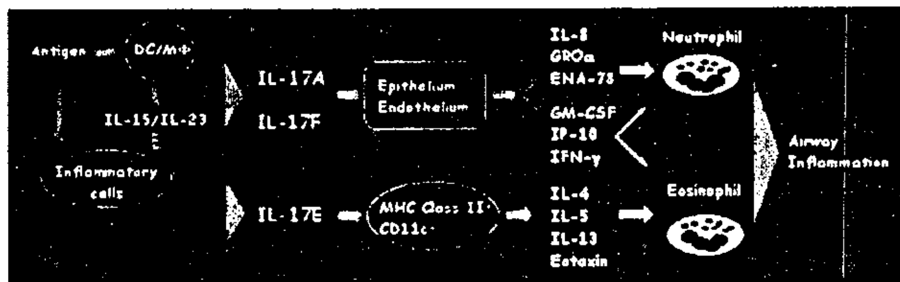

FIG 1. Working model for the role of IL-17A, IL-17E, and IL-17F in the expression of airway inflammation. *DC*, Dendritic cell; *MΦ*, macrophage.

either recombinant cytokines or gene expression constructs encoding IL-17A or IL-17F. In the first of this series of studies, intratracheal administration of IL-17A significantly increased the absolute number of neutrophils in bronchial alveolar lavage fluids (BALFs).[34] Also, expression of IL-17A via adenovirus-mediated gene transfer resulted in massive peripheral neutrophilia associated with increased level of G-CSF and enhanced granulopoiesis.[35] This effect is specific, because pretreatment of mice with a neutralizing antibody directed against IL-17A significantly reduced pulmonary neutrophilia.[36] Also, in a rat model, IL-17A induces endogenous tachykinins, which in turn, through natural killer 1 receptors, mediate neutrophil recruitment.[37]

Similar approaches for introducing the *IL17F* gene at the mucosal sites have been used, and have led to a similar conclusion. Using an adenoviral gene transfer strategy, overexpression of IL-17F in the mouse airways also leads to an increased number of neutrophils in BALFs.[38] However, using a different model, Oda et al[39] recently provided evidence that overexpression of IL-17F through intratracheal delivery of the IL-17F gene results in an increase in the number of neutrophils and macrophages in the airways. Interestingly, profiling of gene expression for 67 chemokine and 96 cytokine genes showed that genes known to be involved in chemotaxis and activation for monocytes/macrophages and neutrophils, including IL-6, GM-CSF, MCP-1/2, and keratinocyte-derived chemokine (a CXC chemokine), are the most prominently upregulated genes in IL-17F–transduced mouse lungs. In a murine model of asthma, overexpression of IL-17F provided an additive effect on antigen-induced allergic inflammatory responses, characterized, in part, by an increased level of neutrophilia.[39]

In these murine models, eosinophil number was not significantly enhanced in comparison with that seen in mock control mice or mice receiving antigen challenge alone. Also, no effect on the levels of T_H2 cytokines (IL-4, IL-5, and IL-13) was noted. This selective activity of IL-17A and IL-17F is consistent with the fact that human IL-17F is incapable of inducing CC chemokines, such as eotaxin and RANTES, which are potent chemoattractants for eosinophils, in primary bronchial epithelial cells.[3] To support further the involvement of both cytokines in neutrophil recruitment *in vivo*, a recent study of mice deficient for IL-23, a potent inducer for IL-17A and IL-17F, shows that those mice are unable to develop neutrophilia in the tissues.[23] Moreover, in our recent unpublished study, an additive effect on the induction of IL-6 and IL-8 has been observed when the cells are treated with IL-17F in combination with either T_H2 cytokines (IL-4 and IL-13) or T_H1 cytokine (IFN-γ).

In obstructive airway diseases, including bronchial asthma and chronic obstructive pulmonary disease (COPD), accumulation of neutrophils in the airways has been a major characteristic.[40,41] Neutrophil recruitment into the airways is regulated through the chemokine network, and bronchial epithelial cells and vascular endothelial cells are important cellular sources of CXC chemokines. Increased expression of the 3 CXC chemokines IL-8, GROα, and ENA-78 has been found in various human diseases, including acute respiratory distress syndrome and bacterial pneumonia, in which abundant neutrophils are a major feature.[42] The potent stimulatory activity of both IL-17A and IL-17F on the expression of cytokines and chemokines suggests their intimate associations with the recruitment and activation of neutrophils and potential contribution to the pathogenesis of these diseases. Further, the induction of GM-CSF from epithelial cells by IL-17A and IL-17F is noteworthy, because among its pleiotropic functions, GM-CSF has multiple effects on neutrophil effector functions, including the induction of 5-lipooxygenase, superoxide generation, degranulation, and cytotoxicity.[43]

Involvement of IL-17A and IL-17F in pulmonary allergic responses

Allergic asthma is characterized by a local T_H2-biased immune response; changes of remodeling, including epithelial hypertrophy; subepithelial fibrosis; goblet cell hyperplasia/metaplasia; and airway hyperreactivity (see review[44]). One of the most prominent features of airway remodeling is subepithelial fibrosis, which serves as a potential marker for disease severity.[45] Current studies have provided evidence for the involvement of IL-17A and IL-17F in these pathological processes. It has been reported that IL-17A is able to induce the expression of 2 mucin genes, *MUC5AC* and *MUC5B*, in bronchial epithelial cells,[46] and that an increased expression of IL-17A is associated with enhanced mucin gene expres-

sion *in vivo*.[47] In a recent study of a mouse model, overexpression of IL-17F also resulted in goblet cell hyperplasia and mucin gene expression, but only when the mice were challenged with antigen, and increased goblet cell hyperplasia was seen only in the small airways.[39] Although it is unclear currently whether IL-17F has a direct effect on the expression of mucin genes, these results suggest that in addition to IL-13, both IL-17A and IL-17F may be important contributors to pulmonary mucus hypersecretion.

In addition to their role in the induction of CXC chemokines, IL-17A and IL-17F have been shown to induce several profibrotic cytokines. For example, in fibroblasts isolated from bronchial biopsy of subjects with asthma, IL-17A enhances the production of 2 profibrotic cytokines, IL-6 and IL-11,[48] and IL-17F has been shown to be able to induce the expression of TGF-β in human umbilical vein endothelial cells (HUVECs[8]). TGF-β is a profibrotic cytokine and has been implicated in the extracellular matrix changes observed in fibrosis. IL-11 is produced by many cell types, including human lung fibroblasts and alveolar and airway epithelial cells, and is involved in subepithelial fibrosis.[49] In addition, increased expression of both TGF-β and IL-11 was found in the airways of patients with asthma and correlated with disease severity.[18] Although direct proof is needed, the *in vitro* activity of IL-17A and IL-17F in inducing profibrotic cytokines and increased expression of IL-17A, IL-11, and TGF-β in subjects with asthma[18] suggest their potential role in airway remodeling.

