行政院國家科學委員會專題研究計畫　成果報告

研究幽門螺旋桿菌抑制宿主免疫反應之新基因

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Abstract

Cytokines induced by *H. pylori* and the intricate balance of proinflammatory and anti-inflammatory cytokines are relevant to the outcomes of *H. pylori* infection. Transforming growth factor-beta (TGF-beta) and interleukin (IL)-10 are two vital anti-inflammatory cytokines that regulate mucosal immune events in various inflammatory and infectious diseases. To elucidate whether host-bacteria interaction influence TGF-beta and IL-10 production, we investigated the expression of TGF-beta and IL-10 in various mammalian cell lines preincubated with *H. pylori* and other enteric bacteria. The amount of TGF-beta protein, but not IL-10, was significantly increased after stimulation of epithelial cell lines with *H. pylori*, while non-*H. pylori* enteric bacteria such as *E. coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, group B *Salmonella*, *Shigella flexneri* and *Pseudomonas aeruginosa* did not induce TGF-beta production. Different *H. pylori* strains isolated from gastritis, peptic ulcer and gastric cancer or cagA or vacA isogenic mutants showed similar effects of TGF-beta induction, indicating such effect was a constitutional characteristic of *H. pylori* and independent of cagA and vacA status. Pretreatment with boiling and proteinase K suggest the responsible factor is *H. pylori* soluble proteins. The soluble fraction of *H. pylori* has also been demonstrated to lead to TGF-beta production in primary gastric epithelial cell culture and mononuclear cells. These results implicate the presence of a protein factor (termed TIP for TGF-beta inducing protein) with activity of inducing production of TGF-beta by both epithelial and mononuclear cells. In view of the multiple effects of TGF-beta, TIP of *H. pylori* might mediate the immune response and contribute to the pathogenesis of *H. pylori* infection. Finally, we found that Hsp60 is the major factor to induce TGF-beta expression.

Purpose

Define TGF-beta inducing factor of *H. pylori.*

Introduction

Helicobacter pylori (*H. pylori*) are gram-negative bacilli that infect about half of the world's population. The majority of infected patient remain in asymptomatic gastritis, while 10-15% develop peptic ulcer, gastric carcinoma, and B cell mucosa-associated lymphoid tissue (MALT) lymphoma. The variable clinical outcomes of H. pylori infection were attributed to variations in bacterial virulence factors as well as differences in host immune responses. In particular, the immune response against *H. pylori* virulent factors might provide a direct linkage to the development of gastroduodenal diseases.

In the early stages of infection, *H. pylori* induces production of chemokines

including RNATES, GROα, MIP1α, ENA-78, MCP-1 and IL-8, as well as secretion of proinflammatory cytokines such as IL-1, IL-6 and TNF-α. The induction of chemokines or proinflammatory cytokines will attract neutrophils, monocytes, macrophages or dendritic cells migrating to inflammatory area and activate a number of innate inflammatory responses. Neutrophils participating in gastric inflammation are related with the clearance of *H. pylori*. Mucosal macrophages and dendritic cells, the monocyte-derived cells could capture and digest pathogenic antigen soon after infection of pathogen. Furthermore, specific antibodies against *H. pylori* are detectable in patients' sera that may help eradicate the bacterial infection in gastric mucus. In terms of cell-mediated immunity, evidences have shown CD4+ T-cell plays an important role for protective immunity against *H. pylori*. However, *H. pylori* are not eliminated in the face of detectable cytotoxic lymphocyte, Th1 and antibody responses. It was presumed that *H. pylori* have evolved a number of strategies to circumvent protective immune responses. In this regard, studies have shown *H. pylori* could induce apoptosis of different cells, including gastric epithelial cells, macrophage, and T cells. Moreover, the local cytokine milieu, particularly the intricate balance between proinflammatory and anti-inflammatory cytokines, can influence T cell development and then the efficacy of immune responses and gastric pathology. An early and persistent Th1-dominated CD4 response appears to be critical in the prevention of the chronically infected state but may lead to more severe gastric inflammation. In contrast, the development of chronic infection is linked to weak or absent *H. pylori*-specific Th1 response and to the presence of Th2-type cytokines but only minimal gastritis was found under such condition. Among known Th2 cytokines, IL-4 and IL-5 are virtually absent when gastric lymphocytes from H. pylori infected patients were evaluated. Recently, two anti-inflammatory cytokines, TGF-beta and IL-10, have been reported to exert potent anti-proliferative and anergy-inducing effects on CD4 cells in other infectious diseases. Such pathogen-stimulated IL-10 or TGF-beta production by innate cells might play a vital role in the prevention of infection-induced immunopathology or prolongation of persistence by suppressing Th1 responses. However, the roles of TGF-beta and IL-10 in *H. pylori* infection are less well known. Few studies have investigated the effects of H. pylori on TGF-beta and IL-10 production and controversial results were observed.

