The capsaicin- and Helicobacter strains-induced cellular mechanisms of the gastric mucosa in humans and animals

Ph.D. dissertation

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Abbreviations

BAO = basal acid output

Cag A = cytotoxin associated gene A

CCR = CC-chemokine receptor

CD = cluster of differentiation

cDNA = complementary deoxyribonucleic acid

CINC-2 beta = cytokine-induced neutrophil chemoattractant 2 beta

CNS = central nervous system

CSPASF = capsaicin sensitive primary afferent sensory fibres

DOB = Delta Over Base

E. coli = Escherichia coli

eotaxin = eosinophil chemotaxis inducing protein

FBS = foetal bovine serum

GAS = gastric acid secretion

GI = gastrointestinal

GM = gastric motility

GM-CSF = granulocyte-monocyte colony stimulating factor

GRO-alpha = growth related oncogen alpha

GSM06 = gastric surface mucous epithelial cell line 06

H. felis = Helicobacter felis

hIFN-gamma = recombinant human interferon gamma

H. pylori = Helicobacter pylori

hTNF-alpha = recombinant human tumor necrosis factor alpha

ID_{50} = inhibitory dose causing 50% inhibition

IFN-gamma = interferon gamma

i.g. = intragastrically

IL = interleukin

IRIS = Infra Red ISotope analyser

MALT = mucosa associated lymphoid tissue;

MCP-1 = monocyte chemotactic protein 1

M-CSF = monocyte colony stimulating factor

MIP1-alpha/beta = macrophage inflammatory protein 1 alpha/beta

mRNA = messenger ribonucleic acid

RANTES = regulated on activation normal T-cell expressed and secreted

RT-PCR = reverse transcriptase - polymerase chain reaction

RTX = resiniferatoxin

SV40 = Simian virus 40

TGF-beta = transforming growth factor beta

Th1 = inflammatory T lymphocyte

Th2 = helper T lymphocyte

TNF-alpha = tumor necrosis factor alpha

Vac A = vacuolating toxin gene A

The effect of intragastric capsaicin on gastric secretory parameters, gastric motility and glucose absorption in humans

1 Introduction

1.1 Capsaicin

Capsaicin (8-methyl-N-vanillyl-6-noneamide) is the pungent alkaloid of the fruits of those about 200 species which belong to the genus Capsicum. These fruits exist under well known names, e.g. red/hot paprika/pepper/chilli, black pepper, and are world widely consumed and used as spices for cooking. Pharmakokinetic studies showed that after applying capsaicin per orally, it is absorbed in the gastrointestinal (GI) tract through a non-active transport into the portal vein, then the majority is excreted in the urine without metabolisation, the minority is metabolised in the liver by mixed function oxidase system, conjugated with glucuronid, and then excreted in the urine (Pimparkar, 1972).

1.2 Capsaicin sensitive primary afferent sensory fibres (CSPASF)

From pharmacological aspect capsaicin has the unique feature to cause a short, initial stimulation, and then - by desensitisation - blockage of a subset of mammalian afferent neurons with Aδ and C fibres (Holzer, 1991). Therefore they are called capsaicin sensitive primary afferent sensory fibres (CSPASF) (Szolcsányi, 1978). The desenzitising concentration is about 0.1 mM in rats (Szolcsányi, 1975). Because of this neuron blocking effect, capsaicin is used as a therapeutic drug with topical analgetic effect in different neuralgias (Bernstein, 1988).

CSPASF are present only in the afferents of the vagal, trigeminal nerve, and found in the spinal afferents of dorsal root ganglia (Holzer, 1998a). About 85% of the vagal nerve fibres are afferents, and about 10% of these afferents are capsaicin-sensitive. In the case of spinal nerves, about 80% of spinal afferents are sensitive to capsaicin (Dockray, 1992). They contain bioactive peptide neurotransmitters as

CGRP, tachykinins - SP and NKA -, which are released from the nerve terminals under orthodromic capsaicin stimulation of the receptors, or antidromic electric stimulation of the nerve. After released these neurotransmitters exert different local vascular tissue reactions and motor functions in the skin, mucosal surfaces, heart, etc. (Holzer, 1998b; Szolcsányi, 1996). Because of this unique feature of these fibres to parallely signal the sensory stimulus from the nerve ending to the central nervous system (CNS), and exert efferent function too, the term and concept of dual sensory-efferent function was introduced for these fibres (Szolcsányi, 1984). Another fundamental step in this field was the discovery, and later the cloning of the capsaicin (vanilloid) receptor (Caterina, 1997).

1.3 The effect of capsaicin and role of CSPASF in the GI tract

Although the role of CSPASF was thoroughly investigated first in the context of neurogen inflammation in the skin (Holzer, 1998b), CSPASF have been found in the nerves innervating many organs, among them the organs of the whole GI tract in animals (Barthó, 1999; Maggi, 1988a,b,c; Holzer, 1982). The functions of the CSPASF may be investigated in two ways. The *direct* approach means the application of capsaicin topically in a concentration (<0.1 mM) which stimulates the nerve endings of CSPASF. The *indirect* method is based on the neurotoxic feature of high concentration (>0.1 mM) capsaicin. In this latter way the neurodegenerative dose of capsaicin is applied either neonatally, or in adult age systemically, or topically around the nerves or ganglia (Abdel-Salam, 1997b). Then the abolished, inhibited or aggravated effects are considered to indicate the role of CSPASF in the process under investigation.

The investigations in animals revealed that the CSPASF have very wide range of important roles including vascular, secretory and motor functions in the GI tract (Holzer, 1998a). They take part in the gastroprotection (Abdel-Salam, 1995a,b,d; 1997a; Szolcsányi,1990; Gray, 1994; Holzer, 1989a), in healing of ulcers (Takeuchi, 1994), in acid-induced bicarbonate secretion (Takeuchi, 1992), in enterogastric reflexes (Cervero, 1982). Capsaicin furthermore was found to dilate

arterioles in the gastric submucosa (Chen, 1992) and in the mesenteric bed (Manzini, 1988), to increase gastric mucosal blood flow (Szolcsányi, 1981), to stimulate mucus output (Toh, 1955; Kang, 1995a), both to inhibit (Abdel-Salam, 1995c, 2000) and increase (Makara, 1965) gastric acid secretion, to cause neurogenic inflammation in the gallbladder (Lundberg, 1984).

1.4 The innervation of the stomach

The secretory and motor function of the stomach is under neuronal and hormonal control. Both of them include stimulating and inhibiting factors, which are in balance under physiological circumstances to provide an appropriate secretion and emptying for the digestion. The nerves innervating the stomach are part of the autonomic nervous system and composed of intrinsic and extrinsic neurons. These latter are made up of afferent and efferent fibres, which run in the vagal and spinal nerves (Jass, 1984). Among the afferents of the vagal and spinal nerves are found CSPASF too (Dockray, 1992). The intrinsic neurones are not capsaicin-sensitive (Green, 1988).

1.5 The effects of capsaicin, and role of CSPASF in the stomach

1.5.1 Gastric acid secretion (GAS)

Gastric acid secretion is a result of numerous neural (sympathetic, parasympathetic), hormonal (e.g. gastrin), and paracrin (e.g. somatostatin) mechanisms (Hersey, 1995). The capsaicin-containing spices have been considered for long time to be harmful to the gastric mucosa on the basis of feeling discomfort after consuming them, however the ulcer patients were advised to avoid using these spices without any scientific basis (Schneider, 1956; Solanke, 1973). Attempts to scientifically clarify the effect of capsaicin containing spices on GAS have already

performed in the 1930s (Varga, 1938). Since that time a number of observations were carried out either in animals or in humans. In these investigations usually big amounts of different capsaicin containing spices were used, between 1 and 30 g, which doses are around and above the dose of the everyday consumption of these spices in certain countries (Desai, 1973). Since red pepper contains about 0.1-1 % capsaicin (Szolcsányi, 1977), the dose of capsaicin in these studies ranged between 1 and 300 mg.

1.5.2 The effects of capsaicin on, and role of CSPASF in GAS

1.5.2.1 Animals

The investigations of the effect of capsaicin on, and role of CSPASF in GAS showed contradictory results in animals. However there have been performed experiments with intragastric capsaicin application resulting increased (Makara, 1965), not altered (Toh, 1955), or decreased (Lippe, 1989) gastric acid secretion depending on the dose, concentration and duration time of capsaicin application, most of the experiments were performed by the indirect way causing degeneration (Evangelista, 1989) or functional ablation (Raybould, 1989) of the nerves, and the effects of these interventions on stimulated gastric acid secretion by different secretory stimulants was studied (Raybould, 1989; Evangelista, 1989). In these circumstances the stimulated gastric acid secretion was found inhibited (Raybould, 1989), not changed (Esplugues, 1990), or even increased (Lloyed, 1993).

Our laboratory group previously found that small dose (0.25-1 ug/kg) of capsaicin and capsaicin analogue resiniferatoxin (RTX) decreased basal GAS in dose-dependent manner in pylorus ligated rats (Abdel-Salam, 1995c). The pentagastrin-stimulated GAS was also inhibited by capsaicin in rats with acute gastric fistula (Abdel-Salam, 1999). Furthermore the pentagastrin-, bethanecol-, and histamine-stimulated GAS was blocked in pylorus-ligated rats by intragastric application of RTX too (Abdel-Salam, 1995b).

1.5.2.2 Humans

As for GAS in humans, many attempts were done to reveal the effect of different doses of capsaicin containing spices and capsaicin itself on gastric secretory parameters from the 1930s (Heupke, 1932; Varga, 1938). These observations produced very contradictory results, from the increased (Desai, 1977), through unaltered (Myers, 1987), to decreased GAS (Heupke, 1932). However the conditions of the observations, as amount of capsaicin or spicy, the duration of study - similarly to animal studies - varied between wide borders, and usually relatively big amount (1-30 g) of spices were used, such doses of capsaicin what amount can be found in the generally consumed foods (Desai, 1973).

Therefore in our study we aimed to evaluate the effect of small (100-800 ug) dose of capsaicin in concentrations between 3.2-26 uM on gastric secretory parameters in human healthy volunteers.

1.5.3 Gastric motility (GM)

The motility of the GI tract is under neuronal and hormonal control. The stomach receives innervation from the central nervous system (CNS) through the extrinsic sympathetic and parasympathetic vegetative efferent motor neurones, which run in the vagal and spinal nerves, however the motility is also affected directly by the capsaicin-sensitive spinal and vagal afferents with dual sensory-efferent function (Holzer, 1998a). The CSPASF in the nerves supplying the organs of the GI tract take part in the control of motility of the whole GI tract, in the oesophagus (Gonzalez, 1998; Barthó, 1999), stomach (Holzer-Petsche, 1989; Kang, 1993; Uno, 1997; Takeuchi, 1994), small (Maggi, 1986, 1987, 1988b,c, 1989a, 1990a; Barthó, 1987) and large intestine (Maggi, 1988a, 1990b; Cervero, 1982; Manzini, 1988), gallbladder (Holzer, 1982; Maggi, 1989b; Lundberg, 1984).

1.5.4 The effects of capsaicin on, and role of CSPASF in GM

1.5.4.1 Animals

As for the effect of capsaicin on, or the role of CSPASF in GM, however there have been performed investigations on intact animals resulting in both increased (Raybould, 1988), or decreased (Kang, 1993) gastric emptying, most of the studies were done on isolated muscle stripes removed from different type of muscle layers of the stomach wall. Contradictory results can be found in the literature, and according to the type of the original muscle layer, and dose of capsaicin, the results show relaxing (Lefebvre, 1991) or biphasic effect (Holzer-Petsche, 1989) on isolated muscle stripes.

1.5.4.2 Humans

In contrast to the investigations in animals we know only a little about the effect of capsaicin in human. From investigations with isolated muscle strips of small and large intestine of operated patients we know, that these parts of the GI tract response to capsaicin in vitro, and capsaicin mainly causes relaxation of these muscle strips (Giuliani, 1991; Maggi, 1990b). But there are only a few objective scientific data in the literature about the function of CSPASN in the human stomach. Although the effect of capsaicin and capsaicin-containing spices on gastric symptoms (Schneider, 1956), mucosa (Kang, 1988), secretion (Myers, 1987) and ulcer-formation (Kumar, 1984) was thoroughly investigated with many controversial results in human, the pharmacology effect of capsaicin on the motor activity of GI tract (especially of the stomach) hardly studied in human yet, moreover these studies did not provide concordant data. While Desai et al. (1977) found increased gastric motility (emptying) after ingestion of red chilli powder, Gonzalez et al. (1998)

measured delayed gastric emptying after giving red pepper sauce to healthy human subjects.

Therefore we measured the gastric emptying after applying small dose (400 ug) capsaicin in concentration of 13 uM in healthy human volunteers.

We used the ^{13}C-labeled octanoic acid breath test with Infra Red ISotope analyser (IRIS, Izinta, Budapest, Hungary) for the gastric emptying measurements. This is a reliable, safe, non-invasive (Ghoos, 1993; Maes, 1994; Veereman, 1996) and well accepted method for measurements of gastric emptying/motility in human.

2 Aims

Our aims were in these studies the followings:

1. To measure the effect of small dose (100-800 ug) of intragastrically given capsaicin (in concentrations of 3.2-26 uM) on GAS in healthy human volunteers.

2. After obtaining the result that capsaicin has an inhibitory effect on GAS in human, we aimed to determine the ID50 for capsaicin on GAS.

3. Then we planned to identify the time for action of capsaicin on GAS.

4. We aimed to measure the gastric emptying rate after intragastric application of capsaicin in the same dose and concentration which was found to be ID50 on GAS.

5. We aimed to measure glucose absorption, insulin, C-peptide and glucagon hormon levels after application of the same dose and concentration of capsaicin as ID50 on GAS.

3 Materials and methods

3.1 Patients

The observations were carried out on healthy volunteers. The volunteers were informed about the details of the investigations, and then subscribed the informed consent in the presence of an independent physician. The study was carried out in the agreement of good clinical practice (GCP). The Regional Ethical Committee permitted the research protocol.

The volunteers had no gastric diseases earlier, and they have not received any drug affecting gastric secretion or motility for at least 72 h before the investigations.

The volunteers were admitted to the First Department of Medicine, then general medical physical, laboratory (blood picture, liver function, kidney function, electrolytes), and abdominal ultrasonographic examinations were performed before the study. The volunteers with negative results in the above mentioned investigations were initiated in the study. The healthy volunteers went over a night starvation, and the observations were started at 8.00 a.m. in the Gastroenterology Laboratory of the First Department of Medicine.

3.2 Capsaicin solutions

A basic 1% (g/g) capsaicin solution was made for the further dilutions as follows. 100 mg capsaicin (Sigma, Budapest, Hungary) was dissolved in 1 ml 96% alcohol, then 1 ml polysorbate (Sigma, Budapest, Hungary) and 8 ml distilled water was added to the solution. Then diluting the appropriate amount (100-800 ul) of this basic capsaicin solution in 100 ml physiological saline, capsaicin test solutions with different capsaicin concentrations were prepared for the investigations.

3.3 Gastric secretory measurements

3.3.1 Patients

The observations were carried out on 10 healthy volunteers, 5 women and 5 men, with the average age of 31 years.

3.3.2 Capsaicin test solutions

100, 200, 400, 800 ul of the basic 1% capsaicin solution (containing 100, 200, 400, 800 ug capsaicin respectively) was diluted in 100 ml physiological saline, and given intragastrically. The concentration of the test solutions therefore were 3.2, 6.5, 13, 26 uM respectively.

3.3.3 Protocols of the investigations

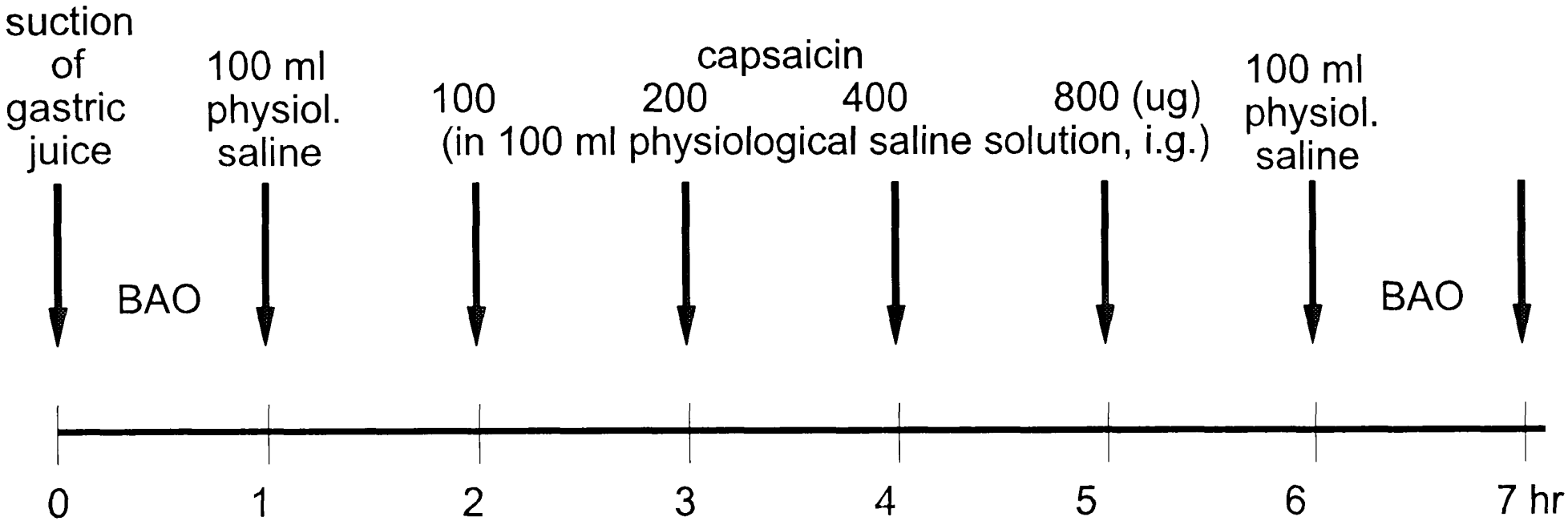
A nasogastric tube was introduced into the stomach of the volunteers. At the start of the observations total gastric content was completely suctioned.

3.3.3.1 Protocol (A) - (Fig. 1.)

The secreted gastric juice was suctioned at every 15 min. for one hour (Basal Acid Output, BAO). Then 100 ml saline was intragastrically given through the nasogastric tube, and gastric juice was suctioned again at every 15 min. for one hour. In the forthcoming hours 100-100 ml test solution containing 100, 200, 400 and 800 ug capsaicin respectively was given into the stomach. Gastric juice was suctioned again after every 15 min. periods. The volunteers received in the last hour

Fig. 1.

PROTOCOL OF INVESTIGATION (A)



The detailed description of the protocol is seen in the text.

100 ml saline solution intragastrically as final control for measuring BAO. The suctioned doses of gastric contents went over the following determinations.

3.3.3.1.1 Volume

Volume (in ml) of the gastric content was measured.

3.3.3.1.2 Gastric acid output

The volume of secreted gastric acid in the gastric content was determined performing a titration of gastric juice with 0,1 N NaOH to pH 7 in the presence of pH titrimeter (Radelkis, Budapest). Gastric acid output (in mmol/h) was calculated and expressed as means ± SEM.