In addition to those mucosal responses, a recent study of IL-17A–deficient mice showed that IL-17A is involved in the activation of allergen-specific T cells. In those mice, decreased levels of IL-4 and IL-5, but not IFN-γ, were seen,[50] which are associated with reduced level of airway hypersensitivity. In addition, a decreased level of IgE was seen in this model.[50] It appears, therefore, that IL-17A not only is involved in the regulation of tissue mucosal response but also has a systemic effect on the development of the T_H2 cell response. The regulatory mechanisms for this interesting observation remain to be explored.

In human beings, studies of IL-17A and IL-17F expression in patients with asthma have been pursued. In patients with asthma, IL-17A is significantly expressed,[18,48] and its level in sputum of patients with asthma correlated negatively with PC_{20} to methacholine.[51] Also, analyses of gene expression in BALF cells from subjects with asthma challenged with allergen or saline control showed enhanced IL-17F gene expression at allergen-challenged sites of study subjects.[7]

Involvement of IL-17A in antimicrobial activity

Although the exact role of the IL-17 cytokine family in host defense against infection remains to be defined, current data primarily on the basis of *in vitro* studies have suggested its involvement in the host defense mechanisms. For example, in a recent study[52] screening a panel of 21 cytokines, IL-17A was the most potent stimulatory cytokine inducing the expression of human β-defensin-2 (hBD-2) and macrophage inflammatory protein 3 (MIP-3). Both β-defensin-2 and MIP-3 are essential components of airway innate immunity.[53,54] The known function of hBD-2 in innate immunity is believed to be related to its antimicrobial activity and to its chemotactic effects on immature dendritic cells and memory T cells.[53] Thus, the stimulatory effects of IL-17A on hBD-2 and MIP-3 expression, in conjunction with its activity in neutrophil recruitment and activation, suggest an important role of IL-17A in host defense from the innate response to adaptive immune mechanisms. It is noted that mice lacking the receptor for IL-17A, IL-17R, are unable to develop full neutrophil response to *Klebsiella pneumoniae* infection in the airways.[55] Also, overexpression of IL-17A in the lungs using a recombinant adenovirus encoding mouse IL-17A (AdIL-17) resulted in enhanced bacterial clearance and survival after challenge with *K pneumoniae*.[56]

Taken together, current evidence suggests that IL-17A and IL-17F are involved in the regulation of pulmonary inflammation at multiple levels, in part through the induction of other cytokines and chemokines. These responses provide important defense mechanisms against infectious pathogens, and functional defects in these responses would increase the susceptibility to specific pathogens. In contrast, exaggerated production of IL-17A and IL-17F may contribute to the expression of airway inflammation and pulmonary hyperreactivity. Moreover, IL-17A has been shown to be able to induce the activity of inducible nitric oxide synthase and nitric oxide production in both mouse and rat endothelial cells.[57] Further, analysis of lung tissues from IL-17F gene–transduced mice[38] showed substantial increases in the levels of IFN-γ and IFN-γ inducible protein 10 (IP-10; a CXC chemokine contributing to the expression of pulmonary allergic responses[58]). These additional functional activities of IL-17A and IL-17F may thus further amplify the inflammatory responses.

Although a comprehensive and comparative analysis of IL-17F and IL-17A has not yet been pursued, IL-17F appears to have biological actions similar to those of IL-17A. So far, the only functional difference reported is the ability of IL-17F (the shorter form) to induce intracellular adhesion molecule 1 (ICAM-1[7]), whereas the induction of ICAM-1 by IL-17A occurs only when IFN-γ is present in the culture.[27] Further, indirect evidence has suggested that IL-17F may use a different receptor.[7] This suggests an incomplete redundancy for these 2 closely related cytokines, but further in-depth comparative studies are required to define the redundant and the distinct function of these 2 cytokines.

Unique function of IL-17E

In comparison with the functions of IL-17A and IL-17F, another member of this family, IL-17E, induces a different set of cytokine genes and is involved in T_H2-type immune response and pathological changes. Transgenic overexpression of IL-17E results in airway eosinophilia and an increase in serum levels of IL-5, IL-13, and IgE.[59,60] Elevated levels of gene expression for several T_H2

cytokines, including IL-4, IL-5, IL-10, and IL-13, were observed in multiple tissues. Also, intranasal administration of IL-17E–expressing adenovirus vector results in the production of IL-4, IL-5, IL-13, and eotaxin, and eosinophilia in lung tissue and BALF.[38] In addition, epithelial cell hyperplasia, increased mucus secretion, and airway hyperreactivity are developed in mice on intraperitoneal administration of recombinant IL-17E.[38] Interestingly, when the induction of T_H2 cytokines was analyzed *in vitro*, the major cell type in response to IL-17E appears to be a non-T/non-B cell population expressing class II MHC and CD11c molecules, a typical accessory cell phenotype.[19] These findings suggest that IL-17E is a unique member of the IL-17 cytokine family and is directly involved in T_H2-associated allergic inflammation.

WORKING MODEL FOR THE ROLE OF IL-17A, IL-17E, AND IL-17F IN THE EXPRESSION OF AIRWAY INFLAMMATION

On the basis of currently available data, it can be envisaged that all 3 members of the IL-17 cytokine family are produced from activated inflammatory cells, including T cells and mast cells (a cell type producing IL-17F and IL-17E), which leads to subsequent recruitment and activation of leukocytes. Although both IL-17A and IL-17F selectively induce endothelial and/or epithelial cell–derived CXC chemokines, such as IL-8, GROα, and ENA-78 for neutrophil influx, IL-17E preferentially induces T_H2 cytokines and eotaxin for eosinophil recruitment. These, together with other inflammatory changes including mucous hypersecretion associated with increased activities of the 3 cytokines, lead ultimately to pulmonary inflammation and tissue injury. The additive effect of IL-17F with either T_H2 cytokines (IL-4 and IL-13) or T_H1 cytokine (IFN-γ) may further amplify leukocyte infiltration and allergic inflammation. Moreover, the induction of IL-17A and IL-17F through dendritic cell/macrophage–derived IL-15 and IL-23 would confer an antimicrobial activity in conjunction with subsequent recruitment/activation of neutrophils and nitric oxide production. IL-17A and IL-17F may also facilitate influx of other inflammatory cells, including eosinophils, and amplify pulmonary inflammatory response through the induction of other cytokines and chemokines, such as GM-CSF, IFN-γ, and IP-10 (Fig 1).

RECEPTORS AND ASSOCIATED SIGNALING PATHWAYS

To date, only the receptors for IL-17A and IL-17E have been characterized. The receptor for IL-17A, IL-17R,[61] was initially discovered in mice by the use of a viral homologue of IL-17A as a ligand, which was found to be able to bind to both human and mouse IL-17A.[2,61] Compared with other known cytokine receptor families, the structure of IL-17R, a type 1 membrane receptor, is quite unique. The cytoplasmic domain of IL-17R lacks conserved signaling domains commonly seen in other cytokine receptors. The IL-17R gene is constitutively expressed in several human cell types, such as a lung epithelial cell line, foreskin fibroblasts, a B-cell line, a myelomonocytic cell line, and an embryonal kidney cell line.[2] The human IL-17A receptor exhibits 69% sequence homology with the mouse counterpart, and direct binding assays of IL-17R–transfected cells and labeled human IL-17A have revealed a relatively low-affinity binding with an association constant value ranging between 10^7 and 10^8 M.[61] This low-affinity binding suggests the existence of an additional binding subunit to form a high-affinity binding complex, because relatively low concentrations (nanogram range) of IL-17A are sufficient to exert its biological activity, but its existence and identity remain a subject of investigation.