To investigate whether *H. pylori* infection could modulate the production of TGF-beta and IL-10, we studied TGF-beta and IL-10 secretion in various mammalian epithelial cells after preinoculation with *H. pylori* and other enteric bacteria. Here, we show that H. pylori may secrete some soluble protein(s) to specifically induce TGF-beta production in gastric epithelial cells and monocytes. Such inducing ability is a constitutional characteristic of *H. pylori* independent of cagA and disease status.

We assume *H. pylori* might utilize this capability to escape from or interfere with T cell effector functions and inflammatory responses.

Materials and Methods

Bacterial strains: The *H. pylori* strains used in this study were obtained from American Type Culture Collection (ATCC 43504) or freshly isolated from gastric biopsy specimens at National Taiwan University Hospital. For the control experiments, bacterial strains including *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Shigella flexneri* and group B *Salmonella* were used.

Epithelial cell cultures: Different epithelial cell lines including gastric cancer (AGS, N87, SUN-1, SUN-16, Hs578T, T-47D, L48, and TSGSH), colonic cancer (colo 320), breast cancer (NIC-H157) and hepatoma (HepG2) were obtained from ATCC (Manassas, VA).

Preparation of soluble extract of H. pylori

Preparation of peripheral blood mononuclear cells (PBMCs) and primary gastric epithelial cell lines

Size exclusion chromatography and assays

Gel filtration and SDS-PAGE

Quantitation of TGF-beta and IL-10 by ELISA

Result

Exposure of human gastric epithelial cell lines to *H. pylori* induces TGF-beta production

To examine the effects of *H. pylori* on TGF-beta production by gastric epithelial cells, *H. pylori* were cocultured with different human gastric cancer cell lines in the initial experiments. As shown in Fig. 1A, quantification of TGF-beta in supernatants after 16h incubation demonstrated a significant increase in TGF-beta compared to that of untreated cells ($p < 0.05$). Three clinical isolates of *H. pylori* isolated from gastritis, duodenal ulcer or gastric cancer were tested in AGS to determine the responding time for TGF-beta production. The expression of TGF-beta increased 5 min after *H. pylori* infection, reached its maximal level at 90 min and remained at such a high level till the end of the experiment (post infection 16h, Fig. 1B). The production of TGF-beta tested in AGS was noted to show a dose-dependent manner (Fig. 1C). The plateau was found to be at MOI of 400:1. Based on these experiments, the following analyses of *H. pylori*-induced TGF-beta production was performed using MOI of 400:1 and incubated for 16h unless otherwise stated.

Induction of TGF-beta production is a constitutional characteristic of *H. pylori,*

irrespective of cagA, vacA and disease status

We next carried out experiments to determine the susceptibility of various cell types or other enteric bacteria to the induction of TGF-beta. As shown in Fig. 2A, *H. pylori* could significantly increase the production of TGF-beta in various mammalian non-gastric cell lines ($p < 0.05$). The induction of TGF-beta in epithelial cells was specific to *H. pylori* since other enteric bacteria such as *E. coli*, *K. pneumoniae*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Shigella flexneri*, and group B *Salmonella*, could not lead to TGF-beta production (Fig. 2B). To study whether there is strain variability with respect to TGF-beta production, various *H. pylori* strains isolated from gastritis, duodenal ulcer, gastric ulcer, or gastric cancer were cocultured with AGS. No significant difference in the mean level of TGF-beta was observed among different disease categories (Fig. 2C). Furthermore, TGF-beta production is similar between cagA-negative isogenic mutants and cagA-positive strains (Fig. 2D), indicating that such a characteristic is independent of cagA status.