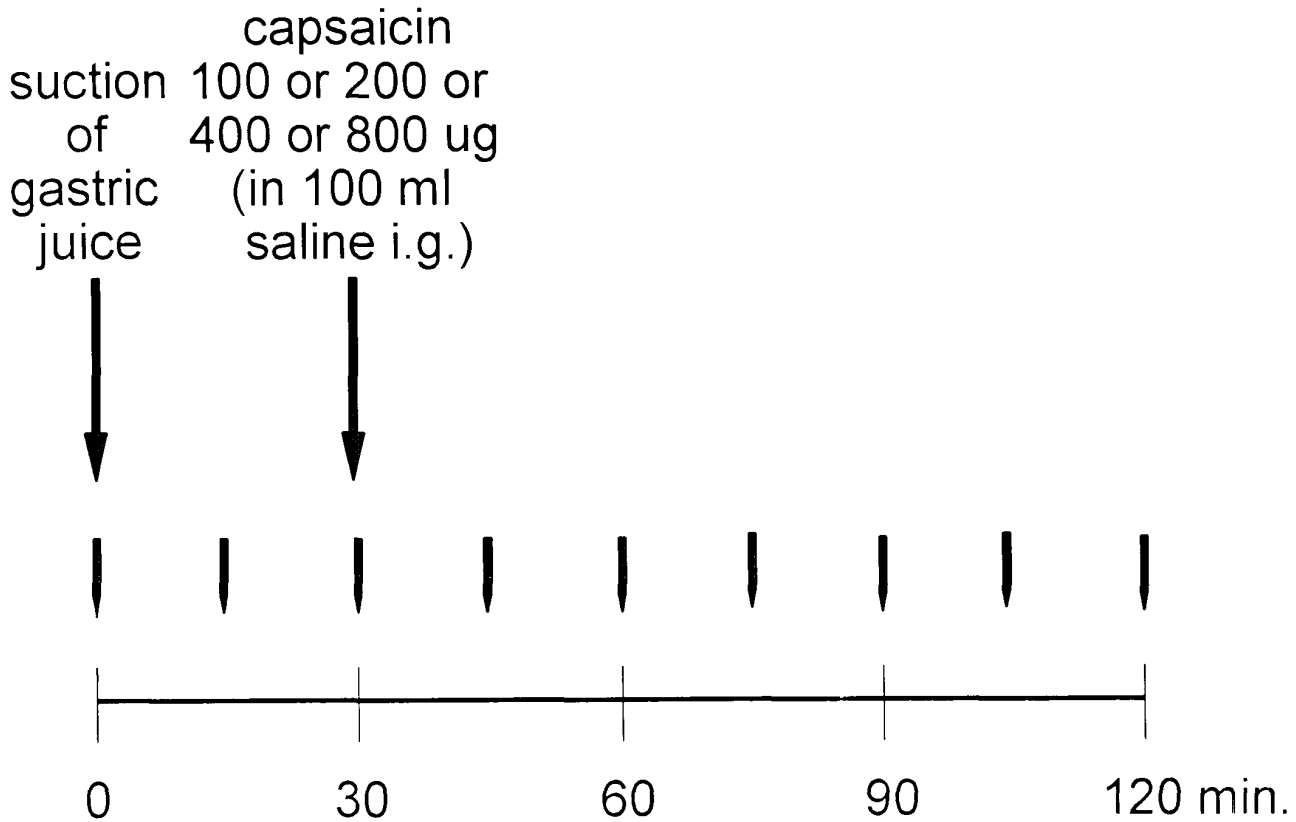
3.3.3.2 Protocol (B) - (Fig. 2.)

In another set of observations the time interval for the action of capsaicin was measured. The gastric content was suctioned at the beginning of the investigation, and at 15 and 30 min. later to determine BAO. Then the appropriate amount of the basic capsaicin solution containing 100 or 200 or 400 or 800 ug capsaicin was given through the nasogastric tube to the volunteers in 100 ml physiological saline solution, and gastric content was collected again at every 15 min. for 2 h.

The same parameters (volume and gastric acid output) were measured as in the previous set of observations.

Fig. 2.

PROTOCOL OF INVESTIGATION (B)



The detailed description of the protocol is seen in the text.

3.4 Gastric emptying measurements

3.4.1 Patients

The observations were carried out on 10 healthy volunteers, 4 women and 6 men, the average of age was 34 years.

3.4.2 Test solutions

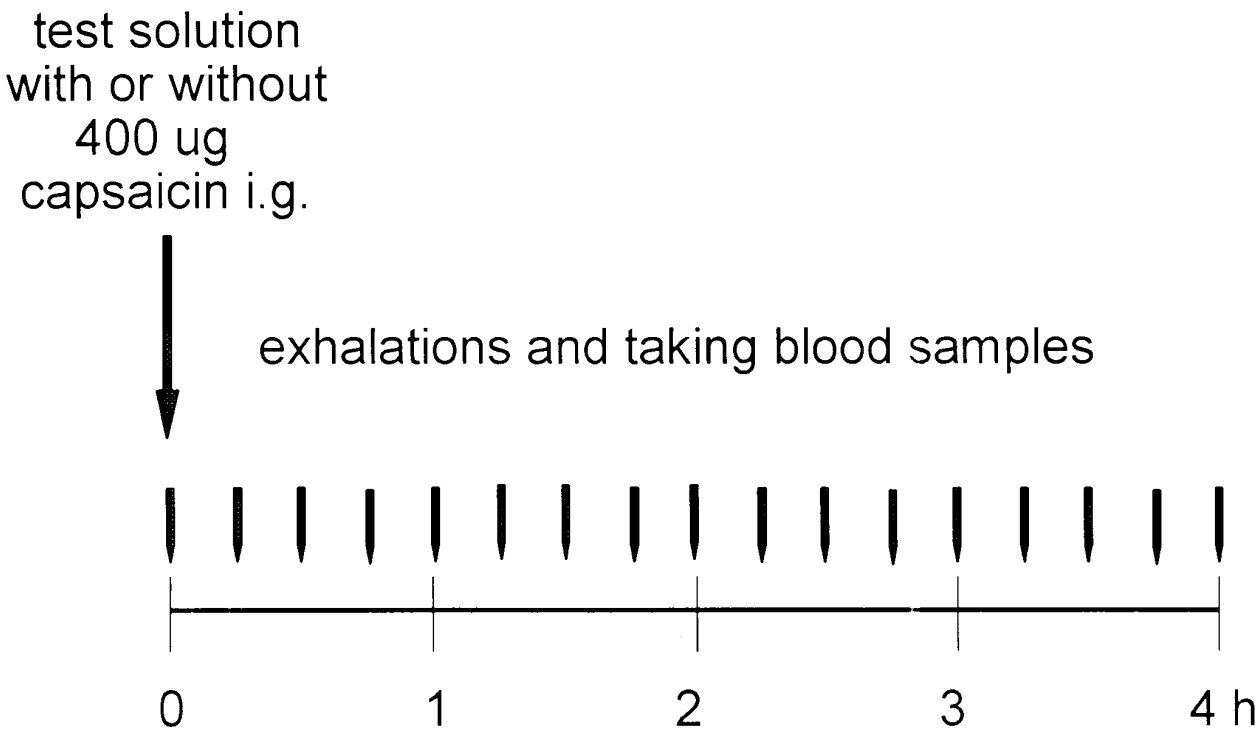
100 mg ^{13}C-octanoic acid (Izinta, Budapest, Hungary) was used for the gastric emptying measurements. This material was given into 100 ml physiological saline, and 75 g glucose was added to the test solution. Gastric emptying measurements were performed on two consecutive days using the same protocol, without (1st day) and with (2nd day) 400 ul of 1% capsaicin solution containing 400 ug capsaicin, in 13 uM concentration.

3.4.3 Protocol of the investigations (C) - (Fig. 3.)

The measurement procedure was the following. Intravenous cannule was introduced into a vein of the forearm of the volunteers. The volunteers first exhaled into a plastic bag with a volume of 0.5 l. This first air sample was considered as reference for the computer. Then the volunteers swallowed the test solution, and exhaled at every 15 min. for 4 hours into similar plastic bags. 10 ml venous blood sample was obtained from the volunteers at every time simultaneously with the exhalations. The IRIS performed the infra-red spectroscopy measurement. The determinations listed below were performed from the blood samples.

Fig. 3.

PROTOCOL OF INVESTIGATION (C)



The detailed description of the protocol is seen in the text.

3.4.3.1 Glucose level

Blood glucose level (in mmol/l) was determined enzymatically (Boehringer Mannheim).

3.4.3.2 Hormone levels

Serum levels of insulin (uIU/ml) (Biochem Immuno System), C-peptide and glucagon (pg/ml) (Byk-Sangtec Diagnostic GmbH) were measured with ^{125}I-labeled Radio Immuno Assay kits.

3.5 Side effects

However the volunteers reported hot feeling in their mouth and/or oesophagus for maximum 2 min. after swallowing the test solution containing capsaicin, they had no other symptoms during the investigations.

3.6 Analysis of data

The four parameters of the gastric emptying curves were analysed by paired Student`s t-test for the comparison of the two means, the results are given as means ± SEM, changes were considered to be significant, when $p < 0.05$.

4 Results

4.1 Gastric secretory measurements

4.1.1 Gastric acid output

Gastric basal acid output (BAO) (measured according to the protocol A) was 2,82 ± 0,20 mmol/h HCl during the first hour. Intragastric application of 100-800 ug capsaicin (concentrations between 3.2-26 uM) dose-dependently inhibited GAS (Fig. 4.).

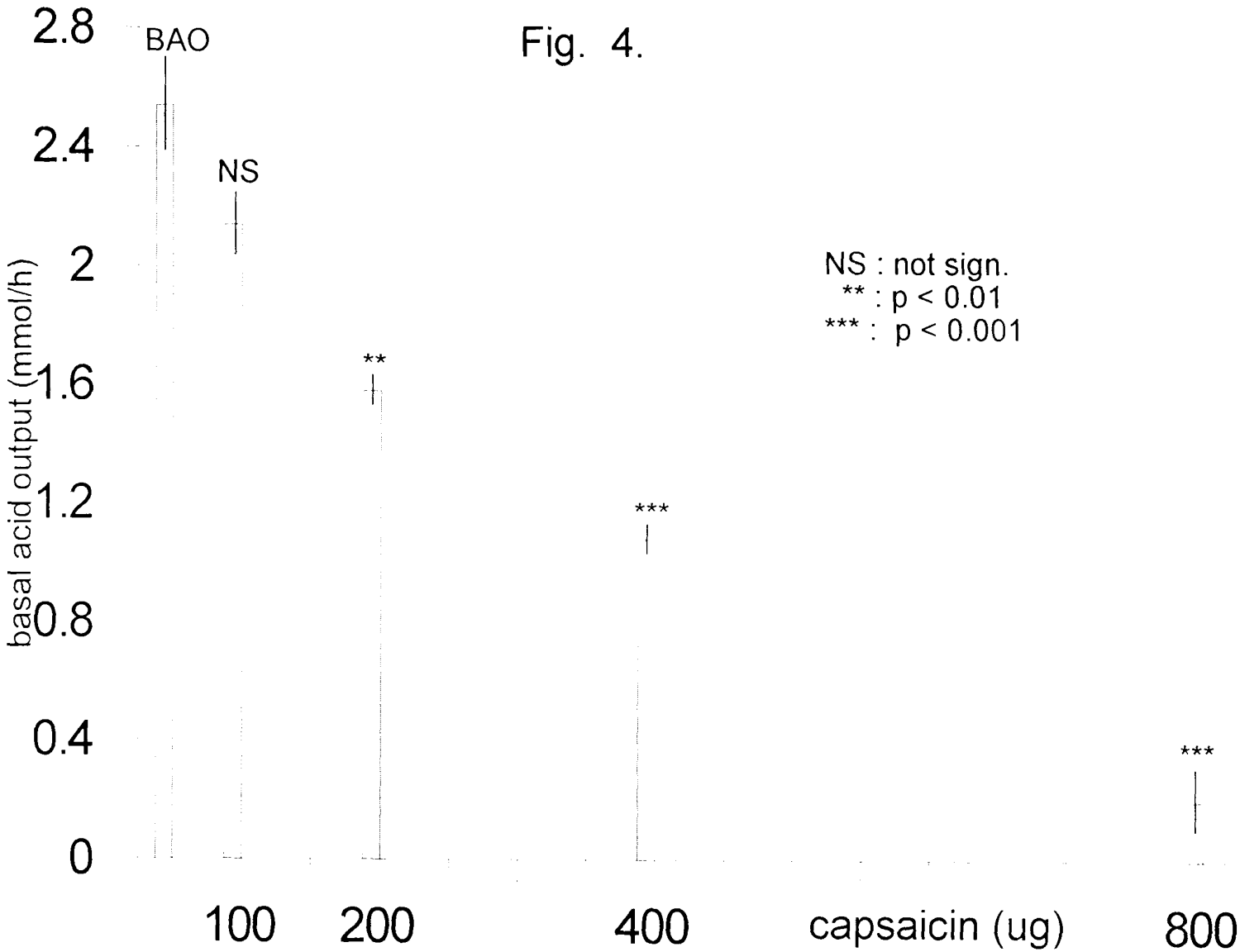
If we determine the maximal inhibition - obtained with 800 ug capsaicin - as 100 %, and represent the inhibitions obtained with the other concentrations of capsaicin (100, 200, 400 ug) in the % of maximal inhibition of BAO, we may identify the ID50, the dose which inhibits GAS with 50%. This dose was found to be about 400 ug for capsaicin on GAS (Fig. 5.).

The time-course curve obtained during the application of intragastric capsaicin in the dose of ID50 (400 ug capsaicin) (measured according to the protocol B) indicate 1 hour time for inhibitory action of capsaicin on GAS (Fig. 6.).

4.2 Gastric emptying measurements

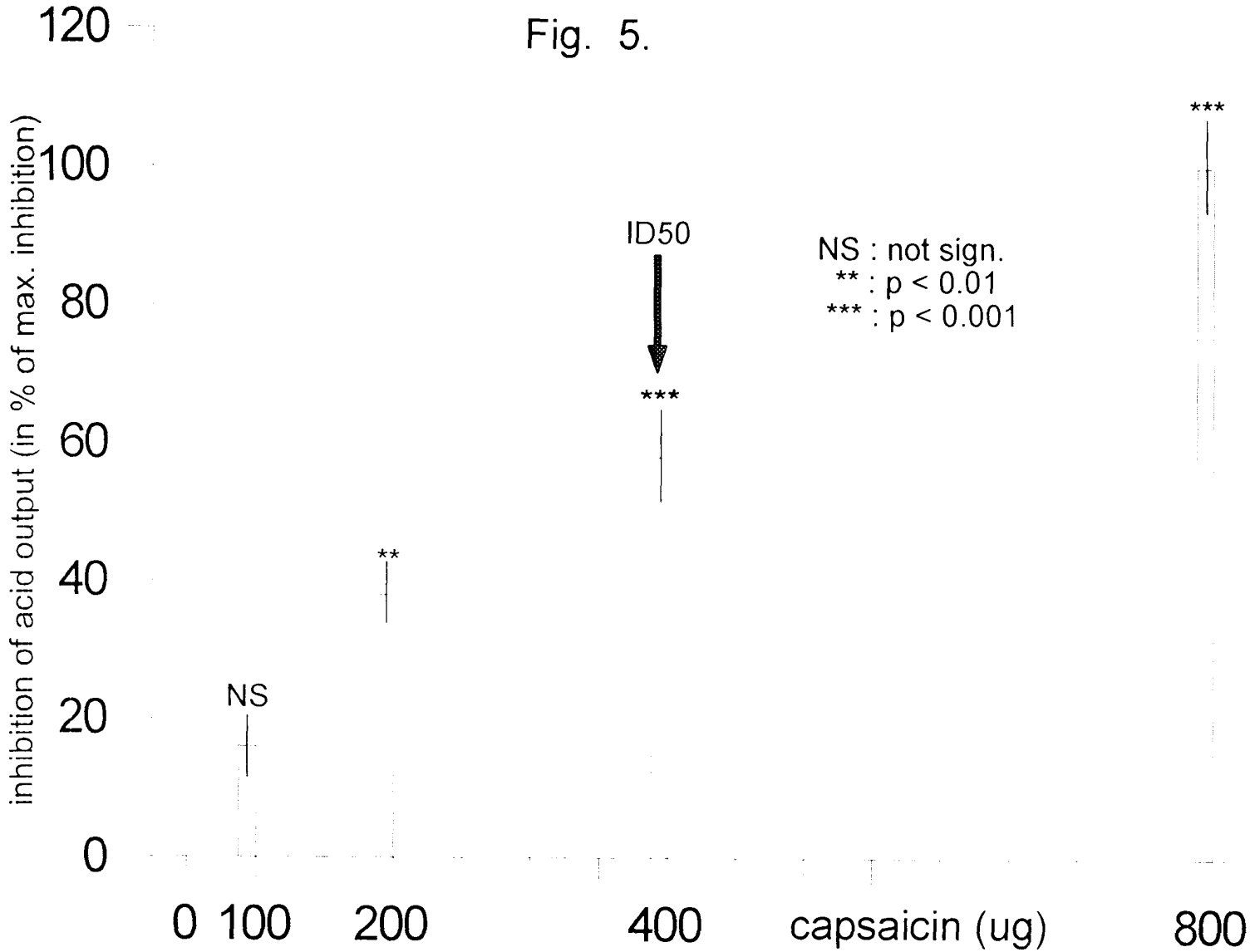
After performing the infra-red spectroscopy measurements the IRIS calculated the Delta Over Base (DOB) values. This value is directly proportional to the ratio of $^{13}CO_2$ and $^{12}CO_2$ ($DOB \sim {}^{13}CO_2/{}^{12}CO_2$) in the exhaled air sample. When the DOB values are represented against the time in a graph, the **gastric emptying curve** is obtained (Fig.7.). On this curve the following four parameters were taken into consideration to characterise the curve and gastric emptying rate: 1.) maximal value of DOB (DOBmax, unit (U)); 2.) time at DOBmax (min.); 3.) slope

Fig. 4.