Studies of the other members of the IL-17 family also suggest the existence of distinct receptors. IL-17E has been identified as a high-affinity ligand for a newly recognized receptor.[3,6] This receptor has also been demonstrated to interact with IL-17B with low affinity.[6] Sequence comparison of the receptors for IL-17A and IL-17E reveals similar overall structure with conservation of several cysteines within the extracellular domains, and also with conserved elements within the intracellular domain, suggesting that these receptors likely engage similar signaling pathways. Indeed, both IL-17A and IL-17E are able to induce the activation of nuclear factor κB (NFκB) and a similar, but not identical, panel of cytokines and chemokines.

To date, the receptor for IL-17F has not been identified, but indirect evidence suggests a distinct cognate receptor. For example, by using the shorter form of IL-17F, Kawaguchi et al[7] performed competitive inhibition experiments to assess whether it interacts with the receptor for IL-17A by measuring the level of IL-17F–induced ICAM-1 gene expression in the presence of varying concentrations of the competitor, IL-17A. The level of IL-17F–induced ICAM-1 expression was not inhibited by the inclusion of IL-17A in the culture, even at a 20-fold higher concentration (2000 ng/mL). Further work is needed to uncover the signaling pathways, including the nature of the putative IL-17 receptor. Four additional IL-17R–related receptors have been identified through sequence homology searches, but the nature of their respective ligands remains elusive.

Although the identity of the receptors for several of the IL-17 family members is still unclear, recent studies of the signaling events associated with IL-17A–induced and IL-17F–induced cellular activation and cytokine expression have been informative. IL-17A is known to activate all 3 subgroups of mitogen-activated protein kinases, the extracellular signal–regulated kinases (ERK1 and ERK2), Jun NH2-terminal kinases, and p38 in several cell types, including bronchial epithelial cells.[26,27] Activation of p38 mitogen-activated protein kinase by IL-17A results in up-regulation of inducible nitric oxide synthase and COX-2

genes.[57] In a monocytic leukemia cell line, U937, IL-17A triggers tyrosine phosphorylation of several members of the Janus kinase family, 1, 2, and 3, and signal transducer and activator of transcription 1, 2, 3, and 4.[62] In the downstream signaling pathway, IL-17A activates transcription factor NFκB, which depends on TNF receptor–associated factor 6, but not TNF receptor–associated factor 2.[63] In addition to IL-17A, IL-17D and IL-17E are able to activate NFκB.[5,6,26]

In primary bronchial epithelial cells and endothelial cells, IL-17F induces the activation of ERK1/2.[31-33] In contrast, no activation of p38 and Jun NH2-terminal kinase kinases was seen at any time point, even after 4-hour stimulation of the cells with IL-17F, despite the high sequence homology between IL-17A and IL-17F. These results suggest, therefore, that the members of the IL-17 cytokine family are capable of activating similar, but not identical, intracellular signaling pathways. It is noted, however, that induction of both IL-6 and IL-8 is not completely inhibited by ERK kinase inhibitors, suggesting that additional signaling pathways are involved in these cytokines induced by IL-17F in these cell types.

CONCLUSION

Taken together, current data from structural and functional analyses have defined a new cytokine family and suggested that this family of cytokines is capable of eliciting profound immunological effects and is, therefore, likely to play an important role in immunoregulation. The diverse tissue expression patterns and pleiotrophic functions of the IL-17 cytokine family with a distinct ligand-receptor signaling system may thus generate a new paradigm within the cytokine network contributing to the maintenance of homeostasis and the expression of inflammatory diseases. Increased understanding of the complexity of the cytokine network, together with characterization of relevant novel genes, would help to uncover the molecular mechanisms of inflammatory diseases. Indeed, members of the IL-17 cytokine family are associated with inflammatory diseases and are able to induce a wide range of cytokines and chemokines that are crucial in the regulation of inflammatory response. Specifically, through memory T-cell responses, IL-17A and IL-17F are able to promote the recruitment of monocytes and neutrophils via the induction of chemokines. This activity, together with the involvement of IL-17A (and likely IL-17F) in the development of the T_H2 responses and with the participation of IL-17E in the expression of T_H2 cytokines, may thus potentiate and amplify allergic inflammation. Further detailed investigation with emphasis on defining the regulatory mechanisms of its expression, the ligand-receptor signaling pathways, and the pathways leading to the concerted effect with other cytokines would be important in defining the molecular basis of this cytokine family's involvement in immunoregulation.

Therapeutic implications

Current data support the notion that IL-17A and IL-17F are involved in host defense against bacterial infection. The use of recombinant proteins (and the derivatives thereof) and/or the strategy for correcting the deficiency of both cytokines would have a prophylactic and therapeutic potential.

The finding that exaggerated response of both IL-17A and IL-17F results in neutrophilic inflammation offers an experimental basis for developing strategies to antagonize the dysregulated cytokine response in diseases such as asthma and COPD in which neutrophilic inflammation is important in lung injury.

Upregulated IL-17E activity is associated with systemic and localized T_H2 responses, offering an attractive therapeutic target for modulation in T_H2-associated allergic diseases.

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Cannabinoid Receptor Agonists Inhibit Sensory Nerve Activation in Guinea Pig Airways

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We examined the effects of cannabinoid receptor agonists on various respiratory reactions induced by the activation of capsaicin-sensitive afferent sensory nerves (C-fibers). (R)-(+)-[2,3-dihydro-5-methyl-3-[(4-merpholino)methyl]pyrrolo-[1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphthyl)methanone (WIN 55212-2) dose-dependently inhibited electrical field stimulation- and capsaicin-induced guinea pig bronchial smooth muscle contraction, but not the neurokinin A-induced contraction. A cannabinoid CB2 receptor antagonist, (N-[(1S)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide) (SR 144528), reduced the inhibitory effect of WIN 55212-2, but not a cannabinoid CB1 antagonist, [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride] (SR 141716A). A cannabinoid CB2 agonist, JWH 133, also inhibited electrical field stimulation-induced guinea pig bronchial smooth muscle contraction and its inhibitory effect was blocked by SR 144528. The inhibitory effect of WIN 55212-2 on electrical field stimulation-induced bronchial contraction was reduced by the pretreatment of large conductance Ca^{2+}-activated K^+ channel (Maxi-K^+ channel) blockers, iberiotoxin and charybdotoxin, but not other K^+ channel blockers, dendrotoxin or glibenclamide. A Maxi-K^+ channel opener, 1-(2'-hydroxy-5'-trifluoromethylphenyl)-5-trifluoromethyl-2(3H) benzimidazolone (NS1619), inhibited bronchial contraction induced by electrical field stimulation. WIN 55212-2 and JWH 133 blocked the capsaicin-induced release of substance P-like immunoreactivity from guinea pig airway tissues. These findings suggest that WIN 55212-2 inhibit the activation of C-fibers via cannabinoid CB2 receptors and Maxi-K^+ channels in guinea pig airways.