The IL-10 level is not influenced by *H. pylori*

In contrast to TGF-beta, there was no alteration of IL-10 level after coculture of *H. pylori* with various mammalian cell lines (Fig. 3). These data suggest that IL-10 production in epithelial cells was not affected by *H. pylori* infection in this in vitro model.

Soluble proteins from *H. pylori* are responsible for the induction of TGF-beta

To characterize the bacterial components responsible for TGF-beta production, AGS was stimulated with live and dead *H. pylori*, soluble and insoluble fraction, respectively. As shown in Fig. 4, only the insoluble fraction could not induce TGF-beta production. To further determine whether the modulatory factors present in *H. pylori* preparations were protein or nonprotein factors, the soluble lysate was pretreated with boiling. After such treatment, the capability of induction of TGF-beta was significantly decreased (Fig. 4). Furthermore, digestion of the supernatant with proteinase K completely abrogated TGF-beta production, indicating soluble proteins account for the inducing effect.

The soluble fraction of *H. pylori* preparations is capable of inducing TGF-beta production both in mononuclear cells and primary gastric epithelial cell line

Although epithelial cells from cancer cell lines could be stimulated to express TGF-beta, whether the normal gastric epithelial cells and PBMC can respond to *H. pylori* is unknown. Therefore, the human primary cultured gastric epithelial cells were incubated with *H. pylori* or the soluble supernatant to monitor the secretion of

TGF-beta into medium. The results revealed that *E. coli* could not induce the TGF-beta secretion of human primary cultured gastric epithelial cells. In contrast, *H. pylori* and the soluble proteins derived from *H. pylori* could induce the TGF-beta secretion for the human primary cultured gastric epithelial cells as well as PBMC (Fig. 5A & 5B).

Partial characterization of the TGF-beta inducing factor(s)

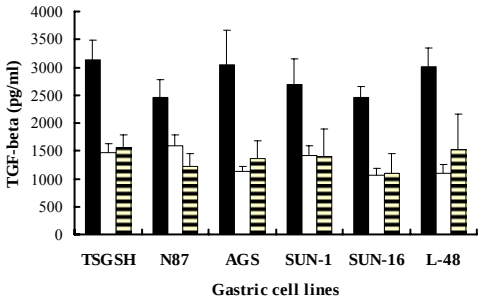
After gel filtration of *H .pylori* supernatants, fractions of TGF-beta inducing factor have different abilities for TGF-beta induction. As the result showed, pooled fractions (1~5) and pooled fractions (6~10) have higher TGF-beta inducing activity (Fig. 6A). Analysis of fractions, containing the higher TGF-beta inducing activities, revealed two major bands in SDS-PAGE (Fig. 6B). Select these two major bands for MALDI-TOF-MS analysis, each highest probability based mowse scored protein is UreB and Hsp60 (Fig. 6C). After cloning UreB and Hsp60 respectively into the protein expression vector (pET30(a)), each clone was induced to express protein. As the result showed, bacteria pellet which expresses the Hsp60 can induce the TGF-beta production but bacteria pellet which expresses the UreB (Fig. 6D).

Discussion

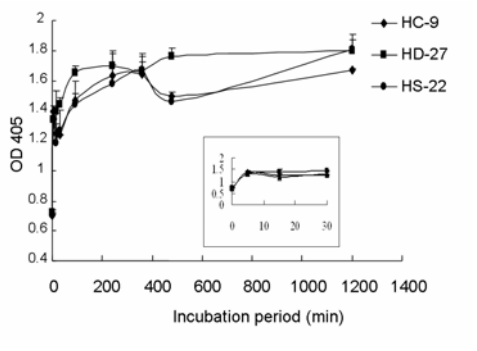
TGF-beta expression is associated with the *H. pylori*. After purification and SDS-PAGE analysis, UreB and Hsp60 were candidates for having TGF-beta inducing activity. But later experiment, we found that Hsp60 is the major factor to induce TGF-beta expression but UreB.