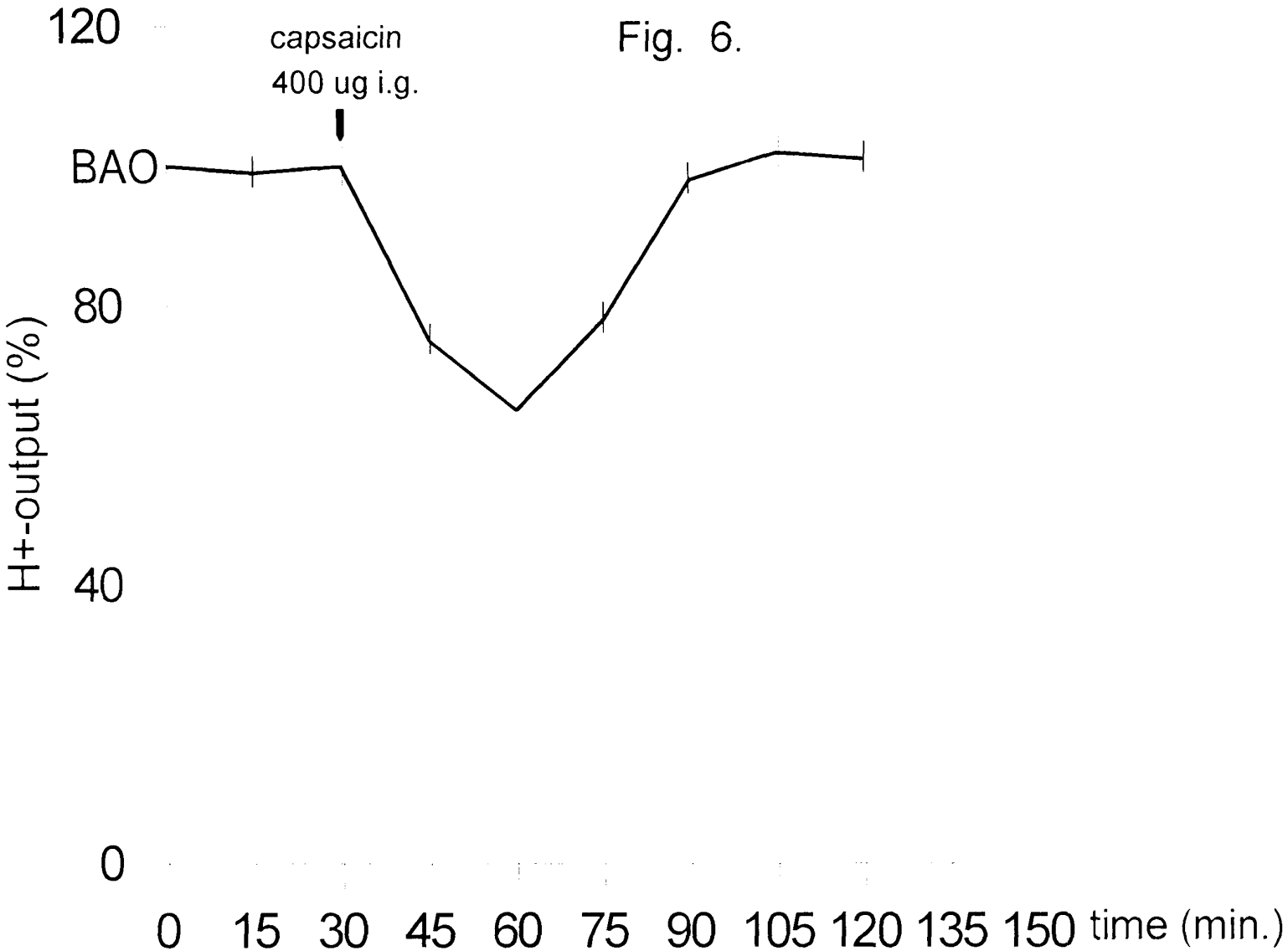
Dose-dependent inhibition of gastric basal acid output after application of 100-800 ug capsaicin i.g. (mean±SEM, n=10)

Fig. 5.



Dose-dependent inhibition of gastric basal acid output after application of 100-800 ug capsaicin i.g. (mean±SEM, n=10)

Fig. 6.



Gastric H^+-output after application of 400ug capsaicin i.g.
(mean±SEM, n=10)

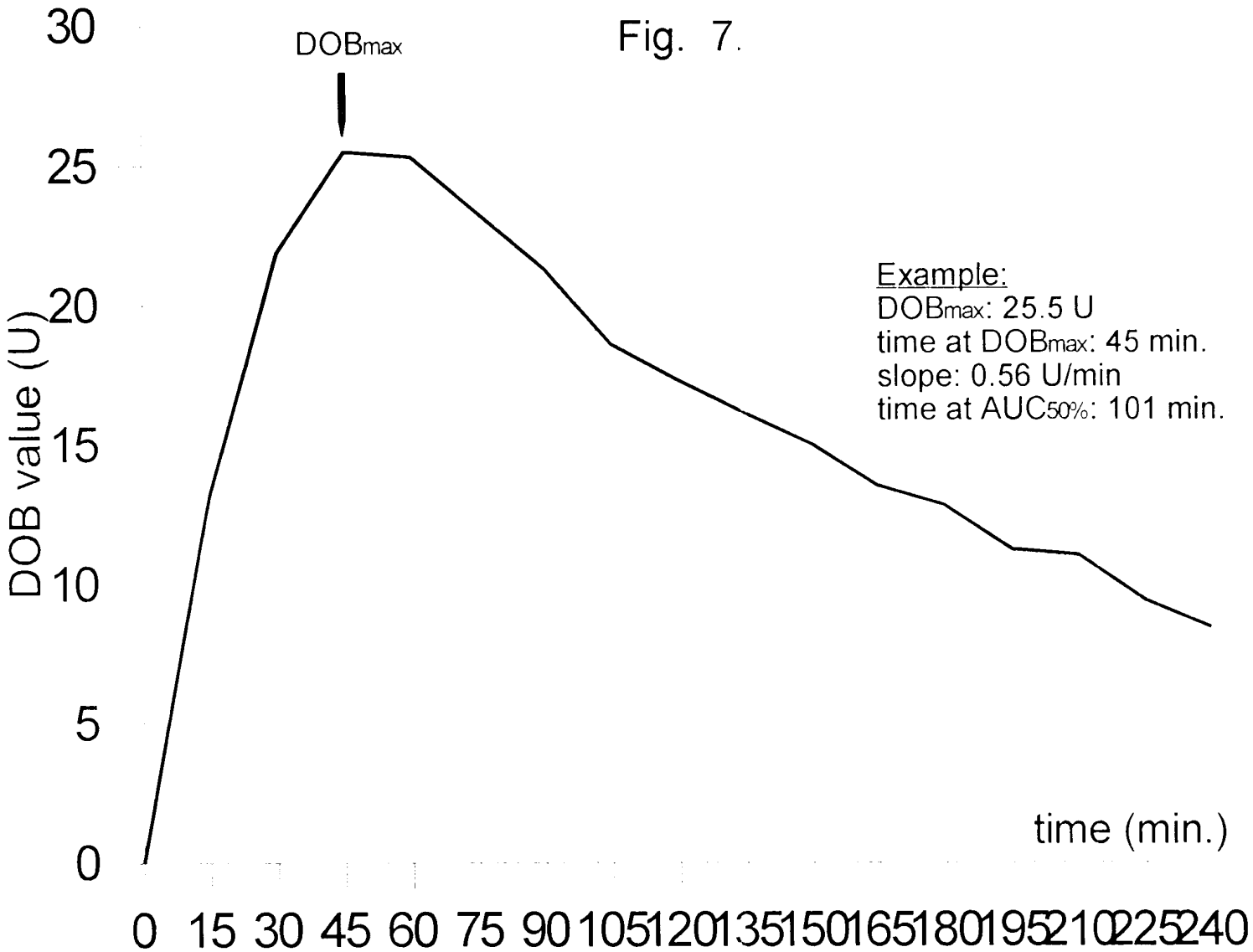
of the rising part of the curve (U/min.); 4.) time at when the 50% of the Area Under the Curve was reached (AUC50%, min.). The DOB_{max} and the slope are directly, while the time at DOBmax and time at AUC50% are inversely proportional to the gastric emptying rate (Fig. 7.).

When we analysed the DOB-curves obtained without and with application of capsaicin in the dose of ID50, we found that the slope of the curves increased significantly from 0.1±0.01 to 0.139±0.014 U/min ($p < 0.05$) after capsaicin (Fig. 8.). The DOBmax decreased from 17.66 U to 15.75 U, but this did not reach significant level (Fig. 8.). The time at DOBmax significantly decreased from 150±18 to 75±12 min. ($p < 0.05$)(Fig. 9.), and the time at AUC50% also significantly decreased from 112±15 to 99±14 min ($p < 0.05$) after application of 400 ug capsaicin (Fig.9.). All of these results indicate that there is an increase in GM during the action of capsaicin.

4.3 Blood glucose and hormone levels

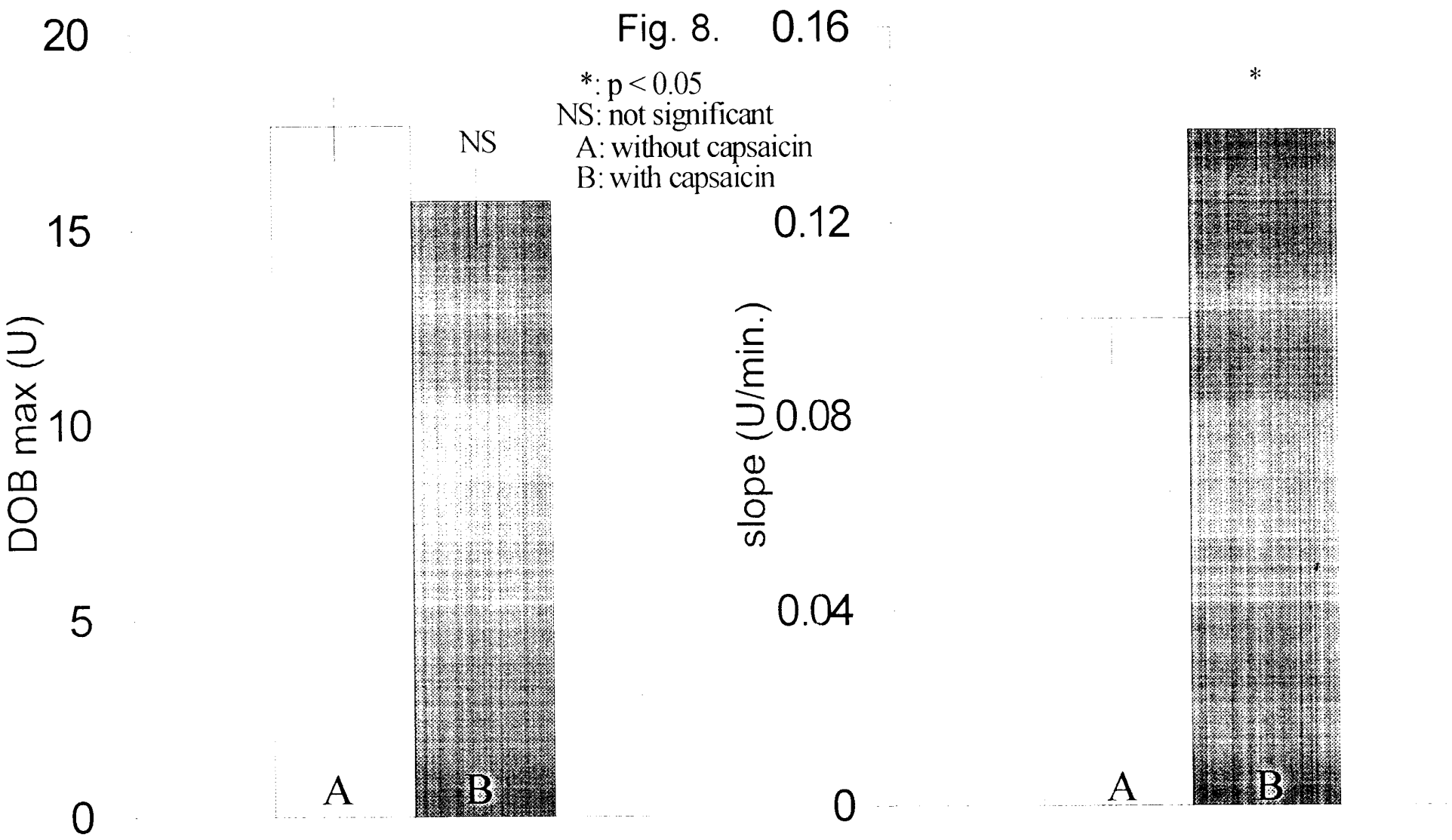
We represented the mean of blood glucose and hormone levels against the time (Fig. 10.). We compared two parameters of these curves obtained without and with application of 400 ug capsaicin: the mean of the *maximum* levels of the curves, and the mean of the *average* levels of glucose and hormones during the whole measured interval. In the case of glucose we compared the mean of the *minimum* glucose levels too, which occured at later time, after a hormonal answer to the elevation of blood glucose level. The measurements of blood glucose levels and the determination of hormones revealed the followings. There was no significant difference between the mean of average and minimum glucose levels obtained without and with application of capsaicin. However we found a slight increase regarding the maximum glucose level in the capsaicin-treated group compared to the untreated group indicating a faster glucose absorption during the action of capsaicin, this did not reach significant level (data are not shown). As for the hormones, the mean of maximum levels of glucagone, but not insulin and C-peptide increased significantly during capsaicin application (Fig. 11.). Similar finding was observed with the mean of average hormone levels. The mean of average glucagon,

Fig. 7.



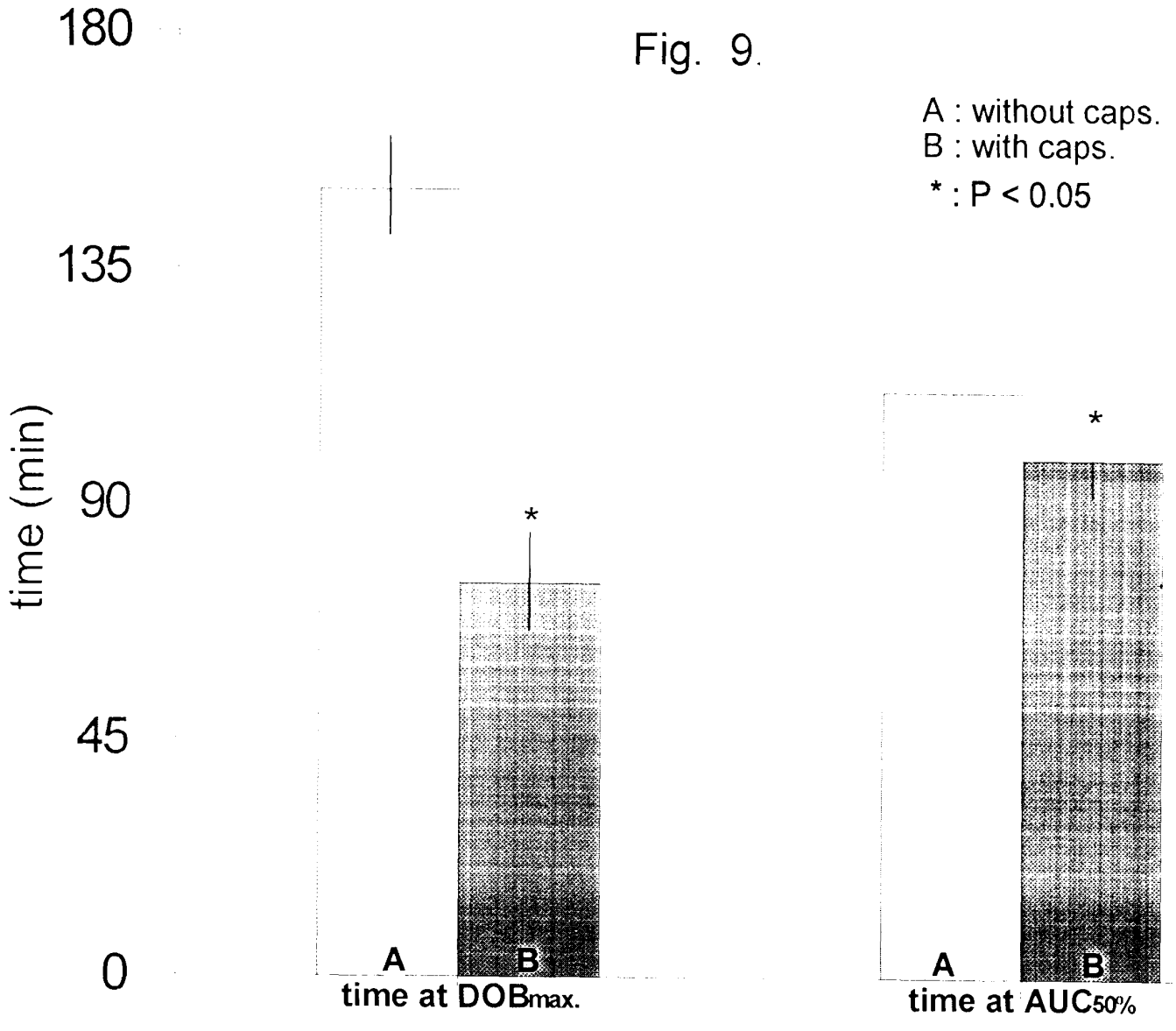
An example for *gastric emptying curve* obtained with the IRIS equipment. See detailed explanation in the text.

Fig. 8.



DOBmax values and slopes of gastric emptying curves obtained during application of of 400 ug capsaicin i.g. (mean±SEM, n=10)

Fig. 9.

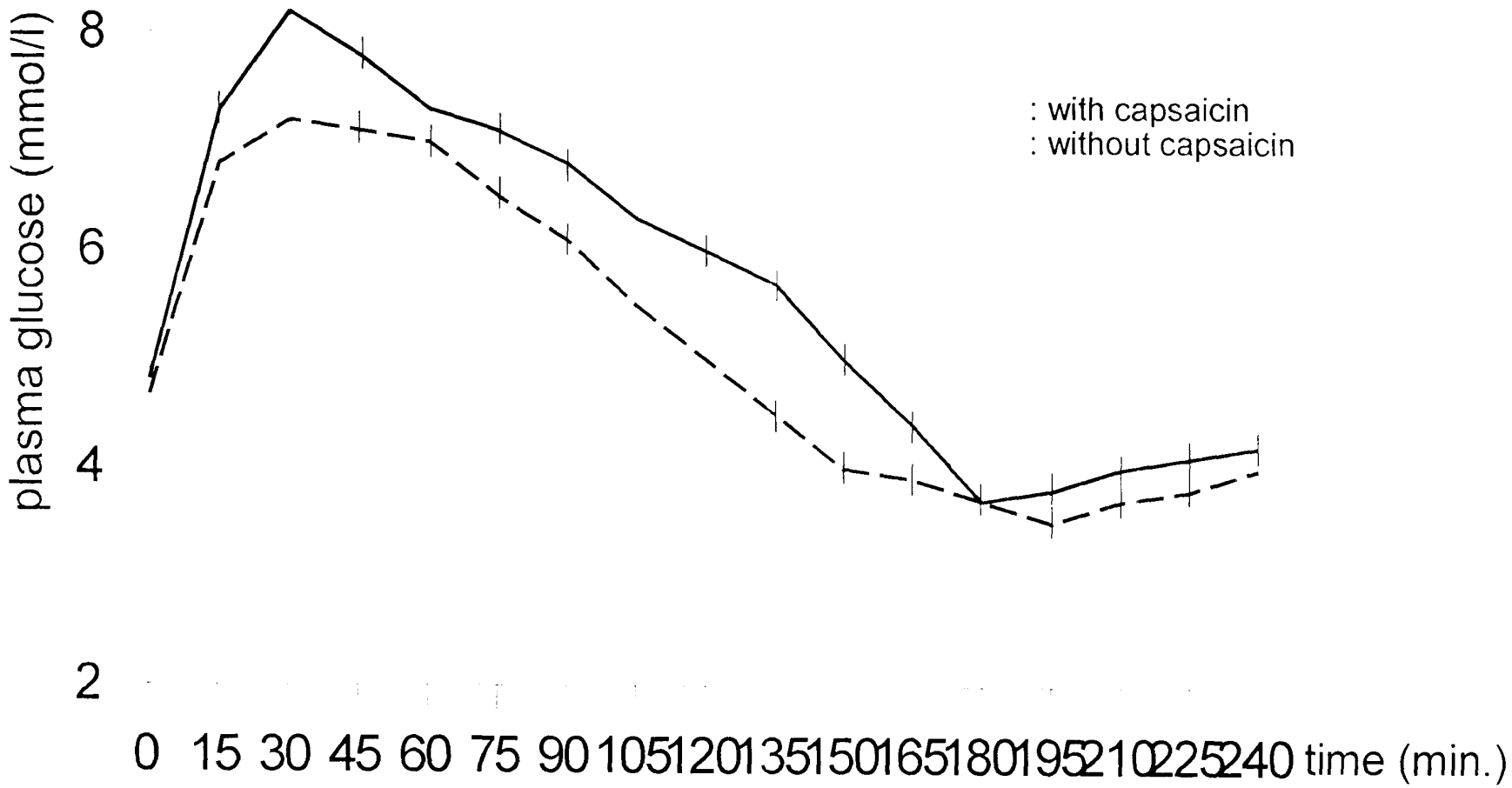


The times at DOBmax and at 50% AUC of gastric emptying curves obtained during application of of 400 ug capsaicin i.g. (mean±SEM, n=10)

but not insulin and C-peptide levels increased during the application of capsaicin (Fig 12)