Keywords: airway; cannabinoid; C-fibers; guinea pig; Maxi-K^+ channels

Several previous studies showed that neurogenic inflammation in the airway must have an important role in the pathogenesis of asthma (1, 2). By using tachykinin antagonists, Murai and coworkers (3, 4) directly demonstrated that airway inflammation is generated by tachykinin released from capsaicin-sensitive afferent sensory nerves (C-fibers), which are stimulated by various types of irritants like cigarette smoke (5), cold air (6), and hypertonic saline (7). These irritants activate C-fibers by opening nonselective cation channels with a high Ca^{2+} permeability (8). Morimoto and colleagues (9) have showed that ω-conotoxin GVIA blocked the activation of C-fibers and the release of substance P from their endings, and suggested that the opening of N-type voltage-dependent Ca^{2+} channels might activate C-fibers in guinea pig airway tissues.

Cannabinoids are a direct class of psychoactive compounds that produce a wide array of effects including hypothermia, depressed motor activity, hypotension, inhibition of intestinal motility, and antinociception (10). The biological effects of cannabinoids are mediated by specific cell surface receptors, CB1 and CB2, in the central nervous system and in peripheral tissues (11–13). Over the last 10 years much evidence has accumulated favoring the concept that cannabinoids are endogenous modulators of neuronal activity in several tissues (14, 15). Cannabinoid receptor agonists have been shown to inhibit the function of Ca^{2+} channels (16, 17).

In this report, we now discovered a cannabinoid receptor agonist, WIN 55212-2 inhibited the electrical field stimulation-induced guinea pig bronchial smooth muscle contraction. We examined to clarify this inhibitory effect of WIN 55212-2 and showed that cannabinoid receptors negatively regulate the release of tachykinins from the endings of C-fibers in guinea pig airways by the inhibition of Ca^{2+} influx to C-fibers via the opening of Maxi-K^+ channels. Our results suggested that cannabinoid receptor agonists may be a valuable tool for the treatment of inflammatory diseases induced by the activation of C-fibers in the airways.

In these experiments, we have used the nonselective agonist WIN 55212-2, the CB2-selective agonist JWH 133, the CB1 receptor antagonist SR 141716A, and the CB2-receptor antagonist SR 144528 as pharmacologic tools with which to characterize the cannabinoid receptor subtype involved in this response. WIN 55212-2 has essentially the same affinity for CB1 and CB2 receptors. The affinities for both receptors are in the nanomolar range, and this agonist exhibits relatively high efficacy at both these receptor type (18). JWH 133 is the most selective CB2 receptor agonist. Its binding affinities (K_i) for CB2 and CB1 receptors are 3.4 ± 1.0 and 677 ± 132 nM, respectively (19). SR 141716A was developed by Sanofi-Synthelabo Recherche (Montpellier, France), and is a highly potent and selective CB1 receptor antagonist (K_i = 5.9 nM for CB1 and > 1 μM for CB2) (20). SR 144528 is also developed by Sanofi that binds with higher affinity to CB2 than CB1 receptors (K_i = 0.6 nM for CB2 and 437 nM for CB1) (21).

METHODS

Animals

Twelve-week-old male Hartley guinea pigs (260–455 g) were purchased from SLC (Hamamatsu, Japan) at least 1 week before the experiments. Animals were housed in a temperature- and humidity-controlled environment under a 12:12 hour light/dark cycle with light on at 7:00 A.M. Animals were allowed free access to food and water *ad libitum*.

Contractile Response of Isolated Guinea Pig Bronchi

The procedure of Fox and coworkers (22) and Patel and colleagues (23) was used with certain modifications. Male Hartley guinea pigs were killed and their bronchi were removed rapidly. A ring preparation with 4 to 5 mm length of the main bronchi (only one bronchi/animal) was mounted in 5-ml organ baths filled with warmed (37°C) and oxygenated (95% O_2, 5% CO_2) standard Tyrode's solution (pH 7.5) containing 137.0 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.05 mM $MgCl_2$, 0.4 mM $NaHPO_4$, 5.6 mM dextrose, 11.9 mM $NaHCO_3$ under a resting tension of 0.5 g. After a 60-min equilibration period, bronchi were stimulated

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by pre-established (data not shown) submaximal concentrations or stimuli of 1 nM neurokinin A, 1 μM capsaicin, or electrical field stimulation in the presence of atropine, propranolol, and phosphoramidon, respectively, 1 μM. Electrical field stimulation was delivered using two parallel platinum wires connected to a stimulator, and square-wave pulses of supramaximal voltage (50 V) and 1 ms pulse duration were applied for 30 s every 30 min at a frequency of 10 Hz. After two reproducible responses were obtained (control response) and the tension of the preparation returned to basal levels by washing, the nonselective cannabinoid agonst, WIN 55212–2, or the CB2 receptor agonist, JWH 133, was added and 10 min later contraction was induced with the same stimuli of neurokinin A, capsaicin, or electrical field stimulation. The contractile response obtained in the presence of drug was compared with the control response. In separate experiments, K^+ channel blockers, the CB1 receptor antagonist SR 141716A or the CB2 receptor antagonist SR 144528 were added 10 min before application of the agonist. Only one concentration of one agonist and/or antagonist was tested per bronchi preparation. All of the drugs employed in this study did not affect the baseline tone.

Substance P Release from Guinea Pig Airway Tissues

The procedure of Ray and coworkers (24) was used with certain modifications. Male Hartley guinea pigs were killed and lungs were perfused (6 ml/min, 37°C) with oxygenated (95% O_2, 5% CO_2) Krebs-Ringer HEPES buffer (pH 7.5) containing 138.0 mM NaCl, 5.6 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 11 mM $NaHCO_3$, 10 mM dextrose, 20 mM HEPES, 30 μM bacitracin, 1 μM phosphoramidon via a cannula that was inserted into the pulmonary artery through the right ventricle. The left atrium was opened to collect the outflow. Fifteen minutes after the start, perfusates from one period (15 min; i.e., 90 ml) were collected on ice in beakers containing hydrochloric acid to give a final concentration of 0.1 M. Each fraction was desalted on Sep-Pak C_{18} cartridges (Waters, Milford, MA) as described for somatostatin (25), and the peptides were concentrated to a final volume of 1 ml. The recovery from the Sep-Pak cartridge was more than 90% for radiolabeled substance P. Chemical irritation of tissues was achieved by perfusion with buffer containing 1 μM capsaicin for 5 min during the second collection period. Substance P–like immunoreactivity was measured by radioimmunoassay. The amount of substance P released by capsaicin was calculated by subtracting the level detected in the first period perfusate from that in the second period perfusate. Drugs were added in Krebs-Ringer HEPES buffer throughout the experiment. After the experiments finished, the lungs, including trachea and bronchi, were dissected out and weighed. The increase of substance P–like immunoreactivity release was calculated in fmol per gram of tissues.

