Antibacterial peptide from *H. pylori*

Colonization of the human stomach by the bacterium *Helicobacter pylori* is a predisposing factor for gastrointestinal illnesses such as gastritis and peptic ulcers[1]. But most infections are asymptomatic, and it has recently been suggested that *H. pylori* may actually have beneficial effects on infected carriers who are heavily exposed to other gastrointestinal pathogens[2]. We find that *H. pylori* possesses antibacterial activity to which it is itself resistant. We have traced this activity to cecropin-like amino-terminal peptides derived from the ribosomal protein L1 (RpL1).

The antibacterial activity was detected when a crude lysate of *H. pylori* was tested in the growth-inhibition zone assay[3]. The activity is protease sensitive, suggesting that *H. pylori*, like some other bacteria[4], could produce one or more antimicrobial peptides[5]. A consensus motif (-(knd)-f-f-k-(kre)-(il)-e-(kr)-f-f-x-(hkrn)-(ivt)-(rkqn)-(dn)-) for 15 N-terminal residues from insect cecropins was used to search the SWISS-PROT database. Only one additional sequence was found: the N terminus of RpL1 from *H. pylori*. A consensus motif for defensins generated no positive hits in the database.

Cecropins are antibacterial peptides which are composed of two amphipathic α-helices joined by a hinge[6]. Unlike several RpL1 proteins from other bacteria, the RpL1 N terminus of *H. pylori* has the ability to form a perfect amphipathic helix. This first helix (residues 2–19) is followed by a second helix (residues 22–38).

Four peptides corresponding to the N-terminal part of *H. pylori* RpL1 were synthesized and tested for antibacterial activity. Two peptides, Hp(2–20) and Hp(22–38), were chosen to correspond to each of the anticipated α-helices. The other two, Hp(2–13) and Hp(2–38), correspond to a truncated first helix and to both helices,

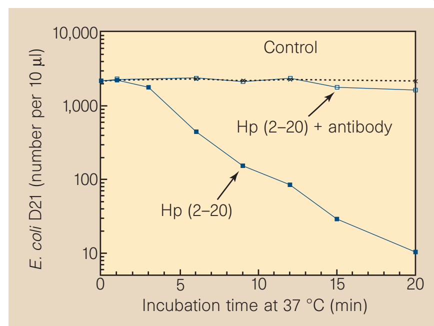

Figure 1 Time curve for the killing of *Escherichia coli* strain D21 by peptide Hp(2–20). Preincubation with affinity-purified antibodies against peptide Hp(2–20) blocked the killing effect. Incubation mixtures contained 2×10^4 bacteria and 0.4 µg peptide in 100 µl phosphate-buffered saline.