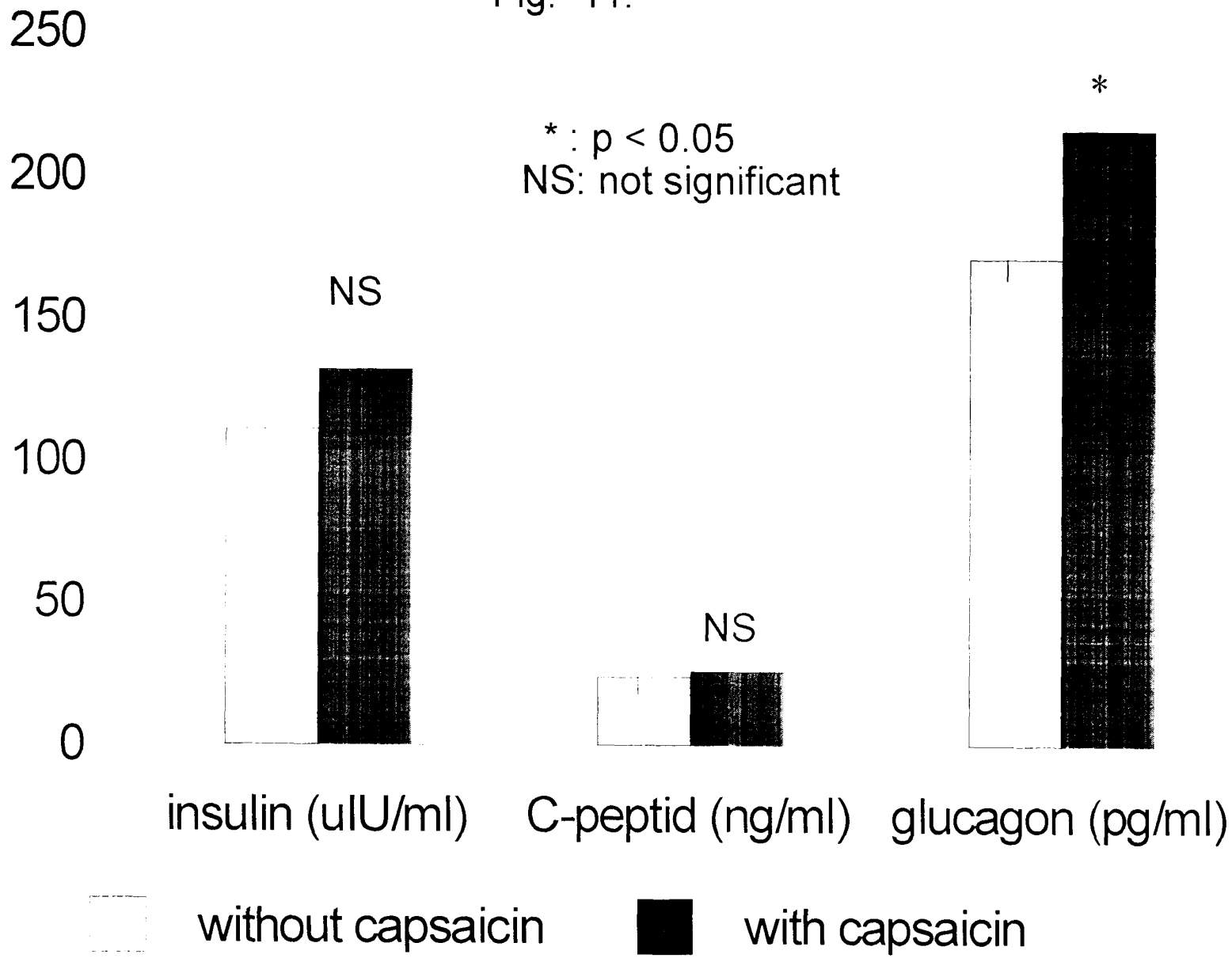
10

Fig. 10.



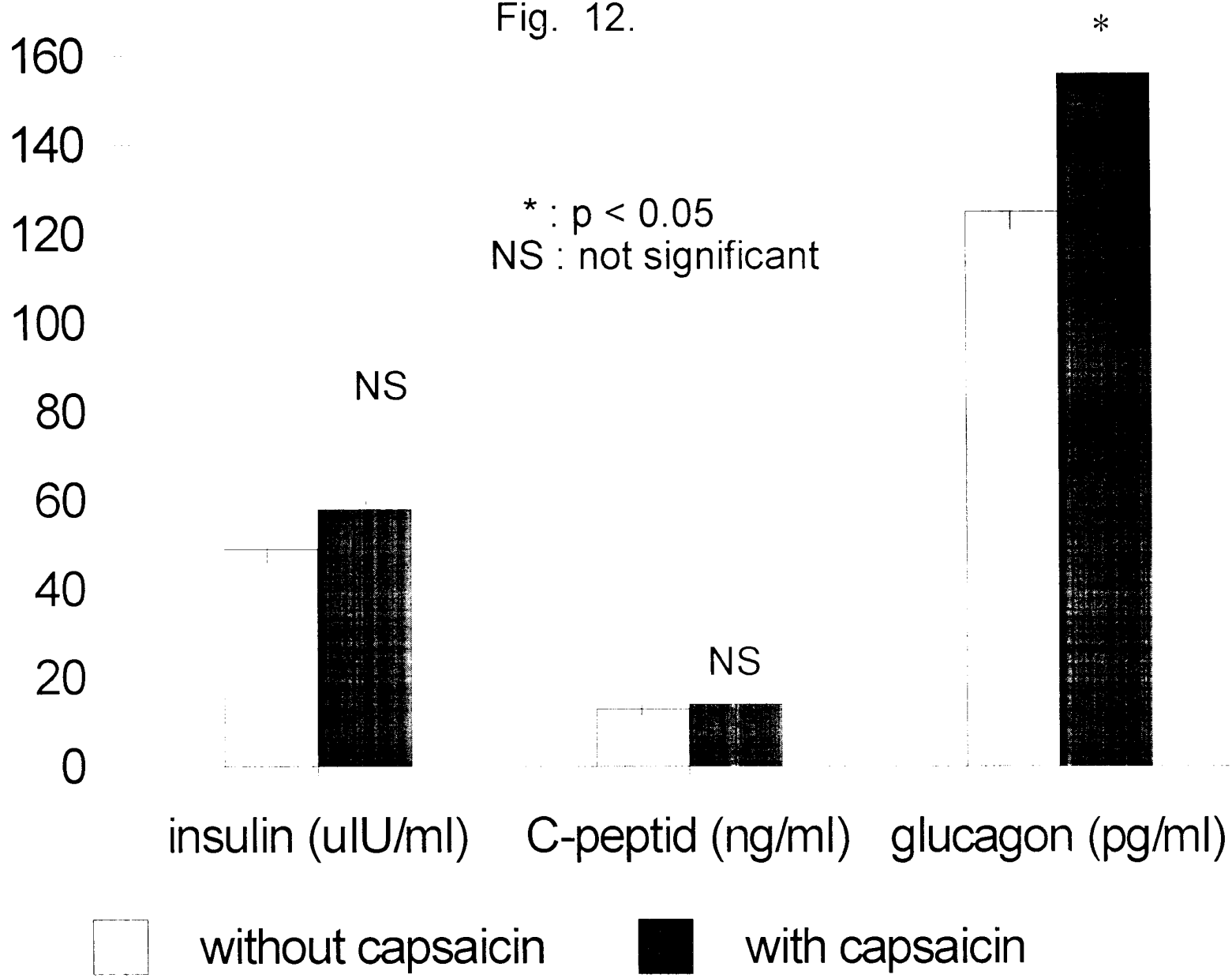
Plasma glucose levels during measurements with without and with application of of 400 ug capsaicin i.g. (mean±SEM, n=10)

Fig. 11.



The maximum levels of insulin, C-peptide and glucagon during the measurements without and with application of of 400 ug capsaicin i.g. (mean±SEM, n=10)

Fig. 12.



The maximum levels of insulin, C-peptide and glucagon during the measurements without and with application of of 400 ug capsaicin i.g. (mean±SEM, n=10)

5 Discussion and conclusion

In these studies we found that intragastric capsaicin inhibits GAS (Mózsik, 1999), and increases gastric emptying rate in healthy humans (Debreceni, 1999).

5.1 GAS

5.1.1 GAS in animals

The effect of capsaicin on GAS in animals have been studied on both direct and indirect way. Neurotoxic dose of capsaicin was applied to the animals systemically in neonatal (Evangelista, 1989; Esplugues, 1990) or adult age (Dugani, 1986), or perineurally (Raybauld, 1989). There is an agreement, that defunctionalisation of CSPASF does not change the basal acid and pepsin output in rats (Lippe, 1989; Raybould, 1989). However the stimulated GAS was affected on different manner by functional ablation of the sensory nerves. Capsaicin desensitisation of CSPASF had no effect on GAS stimulated by histamine (Esplugues, 1990). The pentagastrin evoked secretion was inhibited in adult rats after systemic capsaicin treatment (Dugani, 1986). Similarly the increase in GAS induced by distension of the stomach was reduced in capsaicin-pre-treated rats (Raybould, 1989). In contrast, intraduodenal lipid inhibited GAS induced by intragastric peptone, and this effect of lipid was abolished after perivagal capsaicin treatment (Lloyed, 1993). Intraduodenal acid had the same GAS inhibiting effect, which disappeared after capsaicin-induced ablation of the sensory neurones (Saperas, 1995). It seems that enterogastric inhibitory secretory reflexes involve CSPASF (Holzer, 1998a).

Similar controversial results on GAS were obtained with intragastric application of capsaicin to animals. While intraduodenal instillation of capsaicin increased GAS, intragastric application did not (Makara, 1965). Toh et al. (1955)

also did not find altered GAS after subcutaneous or intragastric capsaicin. In contrast, pentagastrin-stimulated GAS was reduced after intragastric capsaicin perfusion. In the same experiment the aniline clearance was also increased, which indicates that faster remove of intragastric acid from the stomach may be a factor contributing to the reduced GAS (Lippe, 1989). Finally, our laboratory team found that small doses (0.25-1 ug/kg), and very small concentrations (80 nm - 0.33 uM) of capsaicin inhibited GAS in a concentration-dependent manner in pylorus-ligated rats. This effect of capsaicin was the most pronounced in the first hour after pylorus-ligation. The capsaicin analogue RTX had similar effect on GAS. However the same effects of capsaicin and RTX were not reproducible in capsaicin-pre-treated, or in somatostatin-depleted rats pre-treated with cysteamine (Abdel-Salam, 1995c). RTX furthermore inhibited GAS stimulated by bethanecol, pentagastrin and histamine (Abdel-Salam 1995b, 1999).

These discrepancies between the results may be attributable to the various experimental regimens, which indicates that this control system is so sensitive, that even a little difference may change the final result of the regulation.

The dose-dependent inhibitory effect of capsaicin and RTX on GAS cannot be the result of an increased gastric H^+ back-diffusion, as it is hypothesized by Holzer et al. (1998a), because these agents are proved to be gastroprotective, and they reduce gastric H^+ back-diffusion caused by acidified sodium salicylate (Abdel-Salam, 1997c).

5.1.2 GAS in humans

The first publication in the literature reporting that 0.5 g pepper decreased GAS in human was published in 1932 (Heupke, 1932). Since that time - in contrast to the investigations of the effect of capsaicin and capsaicin-containing spices on the gastric mucosa -, only a few observations were performed on this matter resulting contradictory findings. 1 g paprika did not change GAS in human (Sanchez-Palomera,1951), and Viranuvatti et al. (1972) also did not find altered secretion by chilli. In contrast Solanke et al. (1973) found increased GAS after giving

red pepper suspension containing 4 g red pepper to humans. Increased GAS was measured after perfusion of 1.6 g/h red chilli powder into the stomach of humans (Desai, 1977). In another observation the effect of different amount of red pepper on GAS was studied in humans. Interestingly 0.1 g red pepper increased a little, 0.5 g decreased a little, finally 1.5 g increased again significantly GAS (Myers, 1987). When interpreting the contradictory data of capsaicin-effect on GAS, the unique feature of capsaicin, that in higher dose it defunctionalises the CSPASF (Holzer, 1991) should be borne in mind. Since the usual capsaicin contain of red pepper is 0.1-1% (average: 0.5%)(Szolcsányi, 1977), the above mentioned amounts of red pepper (4 g, and 1,5 g) contain about 20 and 8 mg capsaicin in 400 and 100 ml suspension respectively. Since 100 ug/ml is equal to 0.33 mM concentration for capsaicin, this means 0.16 and 0.24 mM capsaicin solution in the stomach. In rats even 0.1 mM concentration of capsaicin could produce desensitisation of the CSPASF (Szolcsányi, 1975). This is supported with the observation, that desensitisation of the oral cavity was found in humans regularly consuming very hot meal (Rozin, 1990).

In our investigations the maximal concentration of capsaicin in the stomach was 800 ug/100 ml, i.e. 26 uM during the measurements, which is far below the defunctionalising concentration.

Our results indicate that capsaicin in this concentration (3.2-26 uM) dose-dependently inhibits GAS in human healthy subjects (Mózsik, 1999). This finding is supported by results of animal experiments (Abdel-Salam, 1995c).

5.2 GM

Gastric emptying is a process controlled by complex mechanisms involving both the nervous (n. vagus, nn. splanchnici, ggl. coeliacum, plexus myentericus, plexus submucosus), and hormonal (gastrin, cholecystokinin, opioids) system. As we mentioned above CSPASN are present in the n. vagus and spinal afferents (nn. splanchnici) innervating the GI tract (Holzer, 1998a).

5.2.1 GM in animals

Functional animal studies served contradictory results regarding the effect of capsaicin on gastric motility. The study of CSPASF with the indirect approach revealed that capsaicin desensitisation does not effect basal GM or gastric emptying (Takeuchi, 1991, Cervero, 1982; Holzer, 1994). However CSPASF seem to take part in enterogastric inhibitory motor reflexes, because the intraduodenal lipid- (Holzer, 1994), acid- (Cervero, 1982; Raybauld, 1993) and distension-induced inhibition of GM was attenuated after capsaicin desensitisation (Holzer, 1992). The direct approach of this question with intragastric capsaicin application resulted in either increased (Raybould, 1988), or decreased (Kang, 1993; Takeuchi, 1991) gastric emptying in intact animals. Shibata et al (1999) found that intragastric, but not intraduodenal application of capsaicin caused contractions in the stomach, duodenum, proximal jejunum and colon. Experiments with isolated muscle stripes from rat and guinea-pig stomachs showed, that capsaicin either relaxed the muscle strips (Lefebvre, 1991; Uno, 1997), or had biphasic effect (Holzer-Petsche, 1989) on these tissue samples.

5.2.2 GM in humans

In contrast to animal studies there is only a few data about the impact of capsaicin on the gastrointestinal physiology in human. However studies from the 1950s were performed to reveal the effect of capsaicin and commonly used spices containing capsaicin on gastric symptoms (Schneider, 1956), mucosal surface (Viranuvatti, 1972; Tyagi, 1974; Graham, 1988; Kang, 1988), secretion (Solanke, 1973; Myers, 1987) and ulcer-formation (Kumar, 1984; Yeoh, 1995b; Kang, 1995b) in human, we still know only a little about the pharmacology effect of capsaicin on the motor activity of GI tract in human.

Maggi`s team performed several investigations on isolated muscle strips removed form human jejunum (Maggi, 1988b), ileum (Giuliani, 1991; Maggi, 1989a,

1990a) and colon (Maggi, 1990b). They found that capsaicin mainly exerts relaxant effect on these tissue samples (Giuliani, 1991; Maggi, 1990b). However there is no publication about isolated muscles stripes removed from human stomach.

Studies on GM after application of spices or capsaicin were performed in humans too. Yeoh et al. (1995a) found that ingestion of 5 g chilli into the oesophagus does not alter oesophageal motility in human. Gonzalez et al (1998) measured delayed oesophageal and gastric motility after giving red pepper sauce to the oesophagus of healthy human subjects. As for gastric emptying rate, in contrast to Gonzalez et al's results, Desai et al (1977) found increased gastric emptying, indicated by greater pyloric loss, after intragastric ingestion of 1.6 g/h red chilli powder in 110 ml isotonic HCl. The contrast between the results of the studies may be attributable to the different time courses, as Gonzalez et al measured gastric emptying only for 180 min., but we did it for 4 h. Furthermore there were differences between the test materials used for the observations. Increased orocecal transit time was found after application of 2 g red pepper in human (Vasquez-Olivencia, 1992). However it is not known which part(s) is (are) responsible for the delayed peristalsis in this study. Similarly to the interpretation of the results of GAS, when making conclusion from the findings of gastric motility studies, the concentration of capsaicin solution should be taken into consideration. In the observations of both Desai et al (1977) and Gonzalez et al (1998) the concentration of capsaicin present in the given solution (about 0,165 and 0,14 mM respectively) was a little above the concentration (0.1 mM) which could produce desensitisation in rats (Szolcsányi, 1975). However the capsaicin used in the form of spicy may not exert such strong effect as similar dose of the pure chemical agent.

It was mentioned that CSPASF are involved in inhibitory enterogastric reflexes (Cervero, 1982; Raybauld, 1993). Nevertheless this does not mean automatically, that capsaicin in the stomach *per se* decrease GM. The situation is more complicated, since intragastric capsaicin inhibits GAS in animals (Abdel-Salam, 1995c) and in human (Mózsik, 1999), and if acid does inhibit GM through CSPASF, then the decreased gastric acid output may be responsible for the faster gastric emptying in our study (Debreceni 1999).

5.3 Glucose absorption and hormon levels

We added 75 g glucose to the test solutions in order to determine the glucose absorption during the effect of capsaicin. Although 75 g glucose *per se* slows down gastric emptying (Nemessányi, 1984), the same dose of glucose was applied in both the capsaicin-treated, and in the control measurements in our study, therefore we may neglect the effect of glucose on gastric emptying, when we assess the effect of capsaicin by comparing the results obtained without and with capsaicin.

As for the glucose and hormone levels and glucose absorption we could not find any data in the literature about the effect of capsaicin or capsaicin-containing spices on these parameters. However our results indicate that capsaicin may increase glucose absorption and consenquently the hormonal answer to the glucose absorption, our data are not enough to draw serious conclusions.