Materials

Substance P, neurokinin A, iberiotoxin, charybdotoxin, and phosphoramidon were purchased from Peptide Institute Inc. (Osaka, Japan). Bacitracin was obtained from Sigma Chemical Co. (St. Louis, MO). Capsaicin, JWH 133, dendrotoxin, and glibenclamide were from Nakalai Tesque Chemical Co. (Kyoto, Japan). 1-(2'-hydroxy-5'-trifluoromethylphenyl)-5-trifluoromethyl-2(3H)benzimidazolone (NS1619) were purchased from Funakoshi Chemical Co. (Tokyo, Japan). (R)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholino)methyl]pyrrolo-[1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphthyl)methanone (WIN 55212–2) was synthesized in Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride] (SR141716A) and [N-[(1S)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide] (SR 144528) were kindly supplied by Sanofi-Synthelabo Recherche. [125I]-Substance P (74 TBq/mmol) and anti-substance P antiserum for the radioimmunoassay were purchased from Amersham Int. Ltd. (Buckinghamshire, UK). All drugs were dissolved in dimethylsulfoxide. In *in vitro* experiments, concentrated solutions of drugs prepared in dimethylsulfoxide were diluted in Tyrode's solution or Krebs-Ringer HEPES buffer. The final bath concentration of dimethylsulfoxide was 0.1%. The solvent alone or 0.1% dimethylsulfoxide, had no effect on the responses in the study.

Statistical Analysis

Results are each given as the means ± SEM of five experiments. Statistical analyses were performed by either ANOVA followed by Dunnett's multicomparison test (contractile response of isolated guinea pig bronchi) or by means of the unpaired Student's *t* test (others). p Values less than or equal to 0.05 were considered indicative of significance.

RESULTS

Effects of Cannabinoid Receptor Agonists on Isolated Guinea Pig Bronchial Smooth Muscle Contraction

The effects of cannabinoid receptor agonists on isolated guinea pig bronchial smooth muscle contraction were examined. After electrical field stimulation (26) and capsaicin (27) in the presence of atropine and propranolol, isolated guinea pig bronchial smooth muscles evoke tachykinin-dependent prolonged contraction. Electrical field stimulation, 1 μM capsaicin, and 1 nM neurokinin A elicited the guinea pig isolated bronchial smooth muscle contraction of 0.25 ± 0.03, 0.31 ± 0.02, and 0.21 ± 0.03 g, respectively. A cannabinoid receptor agonist, WIN 55212–2 (0.0191–19.1 μM), inhibited electrical field stimulation– and capsaicin-induced tachykinin-dependent contraction in a dose-dependent manner (Table 1). On the other hand, neurokinin A–induced guinea pig bronchial smooth muscle contraction was not affected by WIN 55212–2 at dose of 19.1 μM. To identify the cannabinoid receptor subtype that was involved in these effects of WIN 55212–2, we examined the influences of selective cannabinoid CB1 (SR 141716A) and CB2 (SR 144528) receptor antagonists. SR 144528 (10 nM) reduced the inhibitory effect of WIN 55212–2, but not SR 141716A (10 nM) on the guinea pig bronchial smooth muscle contraction induced by electrical field stimulation (Figure 1) and by capsaicin (Figure 2). A selective cannabinoid CB2 receptor agonist, JWH 133 (0.1–100 μM), also dose-dependently reduced electrical field stimulation–induced guinea pig bronchial smooth muscle contraction (Table 2). The inhibitory effect of JWH 133 was also reduced by SR 144528 (10 nM), but not by SR 141716A (10 nM) (Figure 3).

TABLE 1. EFFECT OF WIN 55212-2 ON GUINEA PIG ISOLATED BRONCHIAL SMOOTH MUSCLE CONTRACTION INDUCED BY ELECTRICAL FIELD STIMULATION, CAPSAICIN, AND NEUROKININ A

	% Inhibition of Control Contraction		
WIN 55212-2 (μM)	Electrical field stimulation	Capsaicin	Neurokinin A
0.0191	20.5 ± 7.4	5.4 ± 2.7	N.D.
0.191	44.1 ± 4.8*	26.9 ± 2.5*	N.D.
1.91	56.5 ± 6.5*	50.8 ± 3.1*	N.D.
19.1	75.0 ± 5.8*	73.4 ± 3.5*	−4.9 ± 4.2

Definition of abbreviation: N.D. = not done.
Values are given as the mean ± SEM of five experiments.
* Significantly different from vehicle, p < 0.05.

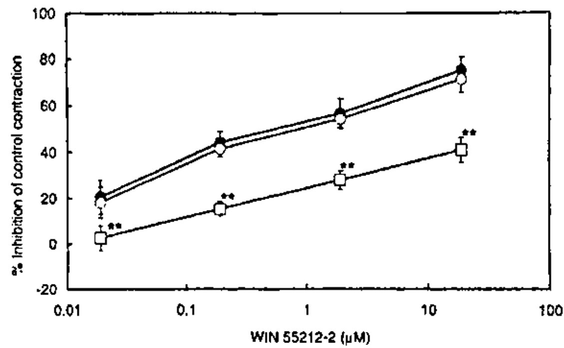

Figure 1. Effects of WIN 55212-2 on isolated guinea pig bronchial smooth muscle contraction induced by electrical field stimulation with cannabinoid receptor antagonists, 10 nM SR 141716A (*open circles*), 10 nM SR 144528 (*squares*) or vehicle (*filled circles*). **Significantly different from vehicle, $p < 0.01$. Values are given as % inhibition of control contraction and are the mean ± SEM of five experiments.

TABLE 2. EFFECT OF A CANNABINOID CB2 RECEPTOR AGONIST, JWH 133, ON GUINEA PIG ISOLATED BRONCHIAL SMOOTH MUSCLE CONTRACTION INDUCED BY ELECTRICAL FIELD STIMULATION AND NEUROKININ A

	% Inhibition of Control Contraction	
JWH 133 (μM)	Electrical Field Stimulation	Neurokinin A
0.1	20.6 ± 3.4	N.D.
1.0	45.7 ± 5.5*	N.D.
10.0	68.3 ± 4.4*	N.D.
100.0	95.6 ± 3.6**	3.1 ± 2.5

For definition of abbreviation, *see* Table 1.
Values are given as the mean ± SEM of five experiments.
* Significantly different from vehicle, $p < 0.05$.
** Significantly different from vehicle, $p < 0.01$.

We examined the influence of K^+ channel blockers on inhibitory effects of WIN 55212-2 on C-fibers. Maxi-K^+ channel antagonists, iberiotoxin (0.1 μM) and charybdotoxin (0.01 μM), greatly reduced the inhibitory effect of WIN 55212-2 on electrical field stimulation–induced guinea pig bronchial smooth muscle contraction (Figure 4). However, a voltage-sensitive A-type K^+ channel blocker, dendrotoxin (1 μM), or an ATP-sensitive K^+ channel blocker, glibenclamide (1 μM), had no effect. Then, a Maxi-K^+ channel opener, NS 1619 (0.0276–27.6 μM) inhibited electrical field stimulation–induced contraction in a dose-dependent manner, but not the neurokinin A–induced contraction at dose of 27.6 μM (Table 3).