Figure 1

(a)


TGF-beta (pg/ml)
4000
3500
3000
2500
2000
1500
1000
500
0
TSGSH
N87
AGS
SUN-1
SUN-16
L-48
Gastric cell lines

(b)


HC-9
HD-27
HS-22
OD 405
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0
200
400
600
800
1000
1200
1400
Incubation period (min)

(c)

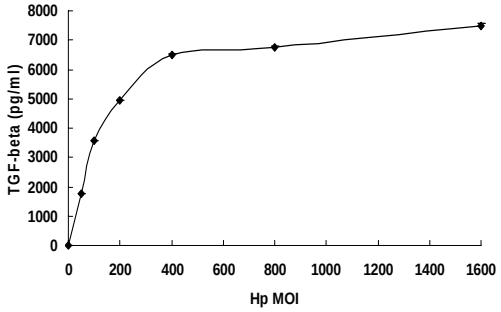
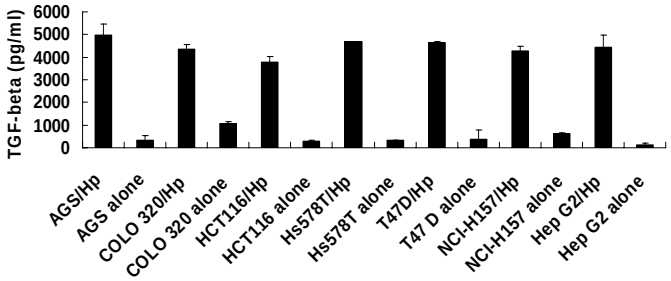
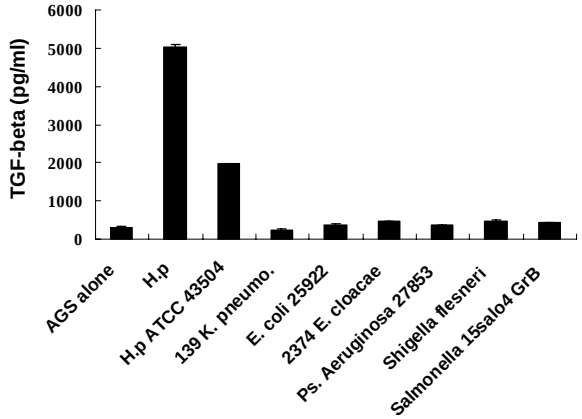

TGF-beta (pg/ml)
8000
7000
6000
5000
4000
3000
2000
1000
0
0
200
400
600
800
1000
1200
1400
1600
Hp MOI

Figure 2

(a)



(b)



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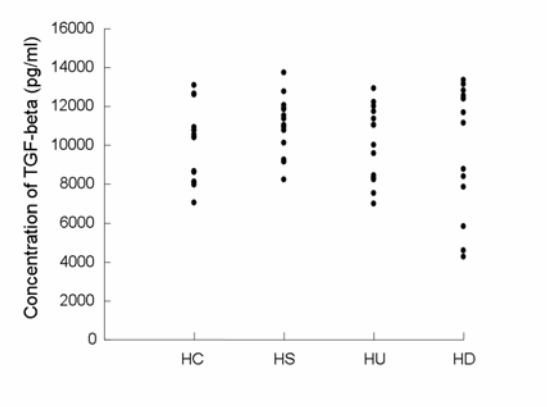


(d)