6 Summary

The results of our studies further increase the knowledge about the effect of capsaicin and capsaicin-containing spices on, and the function of CSPASF in the gastric physiology, namely the gastric secretory parameters, gastric emptying and glucose absorption. These findings strengthen the idea that capsaicin has beneficial effects on the gastric mucosa. The ways how capsaicin is able to defend the gastric mucosa may include - beside the effects of this drug on gastric mucosal blood flow. - the inhibitory action of capsaicin on GAS, and the increase of gastric emptying, by which the time available for acid to cause gastric lesion is shortened.

mRNA expression of cytokines in the normal gastric surface mucous epithelial cell line GSM06 during Helicobacter pylori and Helicobacter felis infection

1 Introduction

1.1 Cytokines, cytokine families

Cytokines are small molecular weight proteins playing important triggering role in the development of immune mechanisms in different diseases via acting on a variety of leukocytes (Schall, 1991; Rollins, 1997, Ward, 1998) and other cells (Dwinell, 1999). They may be classified according to many characteristics, e.g. the structure, cell origin, etc., but the most accepted base for the classification is the role of these molecules. However there is overlapping between these groups, cytokines may be divided into the following main families according to their effects: proinflammatory, antiinflammatory and chemotactic cytokines. The proinflammatory cytokines (TNF-alpha, -beta IFN-gamma, IL1-alpha, -beta, IL-6, IL-8, GM-CSF) are mainly responsible for the activation of the different population of leukocytes, after when these cells come into action against the infective agents by phagocyting them, secreting a variety of cytokines, releasing acute phase proteins, and after all producing an inflammatory response. To maintain a balance during the development of the inflammatory process antiinflammatory cytokines (IL-4, IL-5, IL-10, IL-13, TGF-beta) are released too, which in turn attenuate the effects of the inflammatory cells by inhibiting them. The chemotactic cytokines (chemokines) are responsible for the recruitment of the immune cells from the periphery to the place of inflammation. They may be divided into four groups according the their structure, i.e. the localisation of two of four conserved cystein motifs in the polypeptide chain. This structural classification is in connection with the effect of these chemokines. In the CC-chemokine subfamily the two cysteins are adjacent. The member of this group (RANTES, eotaxin, MCP1-5, MIP1-alpha, -beta, -gamma) are primarily chemotactic for monocytes, eosinophils, basophils, different subgroups of lymphocytes, but not for neutrophils. In contrast the CXC chemokines, in which the two cysteins are separated by another aminoacid, show strong chemotactic activity for the neutrophils, and to a lesser extent for basophils, T lymphocytes (Schall, 1991, Rollins, 1997).

1.2 Source of cytokines

Originally the cells of the immune system were found to express and release most of these proteins (Schall, 1991; Rollins, 1997), however later a wide range of other cells were shown to produce them, including the epithelial cells of the gastrointestinal (Rothenberg, 1995; Jung, 1997; Jedrzkiewicz, 1999; Watanabe, 1997), respiratory (Stellato, 1995, 1999), urogenital tract (Fichorova, 1999), the central nervous system (Janabi, 1999) either constitutively, or during bacterial infection or activation of these cells with proinflammatory cytokines. Significant level of a number of cytokines (IL1-beta, IL-6, IL-8, RANTES, MCP-1, GRO-alpha, TGF-beta, M-CSF) are present even in the human milk (Srivastava, 1996).

1.3 Helicobacter felis

Helicobacter felis (H. felis) is one of the nine species of the genus Helicobacter, and commonly found in, and naturally pathogenic for canine and feline stomach (Lee, 1993). It does not express Cag A and Vac A, however is capable to colonise the stomach of small laboratory animals, and causes mild chronic gastritis with similar type of immune response (infiltration of Th1 dominant lymphocytes) as seen in human H. pylori infection, therefore it is used as a model in these animals to mimic human H. pylori infection, and to investigate the pathomechanism of H. pylori-induced chronic gastritis in human (Mohammadi, 1996; Crabtree, 1998). Resembling to H. pylori, H. felis may also have a role in the development of gastric neoplasm in animal model (Moss, 1999).

1.4 Helicobacter pylori

Helicobacter pylori (H. pylori), another species of the Helicobacter genus, is one of the most widespread pathogenic bacterium, which can be found in about half of the world`s population (Zevering, 1999), and it is in causative relation with such important and common gastrointestinal diseases as chronic gastritis (Blaser,1990), peptic ulcer (Peterson, 1991), mucosa associated lymphoid tissue (MALT)-lymphoma (Parsonnet, 1994) and probably gastric carcinoma (El-Omar, 2000) in human.

1.5 Immune response to H. pylori infection

Since the description of H. pylori in the human stomach (Warren, 1983), the immune mechanism, with which the host tries to eliminate the bacterium, have been thoroughly studied. It was revealed by histology of gastric biopsy samples obtained from H. pylori infected patients that there is a strong infiltration of the gastric mucosa by poly- and mononuclear leukocytes (Genta, 1993) and lymphocytes (Hatz, 1996) during H. pylori infection. Furthermore many cytokines were shown out in the biopsy samples of H. pylori positive patients (see details later). However the cell homogenates of gastric biopsy specimens contain about 10% of immunologically active, therefore cytokine-releasing leukocytes, so it may not be excluded that these are responsible for the cytokine expression found in the biopsy samples. In the last years more data obtained from experiments with tumor originated gastrointestinal cell lines releasing different cytokines have been published indicating that the leukocyte infiltration during H. pylori infection is at least partly due to the result of different proinflammatory and chemotactic cytokines released from the gastric epithelial cells themselves (Watanabe, 1997; Jung, 1997). However these results may not be applied without doubt to normal gastric epithelial cells, because many characteristics of the original cells change during the malignant transformation.

The cytokines found to be expressed in the gastric biopsy samples of H. pylori patients, and in epithelial cell lines represent both the proinflammatory and chemotactic family of the cytokines.

1.6 Cytokines released during H. pylori infection

The proinflammatory cytokine TNF-alpha and IL1-alpha and beta are abundantly expressed during H. pylori infection in the gastric mucosa. Investigations of gastric biopsy samples removed from H. pylori infected patients revealed increased expression of these cytokines (Noach, 1994; Shimada, 1998). Furthermore tumour-originated epithelial cell lines express TNF-alpha and IL1-alpha during bacterial or proinflammatory cytokine stimulation (Jung, 1997).

Among the chemokines which may have role in the immune response during H. pylori infection is the unique chemokine RANTES ("regulated on activation, normal T cell expressed and secreted"). RANTES is a member of the CC(beta)-chemokine family (Nelson, 1993) with chemotactic activity for monocytes, CD45RO+ memory phenotype Th cells (Schall, 1990), and eosinophils (Kameyoshi, 1992). The mRNA expression and protein level was found increased in gastric biopsy samples from H. pylori positive patients (Yamaoka, 1998; Kikuchi, 1999; Watanabe, 1997), and in epithelial cell lines during bacterial stimulation (Jung, 1997).

The eosinophil chemotaxis-inducing chemokine (eotaxin) attracts - beside eosinophils - T lymphocytes (Sallusto, 1997). This chemokine was found to be expressed constitutively in low level in the stomach, intestine, spleen, heart, kidney, but abundantly in the lung of guinea-pig (Rothenberg, 1995). It has important role in the parasitic, allergic gastrointestinal diseases as eosinophilic (parasitic) gastro-enteritis (Hogan, 2000; delPozo 1999), and respiratory diseases, like asthma (Matsukura, 1999) by causing eosinophilia.

Monocyte chemoattractive protein 1 (MCP-1) is responsible for the recruitment of macrophages and granulocytes from the circulation to the inflamed mucosa (MacDermott, 1996). Its expression is greater in biopsy samples of H. pylori infected patients comparing with those of H. pylori negative ones (Shimoyama,

1998; Yamaoka, 1998; Watanabe, 1997). MCP-1 is also expressed in human gastric epithelial cell line with tumor origin during H. pylori activation (Jung, 1997; Watanabe, 1997), and in tumor derived human colonic and gastric epithelial cell lines during proinflammatory cytokine activation (Kolios, 1999, Watanabe, 1997; Warhurst, 1998).

Macrophage inhibitory protein 1 alpha and beta (MIP1-alpha and MIP1-beta) are neutrophil and macrophage attracting agents (Wilson, 1990). The expression of MIP1-alpha is also upregulated during H. pylori infection (Ando, 1998), furthermore reduced after H. pylori eradication (Sato, 1999). These chemokines are secreted during Th1-type immune response (Schrum, 1996) which can be seen in H. pylori infection too (Mohammadi, 1996; Crabtree, 1998).

As the above described data show many gastrointestinal epithelial cell lines with tumor origin, and the cell mass of biopsy samples of H. pylori infected patients express proinflammatory and chemotactic cytokines during bacterial or proinflammatory cytokine stimulation, but it has not been proved yet that normal gastric epithelial cells are capable to express the mRNA of these agents during H. pylori and/or H. felis infection.

1.7 Normal mouse gastric surface mucous epithelial cell line GSM06

We used for these experiments the normal (with non tumor origin) mouse gastric surface mucous cell line GSM06. This cell line was established from gastric surface mucous cells of transgenic C57BL/6 mouse transformed with the temperature sensitive form of simian virus 40 (SV40) large T antigen gene. Using the same method several immortalised cell line was established, all in which the cells kept the cell type specific functions and features of the original cells (Yanai, 1991). The GSM06 cells also show the characteristics of normal gastric surface mucous cells, i.e. produce PAS positive granules in the cytoplasm, secrete glycoprotein and glycolipid rich layer on the cell surface, which positively stains by PAS (Sugiyama, 1993), form microvilli-like structures on, and junctional complexes between the cells (Tabuchi, 1996). GSM06 cells - according to the expression of the

SV40 large T antigen - behave differently on different temperature. At permissive temperature (33^0C) GSM06 cells proliferate until reaching confluent monolayer, express SV40 large T antigen, and characterized by having undifferentiated features, e.g. poor production of PAS positive material, however at non-permissive temperature (39^0C) the T antigene becomes inactive and the cells cease to grow. but exhibit differentiated characters, e.g. production of PAS positive material and secretory granules (Sugiyama, 1993; Konda, 1997).

This cell line serves a good model for the intact normal gastric mucosa and it is applicable for the investigations of cyto- and chemokine expression.

2 Aims

We aimed in these investigations to study the immunological background, i.e. the cytokine mRNA expressing ability and pattern of a normal mouse gastric surface mucous epithelial cell line, the GSM06 cell line during infection with different number of H. felis and H. pylori for different time intervals.

1. We aimed to measure the mRNA expression of the proinflammatory cytokine IL1-beta, and chemotactic cytokine RANTES, eotaxin, MCP-1, MIP1-alpha and -beta with RT-PCR during infection with different number of live H. felis for 2 and 4 h.

2. We planned to determine the mRNA expression of cytokines TNF-alpha with Southern-, and IL1-alpha and RANTES with Northern-blotting during live H pylori infection for 24-48 h.

3. We planned to determine the mRNA expression of cytokines TNF-alpha with Southern-, and IL1-alpha and RANTES with Northern-blotting during sonicated H. pylori infection for 24-48 h.

4. After obtaining the result that RANTES is upregulated during H. pylori infection, we aimed to check with Northern-blotting whether RANTES mRNA expression can be induced by bacterium other than H. pylori, namely by Escherichia coli.

5. We aimed to check also with Northern-blotting whether RANTES mRNA expression can be stimulated by recombinant human proinflammatory cytokines TNF-alpha and IFN-gamma either alone or in combination.

3 Materials and methods

3.1 Gastric epithelial cells

The normal gastric surface mucous epithelial cell line GSM06 (Sugiyama, 1993) was used for the experiments. The cells were kindly given by the Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). The cells were maintained in collagen type I-coated plastic dishes (Iwaki Glass, Chiba, Japan) in Dulbecco's Modification of Eagle's Medium/Ham's F12 medium (ICN Biomedicals, Inc., Aurora, USA) supplemented with 10% foetal bovine serum (FBS; Dainippon Pharmaceutical Co., Ltd., Australia). The dishes were kept in humidified, 5% CO2 atmosphere at 37°C for one night after passage, and then at 33°C during the experiments. The medium was changed on the cells every second day.

When the cells reached confluence in the dishes the medium was changed to FBS-free medium. The cells were infected with bacterium, or incubated with cytokines 24 h later.

3.2 Bacteria

ATCC 49179 strain of *Helicobacter felis* (Takeda Chemical Industries, Ltd., Osaka, Japan) was used for the experiments. The bacterium was maintained in Blood Agar Base No.2 with horse serum (5%, v/v) containing amphotericin B (2.5 mg/l), trimethoprim (5 mg/l), polymixin (1,250 IU/l), and vancomycin (10 mg/l). The bacterium-solution used for the infection composed of medium containing 10^5, 10^6, 10^7, 10^8, 10^9 Helicobacter felis/ml medium.

TN2GF4 strain of *Helicobacter pylori* (Takeda Chemical Industries, Ltd., Osaka, Japan) was used for the infections. The bacterium was maintained in the same conditions as H. felis. The bacterium solution used for the first infections

contained 10^5, 10^6, 10^7, 10^8 organism/ml medium. Because we found the 10^8 bacterium/ml medium surely enough to induce mRNA expression, we used this concentration of the organism for the investigations performed later.

To check whether bacterium other than H. felis or H. pylori can induce cytokine mRNA expression, GSM06 cells were infected with JM109 type of *Escherichia coli* (Takeda Chemical Industries, Ltd., Osaka, Japan). The bacterium solution contained 10^8 E. coli/ml medium.

3.3 Stimulating cytokines

To look whether cytokine mRNA expression can be induced by proinflammatory cytokines, GSM06 cells were treated with recombinant human proinflammatory cytokines TNF-alpha (100 ng/ml medium) and IFN-gamma (100 ng/ml medium) for 24 h.

3.4 Sonication of bacteria

The necessary number of H. pylori was frozen in liquid nitrogen (minus 196C), and thawed to room temperature five times. Then ultrasonography was applied to the bacterium homogenate for 4 min.

3.5 Experimental protocols

1. GSM06 cells were infected with 10^5, 10^6, 10^7, 10^8, 10^9 live H. felis/ml medium for 2 and 4 h.

2. GSM06 cells were infected with 10^8 live H. pylori/ml medium for 36 h.

3. GSM06 cells were infected with 10^8 sonicated H. pylori/ml medium for 36 h.

4. GSM06 cells were infected with 10^8 live E. coli/ml medium for 8 h

5. GSM06 cells were treated with recombinant human TNF-alpha (100 ng/ml medium) and IFN-gamma (100 ng/ml medium) both alone and in combination for 24 h.

Cells treated with medium only for 2 h served as control during the mRNA determinations.

3.6 RT-PCR

For analysis of mRNA expression of the cytokines total RNA was isolated from the cells with RNA-isolation kit (Isogen, Molecular Research Center, Inc., Tokyo, Japan) The RNA was kept at -70°C until used. The concentration of RNA was measured by absorbency at 260 nm in relation to that of 280 nm. For RT-PCR 0.5 ug of the RNA was reverse transcribed into cDNA with Superscript Preamplification System (GIBCO BRL. Life Technologies, Inc., Rockville, MD, USA). Total RNA in the reaction mixture was heated at 42°C for 50 min. and 70°C for 15 min., then chilled on ice. PCR-reaction was performed with a mixture containing cDNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM of each of the deoxynucleotide triphosphates, 0.5 uM of each specific primers and 1 U AmpliTaq Gold polymerase (Perkin-Elmer, Branchburg, N.J.). The parameters of the PCR cycles were the following: 95°C for 20 sec., 55°C for 2 min. and 72°C for 1 min. The PCR-reaction was performed with 25 cycles for beta-actin, and with 35 cycles for the cytokines.

The sequences of primers were the following:

for TNF-alpha: forward: 5`-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC
reverse: 5`-GGGTGTGGCAGTCGGCTAAACGATAGAGTATG

for IL1-beta: forward: 5`-ATGGCAACTGTTCCTGAACTCAACT
reverse: 5`-CAGGACAGGTATAGATTCTTTCCTTT

for RANTES: forward: 5`-GAAGATCTCTGCAGCTGCCCT
reverse: 5`-GCTCATCTCCAAATAGTTGA

for eotaxin: forward: 5`-AGAGGCTGAGATCCAAGCAG
reverse: 5`-CAGATCTCTTTGCCCAACCT

for MCP-1: forward: 5`-GGAAAAATGGATCCACACCTTGC
reverse: 5`-TCTCTTCCTCCACCACCATGCAG

for MIP1-alpha: forward: 5`-GAAGAGTCCCTCGATGTGGCTA
reverse: 5`-CCCTTTTCTGTTCTGCTGACAAG

for MIP1-beta: forward: 5`-..CCACAATAGCAGAGAAACAGCAAT
reverse: 5`-AACCCCGAGCAACACCATGAAG

for beta-actin: forward: 5`-GTGGGCCGCTCTAGGCACCAA
reverse: 5`-CTCTTTGATGTCACGCACGATTTC

5 ul of amplified DNA reaction mixture was applied to 1% agarose gel electrophoresis containing ethidium-bromide. ÷X174 DNA/HaeIII marker (GIBCO BRL, Life Technologies, Inc., Rockville, MD, USA) was used for the detection of the size of RNA bands in the gel. The PCR product in the gel was visualised with UV fluorescence.

During the RT-PCR reaction RNA from mouse spleen was used as *positive PCR control for* the different *cytokines* (labeled as „p" in the figures). There was also performed PCR reaction without cDNA as *negative PCR control* to check the PCR reaction and DNA contamination (labeled as „n" in the figures). RT-PCR reaction of the control samples (cells in the dishes treated with only medium for 2 h) with the cytokines' primers and with beta-actin primers were also performed (labeled as „c" and „b" in the figures respectively).

3.7 Southern-blot

8 ul of the PCR-product was electrophoresed in 1% agarose gel, and then transferred to nylon membrane (Hybond, Amersham Pharmacia Biotech, Buckingamshire, United Kingdom) for 4 h. The nucleic acid was cross-linked, the membrane hybridised and then washed twice for 20 min in 2xSSC/0.1% SDS at room temperature, and twice for 15 min. in 0.1% SSC/0.1% SDS at 57C. Then the radiolabeled probes were detected and signal densities were quantified as described above.

3.8 Northern-blot

30 ug of total RNA was separated by formaldehyde containing 1% agarose electrophoresis, and transferred to nylon hybridization transfer membrane (Hybond-N, Amersham Pharmacia Biotech, Buckingamshire, United Kingdom) for overnight. For both Northern and Southern hybridization the nucleic acid was constantly fixed to the membrane by ultraviolet cross-linking by using Stratalinker.

Hybridisations were performed with the 270 bp cDNA fragment of mouse RANTES, 354 bp cDNA fragment of mouse TNF-alpha, 491 bp cDNA fragment of mouse IL1-alpha and 414 bp cDNA fragment of mouse beta-actin. The probes were synthesized in our laboratory through the RT-PCR method, the sequences of probes were confirmed to be identical to published sequences with an automatic DNA sequencing machine.

The probes were radiolabeled with (alpha 32P) dCTP (Amersham Pharmacia Biotech, Buckingamshire, United Kingdom) by using DNA Labelling Beads (-dCTP) (Ready To Go, Amersham Pharmacia Biotech, Buckingamshire, United Kingdom) kit. Hybridisations were performed at 42°C for overnight with hybridization buffer containing 50% formamide, 5xSSC (1xSSC = 0.15 M NaCl, 0.015 M sodium citrate), 5X Denhardt's solution, 1% sodium dodecyl sulfate (SDS), 20 mM phosphate buffered saline, 5 mg salmon-sperm DNA. For Northern blot the membrane was then washed twice in 2xSSC/0.1% SDS for 30 min. at 42°C.

The radiolabeled probes were detected and signal densities were quantified with bioimage analyser (BAS 2000-II Imaging Analyser, Fuji Photo Film Co., Tokyo, Japan).

When evaluating the results obtained with Southern- and Northern-blot, we used the rationalization/normalization of the cytokines' signal densities to the beta-actin signal densities. Because mRNA of beta-actin - independently from the activated or non-activated state of the cells - is linearly proportional to the amount of the total RNA in the sample, the comparison of the signal densities of the cytokines with signal densities of beta-actin obtained with the same amount of the same cDNA, we may normalize the activation state of the cells.