Effects of Cannabinoid Receptor Agonists on Substance P–like Immunoreactivity Release from Guinea Pig Airway Tissues

To clarify the mechanism involved in cannabinoid receptor agonist inhibition of electrical field stimulation– and capsaicin-induced guinea pig bronchial smooth muscle contraction, their effects on capsaicin-induced substance P–like immunoreactivity release from guinea pig airway tissues were examined (Table 4). WIN 55212-2 (0.00287–2.87 μM) dose-dependently inhibited the capsaicin-induced release of substance P–like immunoreactivity from guinea pig airway tissues. JWH 133 (0.1–10 μM) also significantly reduced substance P–like immunoreactivity release. In the presence of SR 144528 (10 nM) and WIN 55212-2 (2.87 μM) or JWH 133 (10 μM), the capsaicin-induced release of substance P–like immunoreactivity from guinea pig airway tissues were 48.3 ± 1.1 (control; 49.6 ± 1.6) or 44.2 ± 2.5 (control; 43.3 ± 2.1) fmol/ g tissue, respectively (not significantly different from control). SR 141716A (10 nM), however, did not reduce the inhibitory effects of WIN 55212-2 and JWH 133 (data not shown).

DISCUSSION

It has been suggested that various stimuli act on irritant receptors in respiratory mucosa and produce various respiratory reactions (e.g., bronchoconstriction, tracheal plasma extravasation, and mucus hypersecretion) via the release of tachykinins from the endings of C-fibers (28). The influx of Ca^{2+} into presynaptic nerve endings through voltage-dependent Ca^{2+} channels is essential for the release of neurotransmitters within the nervous system. Ca^{2+} influx is a key step in excitation-release coupling in C-fibers (8, 29). For asthma research, it is important to examine how Ca^{2+} influx into C-fibers is modulated. Morimoto and colleagues (9) suggested that N-type voltage-dependent Ca^{2+} channels are involved in the activation of C-fibers.

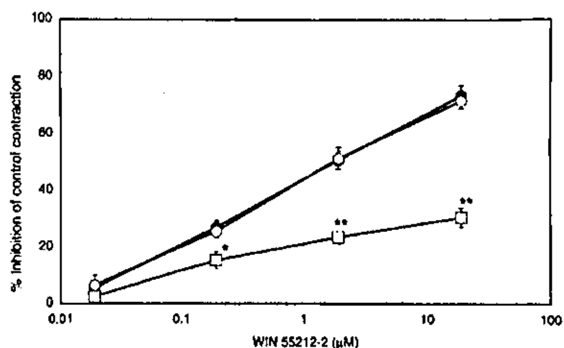

Figure 2. Effects of WIN 55212-2 on isolated guinea pig bronchial smooth muscle contraction induced by capsaicin with cannabinoid receptor antagonists, 10 nM SR 141716A (*open circles*), 10 nM SR 144528 (*squares*) or vehicle (*filled circles*). *Significantly different from vehicle, $p < 0.05$. **Significantly different from vehicle, $p < 0.01$. Values are given as % inhibition of control contraction and are the mean ± SEM of five experiments.

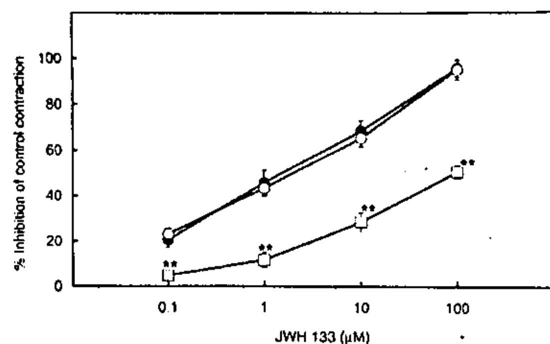

Figure 3. Effects of a cannabinoid receptor agonist, JWH 133 on isolated guinea pig bronchial smooth muscle contraction induced by electrical field stimulation with SR 141716A (*open circles*), SR 144528 (*squares*) or vehicle (*filled circles*). **Significantly different from vehicle, $p < 0.01$. Values are given as % inhibition of control contraction and are the mean ± SEM of five experiments.

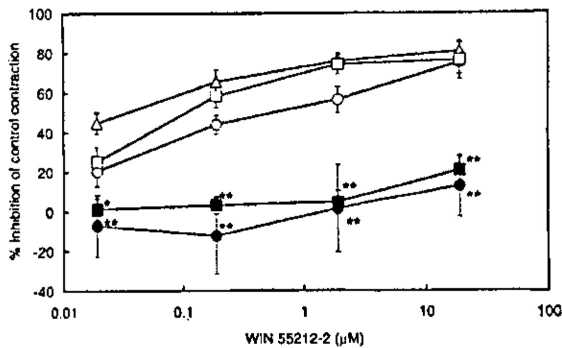

Figure 4. Effects of WIN 55212-2 on isolated guinea pig bronchial smooth muscle contraction induced by electrical field stimulation with K^+ channel blockers, 1 μM dendrotoxin (*open squares*), 1 μM glibenclamide (*triangles*), 0.01 μM charybdotoxin (*filled circles*), 0.1 μM iberiotoxin (*filled squares*) or vehicle (*open circles*). *Significantly different from vehicle, $p < 0.05$. **Significantly different from vehicle, $p < 0.01$. Values are given as % inhibition of control contraction and are the mean ± SEM of five experiments.

TABLE 4. EFFECT OF CANNABINOID AGONISTS ON SUBSTANCE P-LIKE IMMUNOREACTIVITY RELEASE FROM GUINEA PIG AIRWAY TISSUES INDUCED BY CAPSAICIN

Treatment	Increase of Substance P–like Immunoreactivity Release (*fmol/g tissue*)	Inhibition (%)
Control	45.0 ± 2.6	—
WIN 55212-2 0.00287 μM	48.8 ± 1.1	−8.4
WIN 55212-2 0.0287 μM	31.4 ± 1.0**	30.2
WIN 55212-2 0.287 μM	16.3 ± 0.5**	63.8
WIN 55212-2 2.87 μM	3.2 ± 0.4**	92.9
Control	50.1 ± 1.8	—
JWH 133 0.1 μM	32.7 ± 2.6*	34.7
JWH 133 1.0 μM	13.9 ± 1.3**	72.3
JWH 133 10.0 μM	2.3 ± 1.7**	95.4

Substance P–like immunoreactivity release was induced by capsaicin (1 μM). WIN 55212-2 and JWH 133 were added in Krebs-Ringer HEPES buffer throughout the experiment. Values are given as the mean ± S.E.M. of five experiments.
* Significantly different from vehicle, $p < 0.05$.
** Significantly different from vehicle, $p < 0.01$.