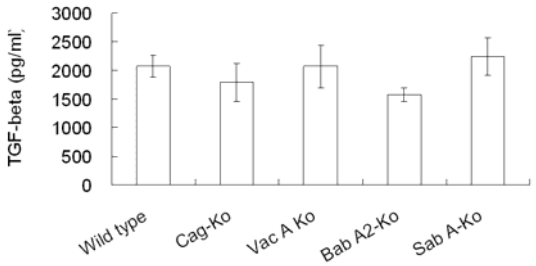


Figure 3

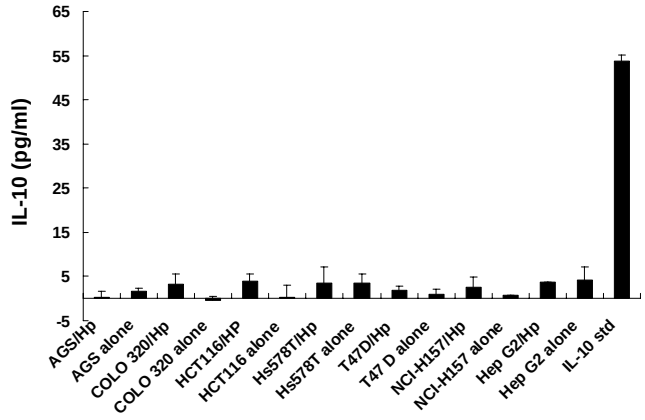


Figure 4

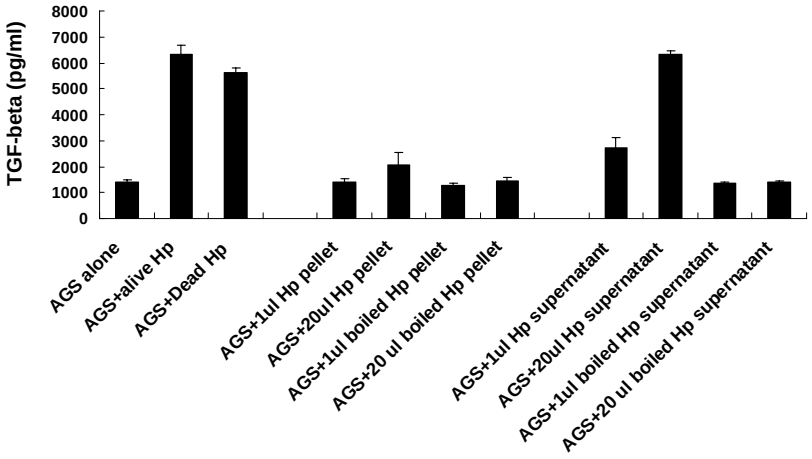
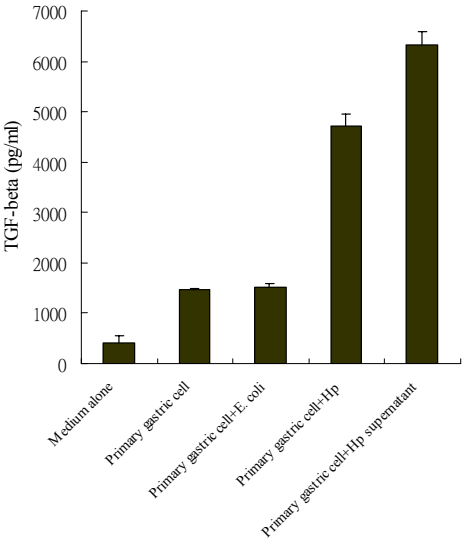


Figure 5

(a)



(b)

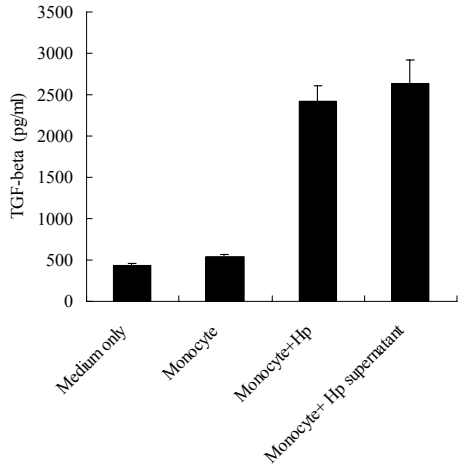
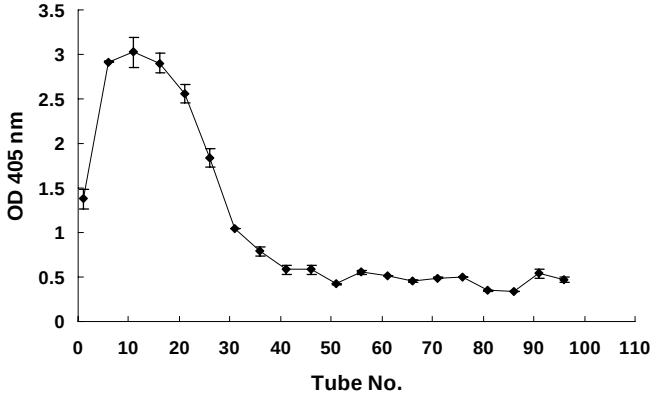
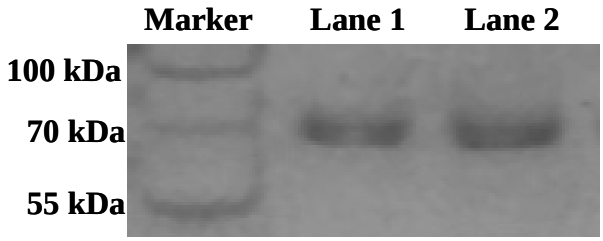