4 Results

4.1 Infection with H. felis

When the mRNA of proinflammatory cytokine IL1-beta, and chemokines RANTES, eotaxin, MCP-1, MIP1-alpha and beta during infection with different number of live H. felis for 2 and 4 h was determined by RT-PCR method, we found that however neither mRNA of cytokines expressed in the control dishes („c" in the figures), i.e. there was no constitutive expression of any of these cytokines in the GSM06 cells (Fig. 1.-6.), the mRNA of each cytokine expressed 2 and 4 h after starting the incubation with the different numbers of bacterium (Fig. 1.-6.).

4.2 Infection with H. pylori

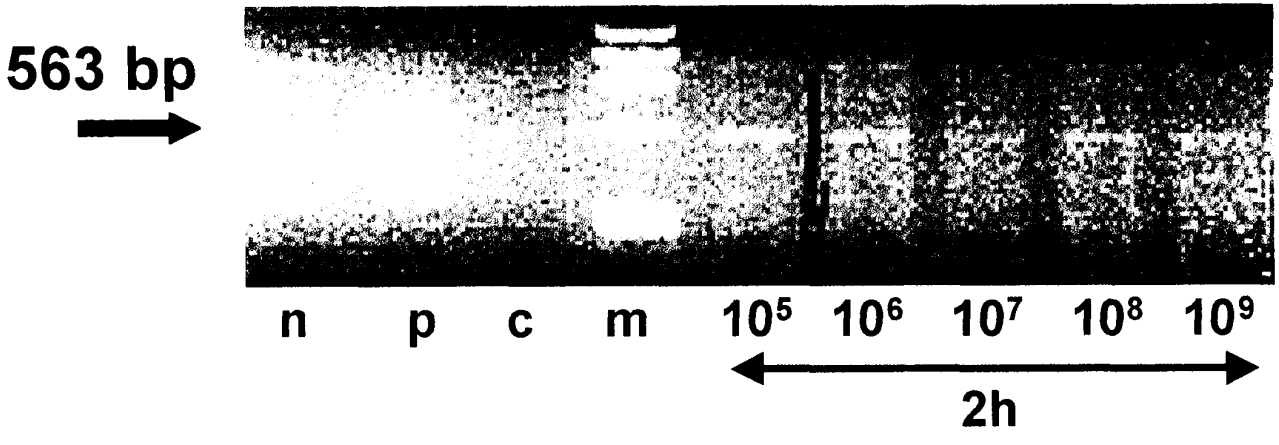
On the Figs. 7.-13. we represented the signal density pictures of cDNA or RNA samples of cells infected for different time intervals, and hybridized with radiolabeled cytokine or beta-actin probes.

4.2.1 Live bacterium

4.2.1.1 TNF-alpha

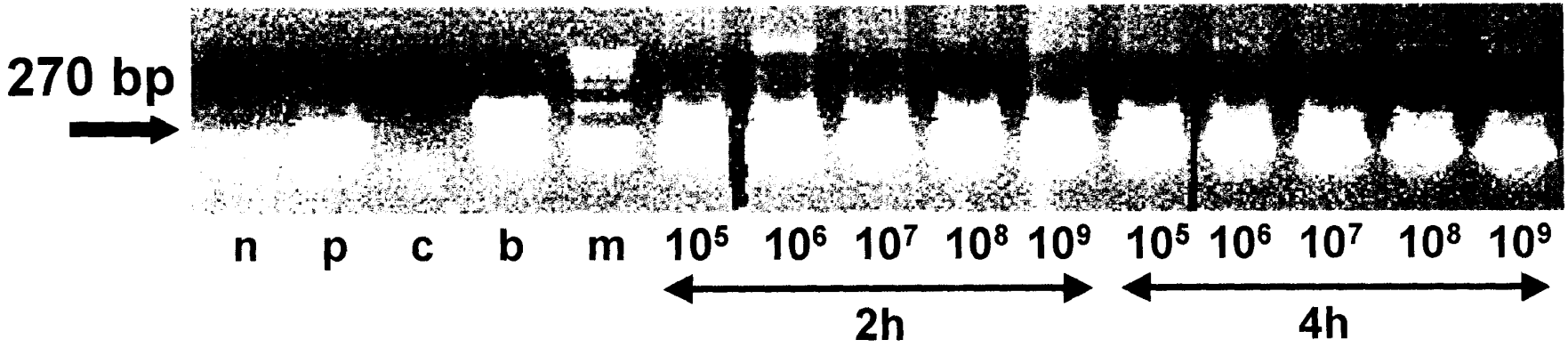
When the mRNA expression of the proinflammatory cytokine TNF-alpha was measured by Southern-blotting in the GSM06 cells, we found a weak constitutive expression. After infection with live H. pylori for up to 36 h, we found that TNF-alpha mRNA expression showed a two stepped increase on Southern blot. The first, but slight elevation appeared at 4 h after the infection, then the expression did not

Fig. 1.



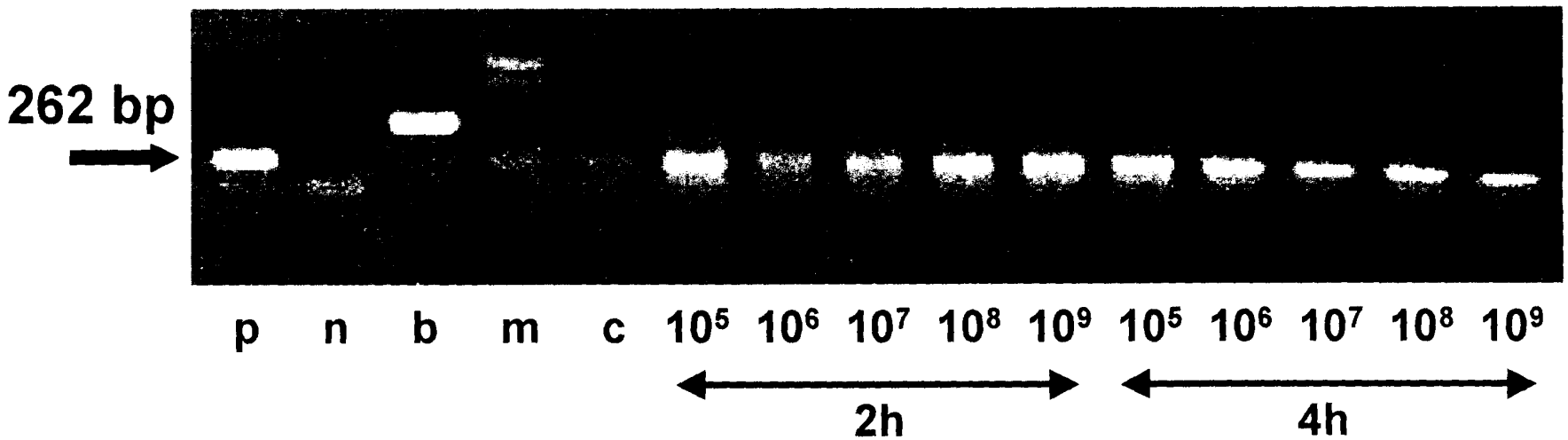
mRNA expression of **IL1-beta** detected by RT-PCR reaction in GSM06 cells during infection with $10^{5\text{-}6\text{-}7\text{-}8\text{-}9}$ *live* H. felis/ml medium for 2 h
n: negative PCR control (without cDNA); p: positive PCR control for IL1-beta (RNA from spleen cells); c: control cells (not treated); m: DNA marker; bp: base pair

Fig. 2.



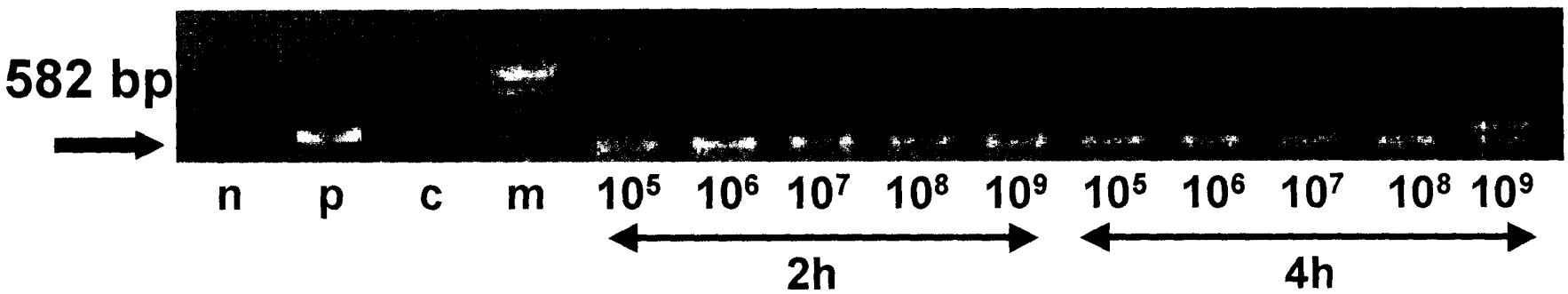
mRNA expression of **RANTES** detected by RT-PCR reaction in GSM06 cells during infection with $10^{5\text{-}6\text{-}7\text{-}8\text{-}9}$ *live* H. felis/ml medium for 2 and 4 h
n: negative PCR control (without cDNA); p: positive PCR control for RANTES (RNA from spleen cells); c: control cells (not treated); b: beta actin; m: DNA marker; bp: base pair

Fig. 3.



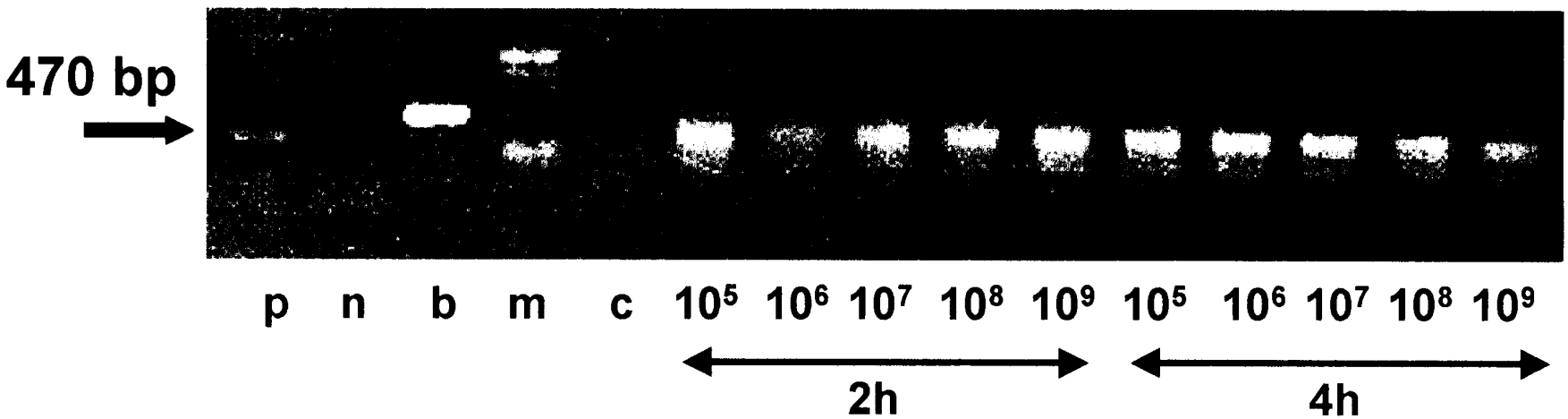
mRNA expression of **eotaxin** detected by RT-PCR reaction in GSM06 cells during infection with $10^{5\text{-}6\text{-}7\text{-}8\text{-}9}$ *live* H. felis/ml medium for 2 and 4 h
p: positive PCR control for eotaxin (RNA from spleen cells); n: negative PCR control (without cDNA); b: beta actin; m: DNA marker; c: control cells (not treated); bp: base pair

Fig. 4.



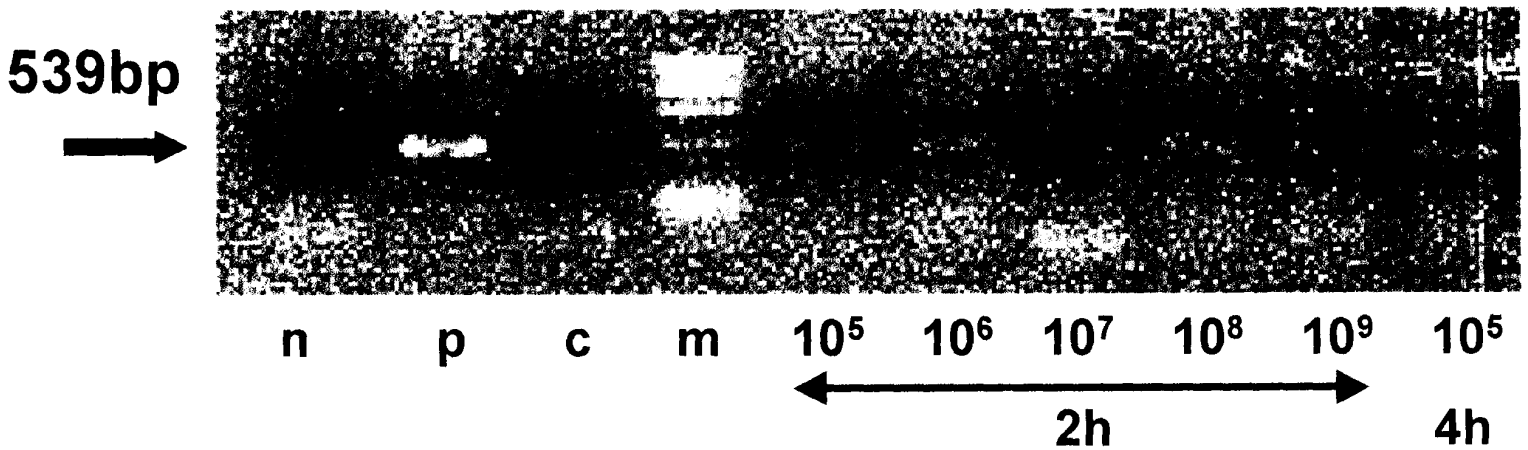
mRNA expression of **MCP-1** detected by RT-PCR reaction in GSM06 cells during infection with $10^{5\text{-}6\text{-}7\text{-}8\text{-}9}$ *live* H. felis/ml medium for 2 and 4 h
n: negative PCR control (without cDNA); p: positive PCR control for MCP-1 (RNA from spleen cells); c: control cells (not treated); m: DNA marker; bp: base pair

Fig. 5.



mRNA expression of **MIP1-alpha** detected by RT-PCR reaction in GSM06 cells during infection with $10^{5\text{-}6\text{-}7\text{-}8\text{-}9}$ *live* H. felis/ml medium for 2 and 4 h
p: positive PCR control for MIP1-alpha (RNA from spleen cells); n: negative PCR control (without cDNA); b: beta actin; m: DNA marker; c: control cells (not treated); bp: base pair

Fig. 6.



mRNA expression of **MIP1-beta** detected by RT-PCR reaction in GSM06 cells during infection with $10^{5\text{-}6\text{-}7\text{-}8\text{-}9}$ *live* H. felis/ml medium for 2 and 4 h
n: negative PCR control (without cDNA); p: positive PCR control for MIP1-beta (RNA from spleen cells); c: control cells (not treated); m: DNA marker; bp: base pair

change. From 18h the expression elevated again, and reached a stronger second peek at 36 h (Fig. 7.). After this the expression tended to decrease at 48 h.

On the graphs of the Figs. 7.-10. we represented the ratio of signal densities of the cytokines and beta-actin. We determined as 100% the ratio of the maximal signal density of the cytokine to the beta-actin density of the same sample.

4.2.1.2 IL1-alpha

IL1-alpha mRNA expression showed also a weak constitutive expression in GSM06 cells as visualised by Northern blot. Furthermore it was also time-dependently elevated during live H. pylori infection. The expression started to increase after 18 h, and then continuously increased until 36 h after the incubation. (Fig. C8.).

4.2.1.3 RANTES

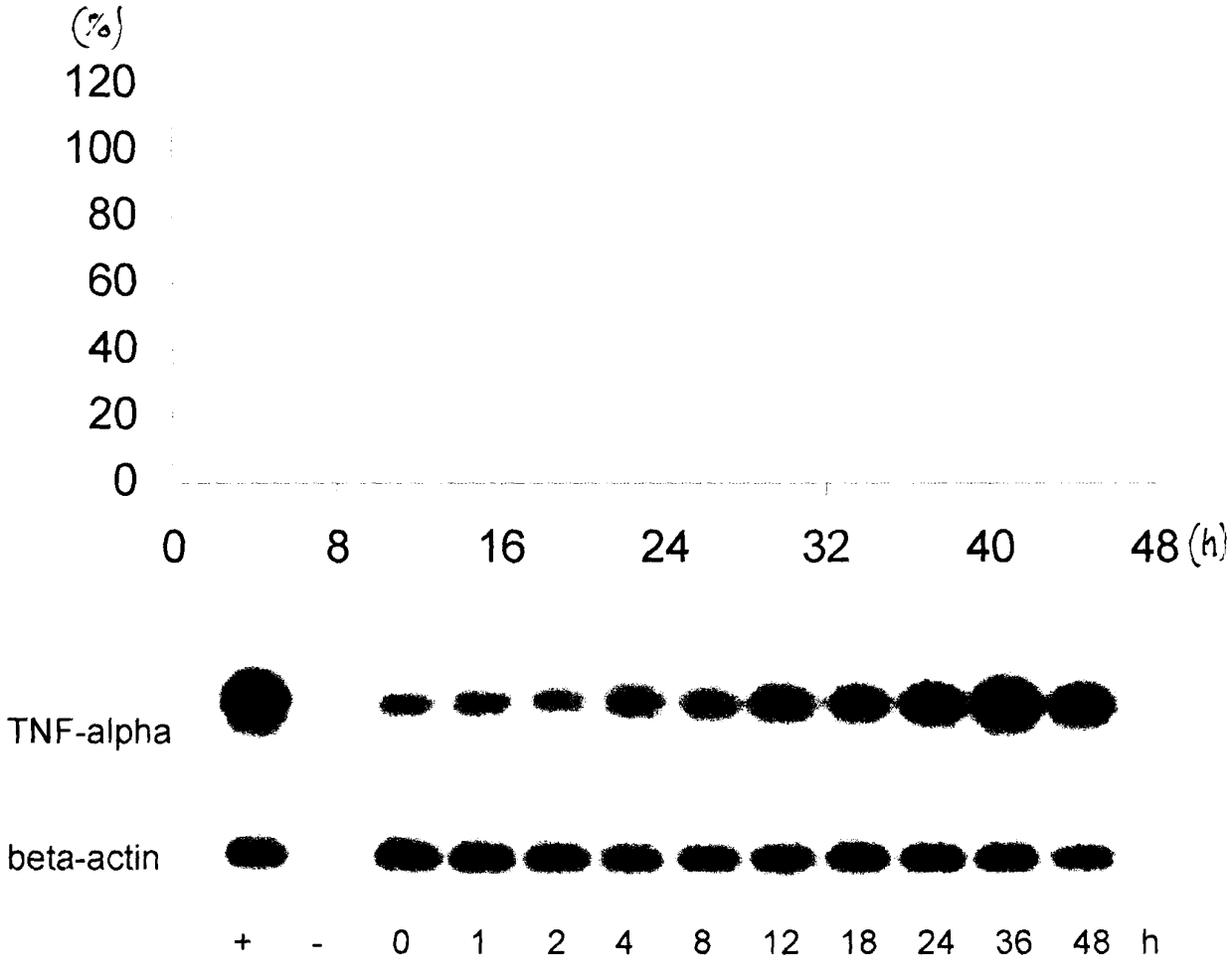
When we measured RANTES mRNA by Northern blot we found that GSM06 cells did not constitutively express RANTES mRNA. However we found that live H. pylori induced a marked and time-dependent increase in the expression of RANTES mRNA as shown by Northern blot during infection with live H. pylori for up to 24 h. The elevation started at 4 h, and continuously increased until 24 h after the infection (Fig. 9.).