WIN 55212-2 has been known to show the cannabismimetic activity and prevent voltage-dependent Ca^{2+} channels (16, 17). In this study, we found that WIN 55212-2 inhibited electrical field stimulation- and capsaicin-induced isolated guinea pig bronchial smooth muscle contraction. This reaction is dependent on tachykinins because it was inhibited by tachykinin receptor antagonists (4, 5, 27). In contrast, WIN 55212-2 did not influence neurokinin A-induced guinea pig bronchial smooth muscle contraction. These results suggested that WIN 55212-2 blocks the release of tachykinins from C-fibers, but does not antagonize the interaction of tachykinins on their receptors. Indeed, WIN 55212-2 did significantly reduce the capsaicin-induced release of substance P-like immunoreactivity from guinea pig airway tissues. Ralevic (30) has reported that cannabinoid-induced inhibition of neurotransmission is mediated by the effector systems such as calcium or potassium channels. Mackie and Hille (17) suggested that inhibitory effect of WIN 55212-2 on voltage-dependent Ca^{2+} channels might be mediated by its interaction with other ion channels coupled either directly or indirectly to G proteins, e.g., potassium channels. Deadwyler and coworkers (31) has shown that WIN 55212-2 increases voltage-dependent potassium-A current in cultured hippocampal cells. It has been reported that ATP-sensitive K^+ channels (32) and Maxi-K^+ channels (28, 33) show inhibitory modulations on the activation of C-fibers. Fox and colleagues (22) reported that NS 1619 inhibited a single C-fiber firing induced by bradykinin and excitatory nonadrenergic noncholinergic contraction induced by electrical field stimulation in guinea pig bronchi at the same dose of 30 μM as shown in our study. Our results well support these evidences and suggest that inhibitory effect of WIN 55212-2 on guinea pig bronchial contraction induced by the activation of C-fibers, may be caused by the inhibition of voltage-dependent Ca^{2+} influx and release of substance P from sensory nerves via the opening of Maxi-K^+ channels.

TABLE 3. EFFECT OF NS 1619 ON GUINEA PIG ISOLATED BRONCHIAL SMOOTH MUSCLE CONTRACTION INDUCED BY ELECTRICAL FIELD STIMULATION AND NEUROKININ A

	% Inhibition of Control Contraction	
NS 1619 (μM)	Electrical Field Stimulation	Neurokinin A
0.0276	35.9 ± 8.4*	N.D.
0.276	55.6 ± 6.6**	N.D.
2.76	66.3 ± 4.3**	N.D.
27.6	69.1 ± 4.2**	−3.1 ± 6.3

For definition of abbreviation, *see* Table 1.
Values are given as the mean ± S.E.M. of five experiments.
* Significantly different from vehicle, $p < 0.05$.
** Significantly different from vehicle, $p < 0.01$.

In the second part of this study, we examined what kind of cannabinoid receptors are involved in the inhibitory effect of cannabinoid receptor agonists on the activation of C-fibers. The biological effects of cannabinoids are mediated by specific receptors, CB1 and CB2, in the central nervous system and in peripheral tissues (11–13). WIN 55212-2 binds to and activates both CB1 and CB2 receptors (18). In this study, inhibitory effects of WIN 55212-2 on electrical field stimulation- and capsaicin-induced isolated guinea pig bronchial smooth muscle contraction, and capsaicin-induced release of substance P from guinea pig airway tissues, were reduced by a specific CB2 cannabinoid receptor antagonist, SR 144528, but not by a CB1 antagonist, SR 141716A. A specific CB2 cannabinoid receptor agonist, JWH 133 (19) also dose-dependently inhibited electrical field stimulation–induced bronchial contraction and capsaicin-induced release of substance P, and its effect was reversed by SR 144528 but not by SR 141716A. According to these facts, it is possible that CB2 receptors exist on the C-fibers in guinea pig airway and that CB2 receptors downregulate the activation of C-fibers and tachykinin release as a physiologic role. It has been reported that the activation of cannabinoid CB1 receptors caused the inhibition of ACh, glutamate, dopamine, noradrenaline, and GABA (34) release from brain slices, and noradrenaline release in the guinea pig lung (35). Recently, it has been reported that an endogenous cannabinoid agonist, anandamide, interacts and activates a nonselective cation channel, TRPV1 receptor, with a high calcium permeability, which is activated by a broad spectrum of stimuli, including capsaicin, heat, and low pH (36, 37). In guinea pig airways, anandamide stimulates sensory nerves by inducing depolarization (38, 39). Our results might support the inactivation of calcium influx in the opposite direction against their reports by the interaction on CB2 receptors, but not CB1 or maybe TRPV1. Indeed, Patel and coworkers (23) reported that activation of the CB2 receptors inhibited sensory nerve activation of guinea pig and human vagus nerve, and the cough reflex in

guinea pigs. They showed the dose-dependent inhibition of a nonselective cannabinoid agonist, CP 55940, and JWH 133 on depolarization of guinea pig and human vagus nerve by capsaicin, prostaglandin E_2, and hypertonic saline. Tucker and colleagues (38), however, indicated CP 55,940 failed to attenuate the excitatory nonadrenergic noncholinergic response in guinea pig airways. Although the same dose of CP 55940 (1 and 10 μM) was used in both studies, their results were discrepant. The reasons for this discrepancy remain to be established, but in our study, a nonselective cannabinoid agonist, WIN 55212-2 and a selective CB2 agonist, JWH 133, significantly inhibited the excitatory nonadrenergic noncholinergic response in guinea pig isolated bronchi and airway tissues in the same micromolar range of doses as the report of Patel and coworkers (23). Some investigators have reported that activation of CB2 receptors was neuroprotective for rat cerebellar granule cells (40) and neuronal expression of CB2 receptor could be regulated by culture conditions (41). These evidences and our results suggest that cannabinoid CB2 receptors exist in nervous system to downregulate Ca^{2+} influx to neuronal cells and the transmitter releases. Especially, in airway tissues, they may act to inhibit the activation of afferent sensory nerves, C-fibers. Calignano and colleagues (42) suggested that the parallel activation of peripheral CB1- and CB2-like receptors inhibited the peripheral pain initiation. They examined the effects of endogenous cannabinoid agonists, anandamide for CB1 and palmitylethanolamide for CB2, and concluded that endogenous cannabinoids might participate in buffering emerging pain signals at sites of tissue injury by the activation of local CB1- and CB2-like receptors. Endogenous cannabinoids may play a physical role to regulate several neuronal responses, including airway tissues.

When excitatory C-fibers are stimulated, not only substance P but other neuropeptides also, e.g., neurokinin A and calcitonin gene-related peptide, are released from their nerve endings and exert various respiratory reactions (43). The inflammatory effects of these neuropeptides on airway tissues may be of pathologic relevance in human bronchial hyperreactivity, and we showed that antiasthmatic compounds, sodium cromoglycate and nedocromil sodium, inhibited hypertonic saline–induced plasma extravasation in guinea pig airways by the activation of C-fibers (7). We conclude that a cannabinoid receptor agonist, WIN 55212–2 which inhibits the activation of C-fibers and the release of these neuropeptides from their endings via the opening of Maxi-K^+ channels by the activation of CB2 receptors, will be a valuable tool for the therapy on airway inflammatory diseases such as asthma.