Figure 6

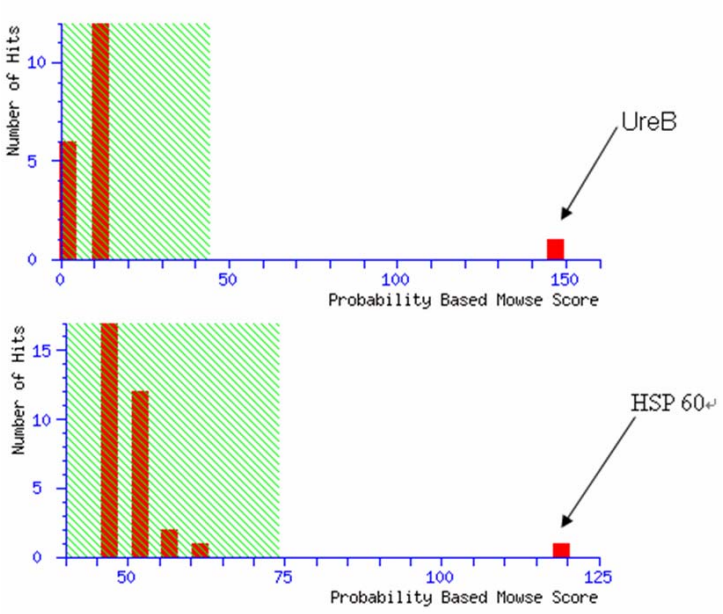
(a)



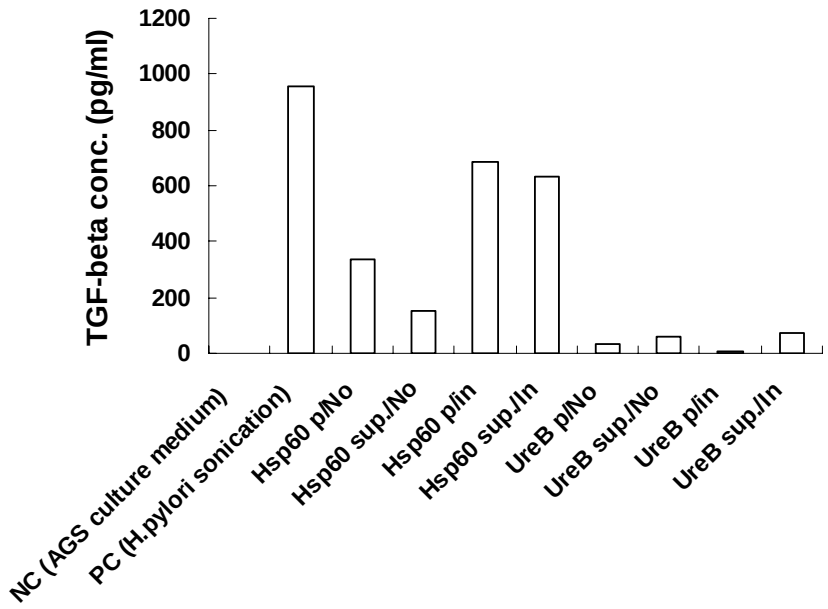
(b)


Marker
Lane 1
Lane 2
100 kDa
70 kDa
55 kDa

(c)


Number of Hits
UreB
Probability Based Mowse Score
Number of Hits
HSP 60
Probability Based Mowse Score

(d)


TGF-beta conc. (pg/ml)
NC (AGS culture medium)
PC (H.pylori sonication)
Hsp60 p/No
Hsp60 sup./No
Hsp60 p/in
Hsp60 sup./In
UreB p/No
UreB sup./No
UreB p/in
UreB sup./In