4.2.2 Sonicated bacterium

4.2.2.1 TNF-alpha

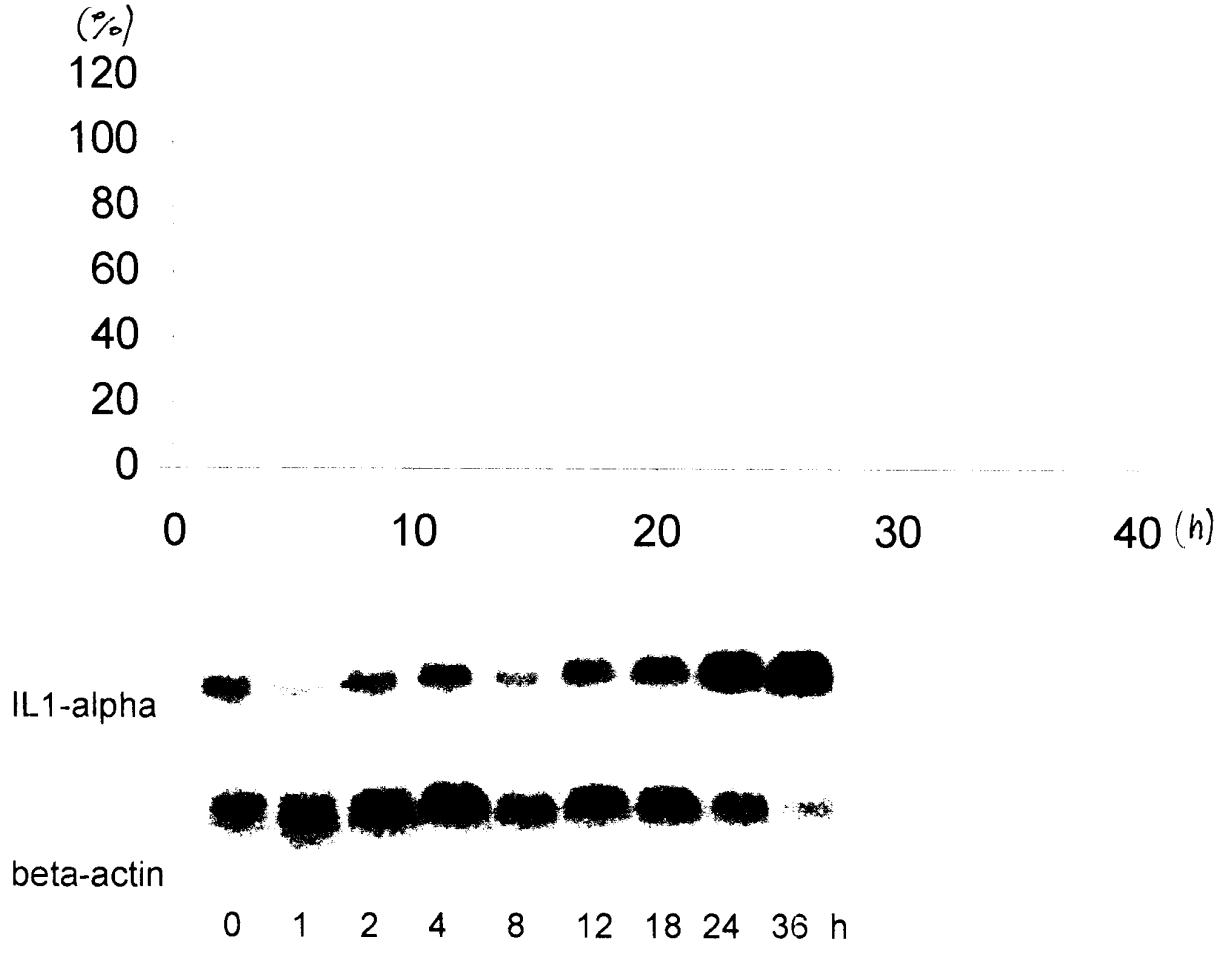
RT-PCR-Southern hybridization showed that TNF-alpha mRNA expression was upregulated during the incubation with sonicated H. pylori with similar pattern as with the live bacterium (Fig. 10.).

Fig. 7.



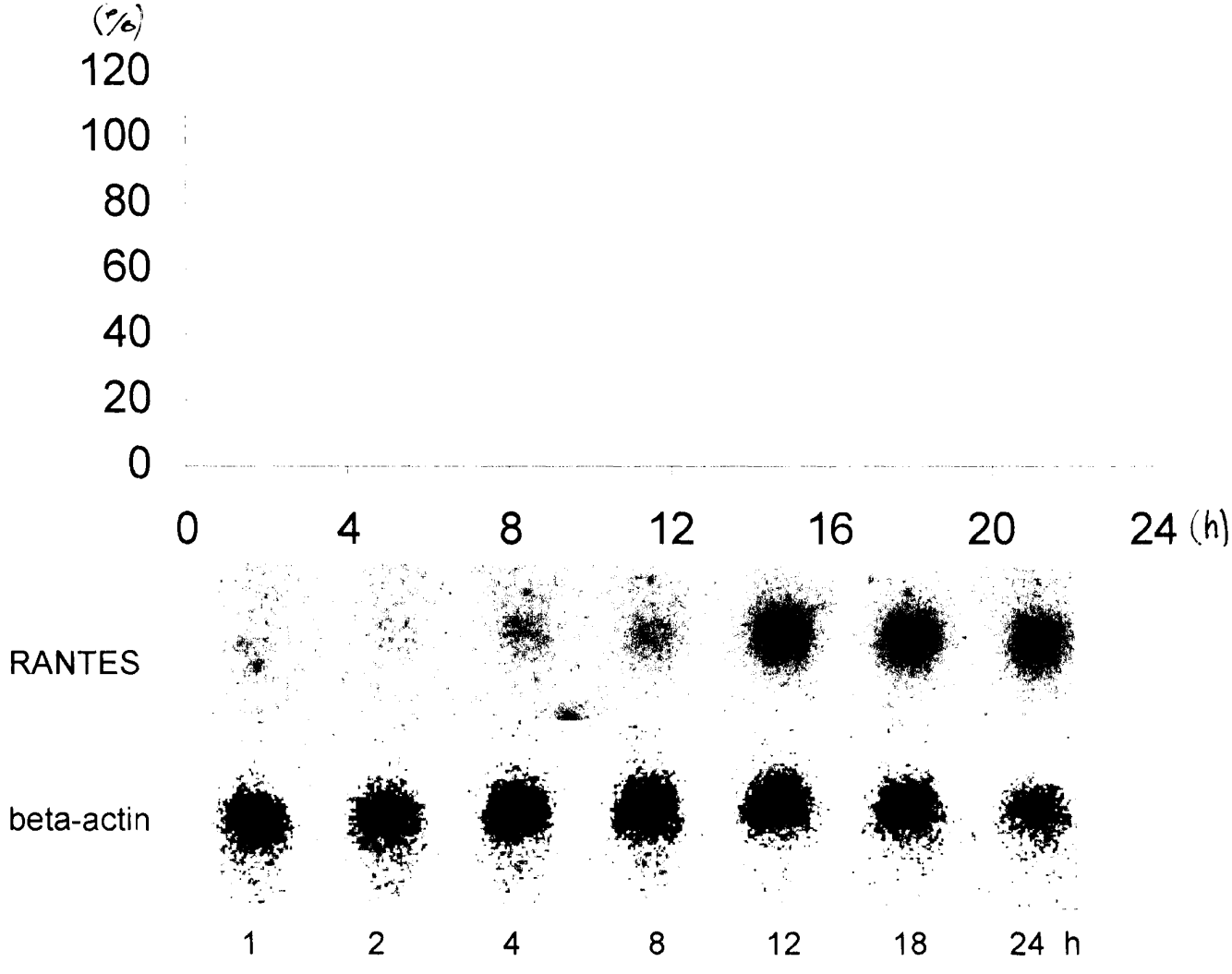
mRNA expression of **TNF-alpha** detected by RT-PCR-Southern blot in GSM06 cells during infection with 10^8 *live* H. pylori/ml medium for up to 48 h
Signal densities of TNF-alpha were rationalized to signal densities of beta-actin
+: positive PCR control for TNF-alpha (RNA from spleen cells); -: negative PCR control (PCR reaction without cDNA)

Fig. 8.



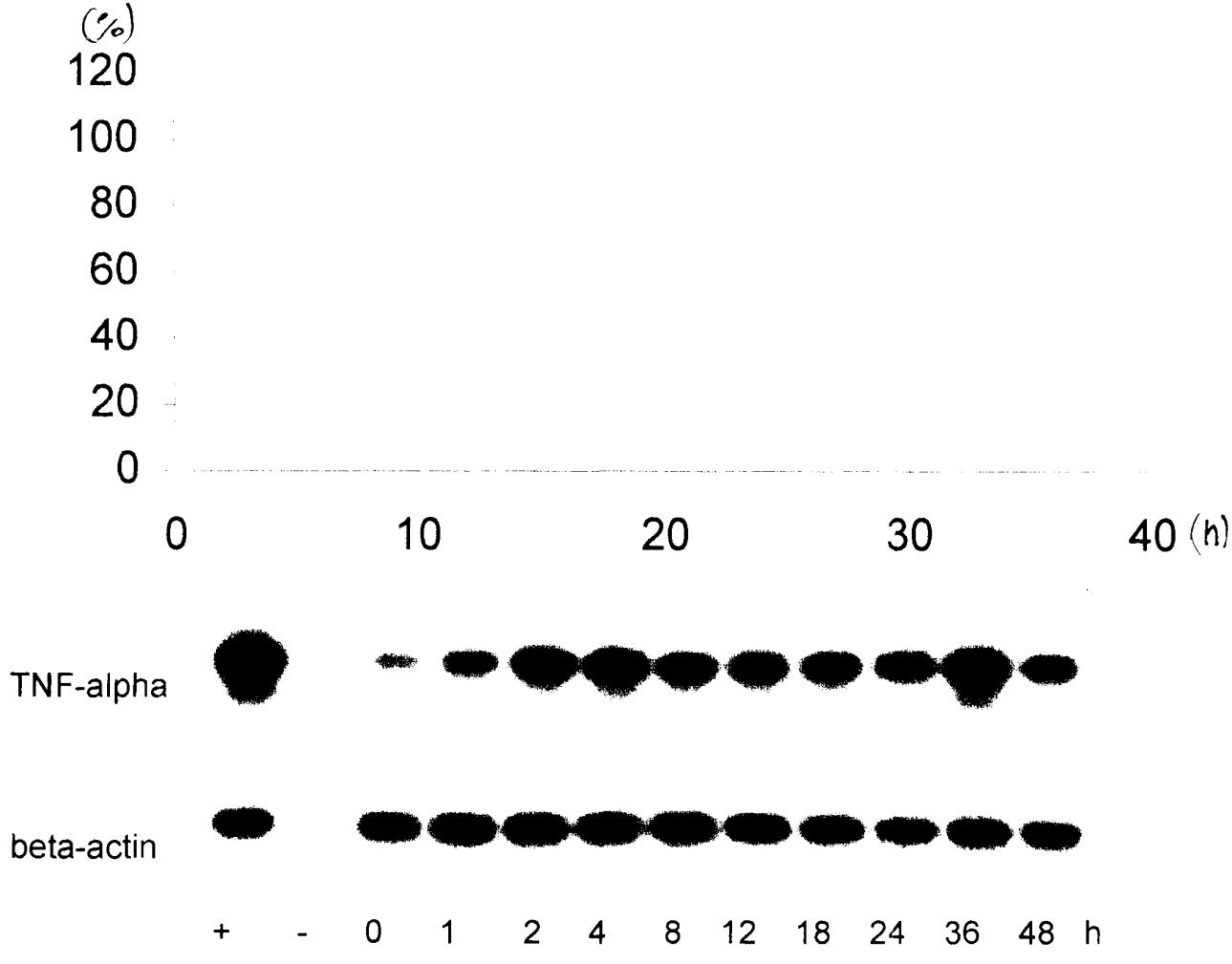
mRNA expression of **IL1-alpha** detected by Northern blot in GSM06 cells during infection with 10^8 *live* H. pylori/ml medium for up to 36 h
Signal densities of IL1-alpha were rationalized to signal densities of beta-actin.

Fig. 9.



mRNA expression of **RANTES** detected by Northern blot in GSM06 cells during infection with 10^8 *live* H. pylori/ml medium for up to 24 h
Signal densities of RANTES were rationalized to signal densities of beta-actin.

Fig. 10.



mRNA expression of **TNF-alpha** detected by RT-PCR-Southern blot in GSM06 cells during infection with 10^8 *sonicated* H. pylori/ml medium for up to 48 h
Signal densities of TNF-alpha were rationalized to signal densities of beta-actin.
+: positive PCR control (RNA from spleen cells); -: negative PCR control (PCR reaction without cDNA)

4.2.2.2 IL1-alpha

The sonicated bacterium did no stimulate the expression of IL1-alpha mRNA visualized with Northern blot (Fig. 11.).

4.2.2.3 RANTES

The components of the sonicated bacterium did not activate the cells to express RANTES mRNA during the 36 h experimental period on the Northern blot (Fig. 12.).

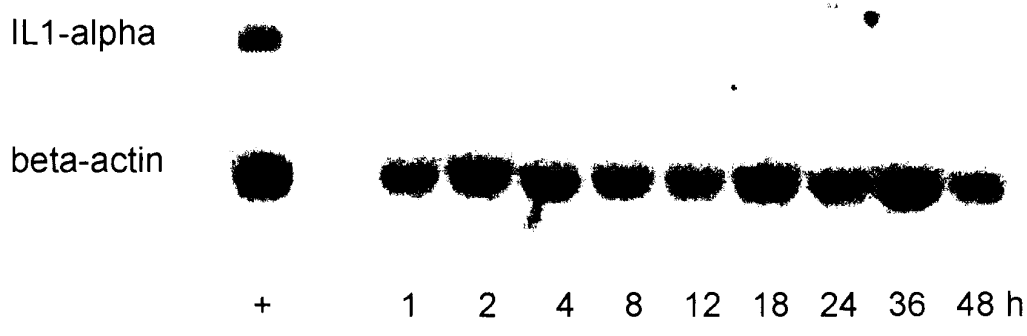
4.3 Infection with E. coli

In contrast to H. pylori, E. coli did not induce RANTES mRNA expression during 8 h as seen on the Northern blot picture (Fig. 13.).

4.4 Treatment with recombinant human proinflammatory cytokines

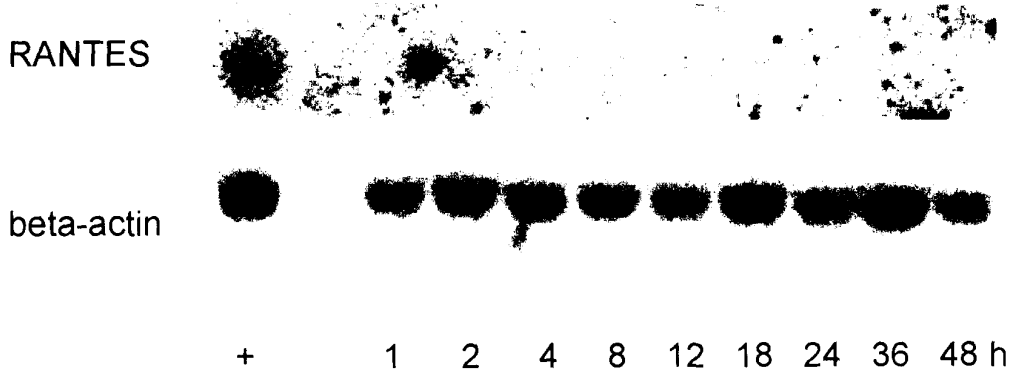
Neither human recombinant TNF-alpha nor IFN-gamma alone or in combination induced RANTES mRNA expression during the 24 h experimental period evaluated by Northern blot (Fig. 13.). The hybridization of the same membrane with RANTES probe was performed twice, therefore two pictures are shown for RANTES on this figure.

Fig. 11.



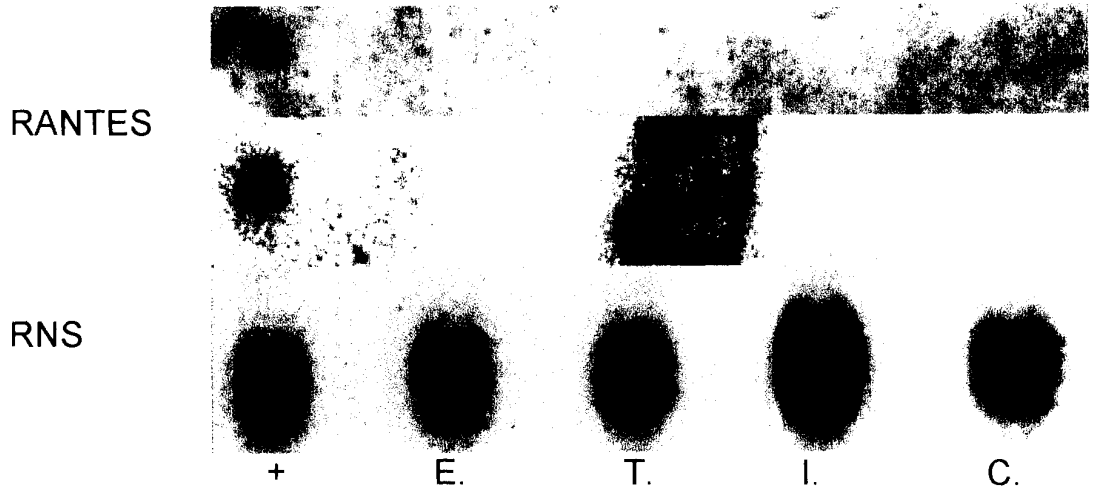
mRNA expression of **IL1-alpha** detected by PCR-Southern blot in GSM06 cells during infection with 10^8 *sonicated* H. pylori/ml medium for up to 48 h
Signal densities of TNF-alpha were compared with signal densities of beta-actin.
+: positive control (RNA from spleen cells)

Fig. 12.



mRNA expression of **RANTES** detected by Northern blot in GSM06 cells during infection with 10^8 *sonicated* H. pylori/ml medium for up to 36 h
Signal densities of RANTES were compared to signal densities of beta-actin.
+: positive control (RNA from spleen cells)

Fig. 13.



mRNA expression of **RANTES** detected by Northern blot in GSM06 cells during infection with 10^8 *live H. pylori*/ml medium for 8 h as positive control (+), with 10^8 *live E. coli*/ml medium for 8 h (E.), with human recombinant *TNF-alpha* (T.), or human recombinant *IFN-gamma* (I.), or with the combination of the two substances (C.). Signal densities of RANTES were compared to signal densities of beta-actin.

5 Discussion

Our present results show that the cells of a normal (non tumor derived) gastric mucous epithelial cell line themselves express the mRNA of a wide range of cytokines with proinflammatory and chemotactic characteristics during bacterial infection with H. pylori and H. felis.