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●教育講演1●

*Ureaplasma*と新生児感染

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Key Words：*Ureaplasma urealyticum*，慢性肺疾患（CLD），
絨毛膜羊膜炎（CAM），サイトカイン，エリスロマイシン

要　旨

ヒトに感染性のある*Mycoplasma*の一種である*Ureaplasma urealyticum*は，妊婦の下部生殖器培養で52%に陽性であったが，陽性例で早産を起こしやすいことはなく，特定の*Ureaplasma*サブタイプが多数を占めることもなかった。新生児保菌例は大部分が母体陽性例で，児保菌陰性例に比べて在胎週数が若く，破水時間が長く，白血球数が多かった。慢性肺疾患（CLD）と新生児の*Ureaplasma*保菌との関連については結論が出ていないが，CLDを厚生省研究班分類に従い感染先行型（Ⅲ，Ⅲ'），非感染型（同Ⅰ，Ⅱ，Ⅴ）に分け検討した結果では，在胎週数が若いほど，出生時の白血球数が多いほどCLDを発症しやすく，非CLDと非感染型CLDでは在胎週数のみに差があり，非CLDと感染型CLDでは在胎週数，PROM，*Ureaplasma*胎盤保菌が，感染型CLDと非感染型CLDの間では，*Ureaplasma*胎盤保菌と白血球数が有意な因子として抽出された。臍帯血のサイトカインの検討では，非感染型に比べて感染型CLD児で炎症性サイトカインが増加，非CLDと非感染型CLDでは差がなかった。CLDの病態を検討する場合，感染・非感染を分けて検討する必要があると考えられる。

はじめに

Ureaplasma urealyticum（Uu）は，広く動植物界に存在し疾患の原因となる*Mycoplasma*の一種である。ヒトでは泌尿生殖器に常在する菌であり，泌尿器感染のみでなく，妊婦の下部生殖器培養で40～80%が陽性であることから，周産期の母体，新生児感染の原因として認識されるようになってきた。細菌学的に*Mycoplasma*は大きさが0.3～0.8 μmの多形性を示すグラム陰性菌で，光学顕微鏡では見えず細菌濾過器を通過するが，ウイルスとは異なり，無細胞培地で増殖し小さなコロニーを形成する。我々は，Uu培養は10B液体培地とA8平板培地がセットになったビオメリュー社のMycoplasma-lyoを用いて行っている。近年ではPCR法が開発され，その検出率は従来の培養法とほぼ同じあるいはむしろ鋭敏であることが報告され，また，DNAの相同性から，*U.urealyticum*は*U.parvum*（serovar1，3，14，6）と*U.urealyticum*（serotype1，2，3）とに分類さ

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表1　母児ともにPCR陽性例28例（検出数29例）

	U.parvum　(93%)				*U.urealyticum*　(11%)	
	serovar				serotype	
	1	3	6	14	1	2
母児	8	11	7	0	1	2
%	28.6%	39.3%	25.0%		4%	7%

表2

(1) 母体保菌の新生児への影響
86例の単胎出生母体
母体膣培養、新生児咽頭・胃液培養
感染パラメーター(臍帯血IgM、WBC、CRP)

(2) 新生児の保菌と慢性肺疾患との関連
266例の新生児
母体膣培養、新生児咽頭・胃液培養
感染パラメーター(臍帯血IgM、WBC、CRP)
保菌とCLD

表3　母体の保菌状況（母体86例）

母体の保菌	Up(+)	Up(-)	
n	45	41	
GA(W)	33.1±3.3	33.6±3.8	NS
BW(g)	1824±551	1840±601	NS
新生児Up(+)	19(42%)	4(9%)	p<0.001
WBC	14127±8075	13721±6725	NS
血中IgM	32.0±49.7	20.4±16.5	NS
破水時間(hrs)	35.4±72.4	14.0±43.6	NS

表4　陽性母体から出生した新生児の保菌状況（母体陽性45例）

新生児の保菌	Up(+)	Up(-)	
n	19	26	
GA(W)	31.4±2.9	34.3±3.0	p<0.05
BW(g)	1726±544	1895±556	NS
WBC	17521±10780	11646±3993	p<0.05
血中IgM	52.0±70.9	16.7±10.8	NS
破水時間(hrs)	72.5±100.1	8.6±16.9	p<0.05

表5　NICU入院児（266例）

新生児の保菌	Up(+)	Up(-)	
n	42	224	
GA(W)	32.2±3.9	34.4±3.2	p<0.01
BW(g)	1780±661	1933±599	NS
WBC	15769±9199	11949±5571	p<0.05
血中IgM	35.2±53.6	19.5±18.1	NS
破水時間(hrs)	54.2±83.0	7.9±34.4	p<0.01
CLD	5(12%)	4(2%)	p<0.001

れるようになった[1]。**表1**は母児ともにPCR陽性例のsubtypeの頻度である。*U.parvum*が93%でserovar3が多かったが，特定のserovarが早産を起こしやすいということはなかった。

I．*Ureaplasma*と新生児

1985年にQuinnが初めて先天性肺炎で死亡した正期産児の剖検肺でUuを証明し，血清中の抗体価の上昇を確認[2]して以来，肺炎，遷延性肺高血圧症や重症呼吸障害例でUuが分離されたという報告が散見されてきた。これらの報告ではむしろ出生直後から呼吸障害のある例にUuが分離され，その呼吸障害とUu保菌の関連を示唆するものであった。我々が行った新生児とUu保菌との関係を**表2～5**に示す[3][4]。第一はNICUに入院した児でかつ妊娠中に母体のUu膣培養が行われた86例の検討である。母体の保菌の有無にかかわらず出生在胎週数に有意差は無い。つまり妊娠中のUu保菌は早産の予測因子とはならない。しかし，新生児のUu保菌は有意に母体保菌例に多い。つまり新生児が保菌するのは，ほとんどの場合，母体が保菌している場合に限られる（**表3**）。陽性母体から出生した新生児の保菌状況をみると，新生児保菌例では，出生在胎週数が有意に少なく，出生時の白血球数（WBC）が有意に多く，破水時間が長かった（**表4**）。つまり，母体が保菌していても，結果的に新生児が保菌しない場合は，早産になりにくいのである。飛躍した考え方をすれば，胎児に保菌すなわち感染をおこし，胎児側からのシグナルで早産になった可能性も考えられる。

NICU入院児266例の検討では，Uu陽性児は同様に出生在胎週数が有意に少なく，出生時のWBCが有意に多く，破水時間が長く，CLDの頻度が高かった（**表5**）。

II．*Ureaplasma*と慢性肺疾患（CLD）

CLDは早産児に発症し，最近の研究からは早産は絨毛膜羊膜炎（CAM）がその原因であり，早産と母体のUu保菌との関連性が報告されている。