5.1 Infiltration and cause of infiltration

The histology of the H. pylori infected stomach shows a considerate infiltration of poly- and mononuclear cells (Genta, 1993) and lymphocytes (Hatz, 1996). This infiltration of the gastric mucosa considered to be at least partly due to the result of cytokine release from the gastric epithelial cells, because in the last few years more and more data has been published about the cytokine (Jedrzkiewicz, 1999; Kolios, 1999; Stellato, 1995; Warhurst, 1998; Yang, 1997; Watanabe, 1997; Jung, 1997) and cytokine receptor expression (Dwinell, 1999; Stevens, 1997; Reinecker, 1996; Ciacci, 1993) by gastrointestinal epithelial cells, indicating that gastrointestinal epithelial cells play an active role in the immune response during bacterial infection.

5.2 TNF-alpha, IL1-alpha and -beta

Among the released cytokines are the proinflammatory cytokines TNF-alpha and IL1-alpha and beta. The mRNA expression of these cytokines is markedly increased during H. pylori infection in the gastric mucosa, high expression of them was found in gastric biopsy samples obtained from H. pylori infected patients (Moss, 1994; Noach, 1994; Crabtree, 1991; Shimada, 1998). Because their increased expression is characteristic for H. pylori infection, and may be the cause and marker

of the gastritis, these cytokines are called H. pylori-related cytokines too (Brzozowski, 1998). The source of these cytokines are probably mainly the chemoattracted (partly by RANTES) and activated neutrophils and monocytes/macrophages are present in the inflamed gastric mucosa, because H. pylori stimulates the IL1-beta and TNF-alpha mRNA expression of human monocytes (Takaishi, 1999), however gastric epithelial cells can also express TNF-alpha and IL1-alpha and -beta mRNA (Jung, 1997; Maekawa, 1997).

In our study normal gastric mucous epithelial cells expressed only TNF-alpha mRNA, but not the others, without bacterial activation, however showed increased mRNA expression of IL1-beta during H. felis, and upregulated mRNA expression of TNF-alpha and IL1-alpha during H. pylori infection.

As for the time course of cytokine expression in our study, the mRNA expression of IL1-beta measured by RT-PCR was upregulated shortly, 2 and 4 h after starting the incubation with H. felis. This is in accordance with previously published results obtained with H. pylori infected human gastric adenocarcinoma cell lines, however there is a difference between the ability of the different cell lines in that respect, what and when they express. Jung et al. investigated the mRNA expression of proinflammatory cytokines in two different tumor derived human gastric epithelial cell lines (SNU-5 and KATO-III) during H. pylori infection, and found that SNU-5 cells expressed IL1-beta at 4 h (Jung, 1997).

TNF-alpha mRNA expression markedly increased at 4 h in our experiment, reached its peek at 36 h, and finally tended to decrease 48 h after starting the incubation with H. pylori as evaluated by Southern blotting. Others found similar early (at 1-3 h) expression of this cytokine in gastric epithelial cells infected with the same bacterium (Jung, 1997; Maekawa, 1997), however one of the two above mentioned adenocarcinoma cell lines, the KATO-III, did not express TNF-alpha even 9 h after H. pylori infection (Jung, 1997). This last data from the literature underlines the argument, that the results obtained with cell lines of different origin cannot be compared without correct interpretation.

In the case of IL1-alpha we found no mRNA expression until 18 h with Northern blotting, but a continuous elevation occurred from this time with a peek at 36 h, then the expression started to decrease during H. pylori infection. In spite of our finding, IL1-alpha mRNA expression increased shortly (1-2 h) after starting the

incubation with this bacterium in human gastric adenocarcinoma cells measured by quantitative RT-PCR. However the mRNA expression was not checked at later interval than 9 h (Jung, 1997). Furthermore when interpreting the data we should be aware of the ability of these molecular biological methods to sensitively detect the presence and/or difference of the expression of a given mRNA in the RNA sample, namely for what they are sensitive. The usual, common RT-PCR method is very sensitive to show out the presence of a small amount of mRNA, i.e. the slightly upregulated mRNA expression. However it cannot quantitatively distinguish the little differences between the mRNA expression of different samples. This is why we did not compare the degree of mRNA expression based only on the RT-PCR results. In contrast, Northern blotting is less sensitive to detect small amount of mRNA in the sample, however - due to the acceleration of the signal by radioactive labeled probe - it is useful to recognise little differences between the mRNA expression of the different samples. Therefore we cannot exclude that GSM06 cells showed expression of IL1-alpha before 18 h, which was weaker than after 18 h, however we could not detect it with Northern blotting.

The incubation time with H. pylori in the two cited studies did not exceed 6 and 9 h. This long-lasting expression of TNF-alpha and IL1-alpha even for 36 h is revealed first in our study.

There is no harmony in the inducibility of the mRNA expression of these proinflammatory cytokines by sonicated H. pylori. Gastric epithelial cells were found to express increased levels of both TNF-alpha and IL1-alpha mRNA 2 h after sonicated H. pylori treatment (Maekawa, 1997). However in our study only TNF-alpha mRNA expression was induced by sonicated H. pylori with the same delayed time-course as by live H. pylori, but not IL1-alpha.

Our observations fit well to the idea that the proinflammatory cytokines have an important role during the H. pylori infection, and indicate how H. pylori may aggravate the gastric damage associated with the infection. This latter is suggested by the observations that IL1-beta induces ulcer relapse in rats (Tominaga, 1998), and that the H. felis infection caused augmentation of water-immersion stress induced gastric mucosal ulceration is accompanied by an increase in IL1-beta mRNA expression (Matsushima, 1999).

5.3 Chemokines

Chemokines are another main group of cytokines attracting different population of leukocytes, however a few of them (e.g. MIP proteins) exert proinflammatory properties too. They may be classified into four subgroups with different target cells according to the adjacent or distant position of four conserved cystein motifs in the polypeptide chain. In the CC-chemokine group two of the cysteins are adjacent. The members of this subgroup (RANTES, eotaxin, MCP-1, MIP1-alpha and beta) attract preferentially monocytes, macrophages, eosinophils, and subsets of lymphocytes (Schall, 1991; Rollins, 1997), and play important role in the inflammatory processes of the gastrointestinal tract by recruiting inflammatory cells from the periphery to the place of the inflammation (Yamaoka, 1998).

5.3.1 RANTES

One of the most important candidates may be responsible for the immune response during H. pylori infection by recruiting leukocytes from the periphery to the place of the infection is the chemokine RANTES. Investigations with gastric biopsy samples removed from Helicobacter pylori infected patients showed upregulated RANTES mRNA, and increased RANTES protein levels compared with those of H. p. negative controls (Shimoyama, 1998; Yamaoka, 1998; Kikuchi, 1999). However the origin of RANTES in these gastric samples is not known, since no data have been published yet about RANTES expression by, or secretion from normal gastric epithelial cells neither in vitro, nor in vivo. According to our results gastric epithelial cells do may be the source of RANTES found in these biopsy samples, since Helicobacter pylori infection markedly upregulated RANTES mRNA expression in normal gastric surface mucous epithelial cells after 4 h, and this expression lasted for 24 h after starting the incubation. Without bacterial stimulation GSM06 cells did not express RANTES mRNA.

The receptors for RANTES belong to the CC-chemokine receptor (CCR) family. Eight CC-chemokine receptors (CCR1-8) have been published to date. They are 7-transmembrane spanning (7-TMS), G-protein coupled receptors, and show considerable promiscuity. Although RANTES can bind to CCR1, CCR3 and CCR5, and all of these receptors are able to bind other chemokines too, the main ligand for CCR5 is RANTES (Rollins, 1997; Ward, 1998). The receptors for RANTES are present on almost every type of leukocytes (Mantovani, 1999), but CCR5 - the main receptor for RANTES - is primarily expressed by memory phenotype CD45R0+ T lymphocytes (Bleul, 1997). RANTES was found to attract selectively CD45RO+ T cells (Roth, 1995), however not to affect other T cell phenotypes (Schall, 1990). Interestingly and notably the phenotypic characterisation of lymphocytic infiltration in gastric biopsy samples from Helicobacter pylori infected individuals showed increased number of CD45RO+ lymphocytes (Hatz, 1996; Kikuchi, 1999). Furthermore neither RANTES protein level, nor the number of CD45RO+ T cells did not decrease even after successful eradication therapy even after one year (Hatz, 1996; Kikuchi, 1999; Sato, 1999).

It should be mentioned too, that Th1 and Th2 cells express different chemokine receptors. CCR5 receptors are preferentially expressed by Th1 cells, the CCR5 seems to be characteristic for Th1 lymphocytes (Kawai, 1999). During H. pylori infection the lymphocyte response is polarised to Th1 cells in the gastric mucosa (Crabtree, 1998).

From the above described data we may speculate that gastric mucous epithelial cells express and release RANTES during Helicobacter pylori infection, which chemokine in turn attracts specific T cell subpopulations - Th1 and CD45RO+ lymphocytes - from the periphery to the area of bacterial invasion. These T cells then can meet the antigens specific for them. After maturing fully they may secrete RANTES abundantly and trigger the immune response to the infection. The observation that T cell transmigration through endothelial layer is enhanced by Th1-type cytokine IFN-gamma, but this effect is dependent both on RANTES produced by endothelial cells, and on CCR5 expressed on Th1, but not on Th2 cells, furthermore that the effect of IFN-gamma was inhibited by anti-RANTES or anti-CCR5 antibody (Kawai, 1999), strengthens this idea. However the effects and roles of this cytokine are more complex, since RANTES - synergistically with eotaxin -

also promote the TNF-alpha- or IL1-beta-induced eosinophil transmigration across endothelial layer through CCR3 (Shahabuddin, 2000). [Shahabuddin S, Ponath P, Schleimer RP (2000) Migration of eosinophils across endothelial cell monolayers: interactions among IL-5, endothelial-activating cytokines, and C-C chemokines. J. Immunol. 164:3847-3854]

As for the time-course, RANTES mRNA detected by Northern-blotting elevated at 4 h, and lasted until 24 h after starting the incubation in our study. The pattern of RANTES mRNA expression found in our study is in accord with previous reports, which showed similar relatively late activation of this chemokine after proinflammatory cytokine activation. RANTES mRNA expression was elevated with a peek at 20 h (Yang, 1997) or at 24 h (Kolios, 1999; Warhurst, 1998) in HT-29 human colonic epithelial cells. RANTES protein secretion was also delayed, it reached its peek at 24 h after TNF-alpha stimulation of HT-29 cells (Yang, 1997). This relative delay in the induction time of RANTES seems to be general, since the same phenomenon was found in other cell types, e.g. in airway epithelial cells, in freshly isolated primer bronchial epithelial cells (Stellato, 1999), fibroblasts (Rathanaswami, 1993), renal epithelial (Heeger, 1992) and mesangial cells (Wolf, 1993). RANTES mRNA expression required *de novo* protein synthesis, as protein synthesis inhibitor cycloheximide blocked the expression (Stellato, 1999). These data suggest that this delayed expression of RANTES mRNA may be due to the synthesis of a protein (transcription factor) after bacterial infection. One of the possible candidates for this role is STAT-1, which has been shown to cooperate with NF-kappa B in the synergistic activation of RANTES gene by TNF-alpha and IFN-gamma in murine fibroblasts. Transcription factors AP-1 and NF-kappa B are known to be involved in the up-regulation of RANTES in epithelial cells too (Stellato, 1999; Moriuchi, 1997; Ohmori, 1997).

The induction of RANTES mRNA expression requires live bacterium, since sonication of H. pylori prevented the effect of bacterium

Furthermore the inducibility of RANTES mRNA expression is not a general feature of gastric epithelial cells for pathogenic bacteria, since E. coli also could not produce the same effect as H. pylori.

Expression of RANTES was inducible in many cell lines by treatment of recombinant TNF-alpha or IFN-gamma either alone or in combination (Kolios, 1999;

Stellato, 1999; Roebuck, 1999; Warhurst, 1998). The synergistic effect between TNF-alpha and IFN-gamma for RANTES expression has been reported in fibroblasts (Rathanaswami, 1993), in human bronchial epithelial cells (Stellato, 1995) and in endothelial cells (Marfaing-Koka, 1995). The mechanism of synergism between proinflammatory agents is discussed in details by Paludan (Paludan, 2000). However using similar doses of these human recombinant cytokines both alone or in combination as in the previously mentioned studies, RANTES mRNA was not inducible in our study. RANTES mRNA expression also did not increase in human colonic T84 epithelial cells after treatment by TNF-alpha or TNF-alpha plus IFN-gamma (Jedrzkiewicz, 1999).

5.3.2 Eotaxin

There is much less data in the literature regarding the presence and role of other chemokines, as eotaxin, MCP-1, MIP1-alpha and -beta in the GI tract, especially in the stomach.

Eotaxin is a potent chemoattractant for eosinophils (Rothenberg, 1995; Rollins, 1997), and for CCR3+ Th2 cells, since its only receptor is CCR3 (Sallusto, 1997) which is preferentially expressed on Th2 cells (Agace, 2000).

It is constitutively expressed in high amount in the lung, and less expression was found in many organs in guinea pig, among them the stomach and intestine (Rothenberg, 1995). However we did not find constitutive expression of eotaxin mRNA in normal mouse gastric epithelial cells. Eotaxin has important role in the pathomechanism of allergic diseases in the respiratory tract as asthma, causing eosinophilia after antigen challenge (Rothenberg, 1995), and in the parasitic diseases of the gastrointestinal tract, since increased eotaxin level was found in parasitic gastro-enteritis (del Pozo, 1999). We found that H. felis infection induced its expression after 2 h.

Eotaxin works in Cupertino and synergism with other members of the chemotactic and proinflammatory cytokine families. A very nice example for this is how eotaxin and RANTES promote - in synergism - the TNF-alpha- or IL1-beta-

induced eosinophil transmigration across endothelial layer (Shahabuddin, 2000). Eotaxin is an essential mediator of the eosinophil trafficking/homing into mucosal tissues (Rothenberg, 1999), and responsible for the release of Th2 related during oral antigen challenge (Hogan, 2000). Since among the members of Th2 cytokines are antiinflammatory cytokines (IL-4, IL-10, IL-13) too, eotaxin may have role in attenuating the immune response during H. pylori infection (Shimada, 1998).

5.3.3 MCP-1

MCP-1 is responsible for the recruitment of monocytes/macrophages and granulocytes from the circulation to the inflamed mucosa (MacDermott, 1996). Its expression is - similarly to RANTES - greater in biopsy samples of H. pylori infected patients comparing with those of H. pylori negative ones (Shimoyama, 1998; Sato, 1999), and in inflammatory bowel disease (MacDermott, 1996). After eradication of H. pylori the expression of MCP-1 decreases (Sato, 1999). Gastric epithelial cells with tumor origin also express MCP-1 mRNA in response to proinflammatory cytokine treatment, and during H. pylori infection (Watanabe, 1997, Jung, 1997). Its role in the development of gastric ulceration is supported with the observation that the IL1-beta caused ulcer relapse is accompanied - beside the macrophage infiltration - by an increased expression of MCP-1 mRNA (Tominaga, 1998), which is also an example for the relation between the different cytokines.

We did not find to be expressed MCP-1 in the control dishes, however 2 and 4 h after starting the incubation with H. felis, upregulation of MCP-1 mRNA expression was detectable in our study.

5.3.4 MIP1-alpha and beta

We found stimulation of MIP1-alpha and -beta mRNA expression 2 and 4 h after infecting the GSM06 cells with H. felis, while the unstimulated cells did not express the mRNA of these chemokines.

MIP1-alpha and MIP1-beta are neutrophil and macrophage attracting agents (Schall, 1991) The mRNA expression of MIP1-alpha - but not MIP1-beta - is also upregulated, and associated with infiltration of mononuclear cells of the gastric mucosa during H. pylori infection, however its expression is reduced after H. pylori eradication (Yamaoka, 1998; Sato, 1999). Ando et al. also found increased mRNA expression in gastric biopsy specimens of H. pylori positive patients comparing with those of negative ones, furthermore the level of MIP1-alpha positively correlated with the histologic grade of activity, inflammation and H. pylori density (Ando, 1998) These chemokines are - similarly to RANTES - secreted during Th1-type immune response (Schrum, 1996) which can be seen in H. pylori infection (Crabtree, 1998).

The results of our studies serve a proof for the possibility that gastric mucosal epithelial cells actively participate in the immune response of gastric mucosa during bacterial infection by expressing different proinflammatory cyto- and chemokines.
From the above described data we may conclude that gastric mucous epithelial cells are active participants of the immune response during H. pylori infection. The relatively early (after 2 h) cytokine release involving a number proinflammatory (TNF-alpha, IL1-alpha and beta) and chemotactic (RANTES, eotaxin, MCP-1, MIP1-alpha and beta, IL8, GRO-alpha) cytokines (Shimada, 1998) from the epithelial cells may induce a wide range of immune cells - mono- and polynuclear cells, eosinophils, and specific T cell subpopulations (mainly Th1 and CD45RO+ T lymphocytes) - to gather from the periphery to the site of bacterial invasion earlier than without this cytokine release. A stable subepithelial chemokine gradient develops after bacterial infection, which promote directional migration of neutrophils toward the site of bacterial invasion (McCormick, 1995). Here the T cells can meet the released antigens are specific for them. After maturing fully they may secrete abundantly the same, and other pro- (IL-2, IL-3, IL-12, GMCSF, IFN-gamma) and antiinflammatory cytokines (IL-10) as the epithelial cells, which in turn regulate - trigger and inhibit - the immune response to the infection. A number of publications indicates that these cyto- and chemokines are released, their receptors are expressed, and their effects are exerted in a very complex way, via induction and inhibition of the release of each other (Tominaga, 1998; Matsushima, 1999; MacDermott, 1996; Schall, 1991; Dwinell, 1999; Crabtree, 1993; Kawai, 1999;

Shimada, 1998). The mechanism of synergism between proinflammatory agents is discussed in details by Paludan (Paludan, 2000).

These data can help us to understand the very complex role of gastric epithelial cells in the immune response to bacterial challenge. Since H. pylori is one of the (if not the) most common pathogen(s) found in human (Zevering, 1999), it is very important to get closer and closer to the key factors having role in the immune process of the gastric mucosa during H. pylori infection.

6 Summary of our new results

In these studies we obtained the following new results.

1. 100-800 ug capsaicin (between 3.2-26 uM concentrations) given intragastrically to healthy human subjects dose-dependently inhibits gastric basal acid secretion.

2. The ID50 on GAS is about 400 ug for capsaicin in human healthy subjects.

3. This inhibitory effect of capsaicin on GAS lasts for about 1 hour after intragastric application into the stomach of human healthy subjects.

4. 400 ug intragastric capsaicin (in 13 uM concentration) increases gastric emptying rate in healthy humans.

5. Blood glucagone level increase shows a faster answer to glucose absorption during the action of 400 ug intragastric capsaicin (in 13 uM concentration).

6. There is no constitutive mRNA expression of the proinflammatory cytokine TNF-alpha, IL1-alpha and -beta, and chemotactic cytokine RANTES, eotaxin, MCP-1, MIP1-alpha and -beta in the normal mouse gastric mucous surface epithelial cell line GSM06.

7. H. pylori or H. felis infection induces the mRNA expression of each cytokine.

8. E. coli does not have RANTES mRNA stimulating effect in these cells.

9. Recombinant human TNF-alpha and IFN-gamma also does not activate the cells to express RANTES mRNA.

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