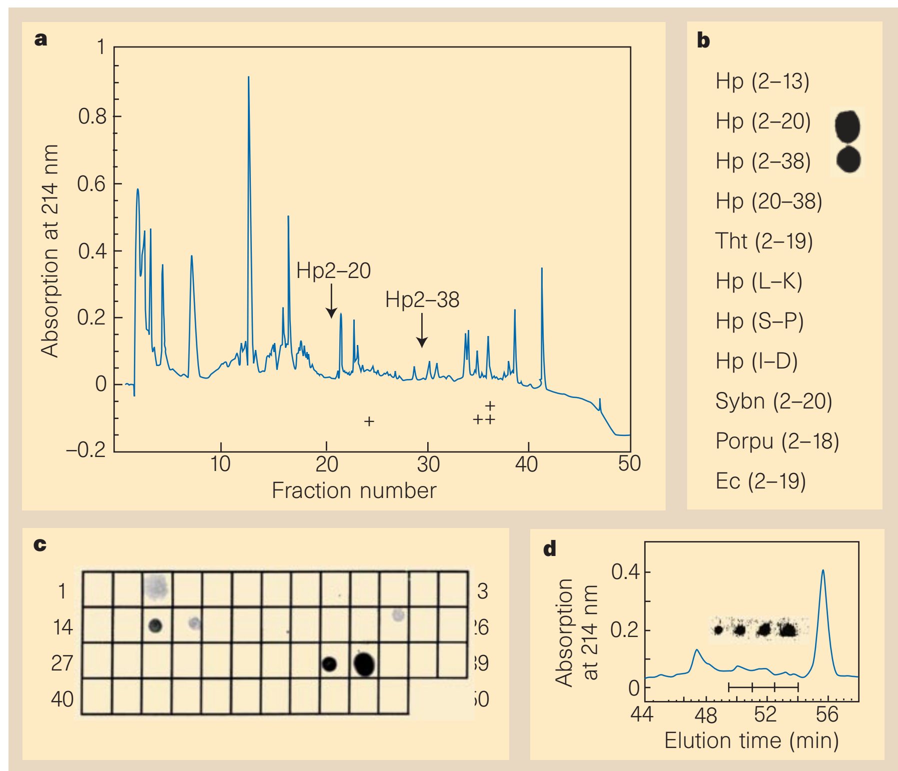

Figure 2 High-performance liquid chromatography separation of antibacterial components in an acetonitrile-extracted *H. pylori* strain MO19 lysate. **a,** Of 50 fractions collected (0.8 ml min^{-1}), concentrated and assayed, antibacterial activity (+) was found in three fractions (main activity in tube 36 (++)). Arrows indicate elution times of the synthetic peptides Hp(2–20) and Hp(2–38). **b,** Reactivity with anti-Hp(2–20) antibody using the dot-blot assay gave three strong and three weak signals. **c,** Specificity of antisera illustrated by a dot-blot assay (fraction numbers are shown). **d,** Magnified part of a rechromatogram showing the main antibacterial activity.

respectively. All peptides except Hp(20–38) exerted antibacterial activity against the Gram-negative *Escherichia coli* strain D21 and the Gram-positive *Bacillus megaterium* strain Bm11. The rate of killing of *E. coli* D21 with peptide Hp(2–20) was 99.5% in 20 minutes. Preincubation of the peptide with purified antibodies raised against the peptide blocked this activity (Fig. 1). The same antibody inhibited the main antibacterial activity of the crude *H. pylori* lysate.

H. pylori RpL1 peptides and cecropins A and P1 were inactive against all *H. pylori* strains tested. It has been suggested that cecropin A binds to the diphosphoryl lipid A moiety of lipopolysaccharide[7]. It may be the lower amount of phosphates in *H. pylori* lipid A[8] that makes it resistant to cecropin.

We extracted the antibacterial agent from the *H. pylori* lysate by using 0.1% trifluoroacetic acid in 60% acetonitrile (final concentration) and fractionated it using reversed-phase high-performance liquid chromatography. Fractions were assayed for antibacterial activity and for reactivity with an affinity-purified anti-Hp(2–20) antibody. Both the main antibacterial activity and the strongest dot–blot signal were obtained in fraction 36 (Fig. 2a). Replacing amino acids in Hp(2–20) abolished all signals (Fig. 2b), indicating that the anti-Hp(2–20) antibody is highly specific. The elution times for the synthetic peptides Hp(2–20) and Hp(2–38) are marked by arrows in Fig. 2a. From these results, we conclude that the main antibacterial agent (fraction 36) is mediated by an N-terminal fragment(s) of RpL1 that is probably larger than the synthetic peptide Hp(2–38). The weak dot–blot signals obtained in fractions 3, 16 and 17 may originate from shorter RpL1 peptides at concentrations too low to have detectable antibacterial activity.

We pooled the active fractions for rechromatography using a flatter gradient to improve the resolution. A magnified part of such a chromatogram with dot–blot signals detected in four fractions is shown in Fig. 2d. Fractions indicated by a horizontal bar were analysed by mass spectrometry, but no clear interpretation of the mass data could be obtained. Our data are compatible with the presence of several N-terminal fragments of RpL1 in the lysate.

H. pylori has been demonstrated to undergo 'altruistic lysis' *in vivo*[9]. We suggest

© 1999 Macmillan Magazines Ltd

that bacterial lysis in the stomach could release L1-derived cecropin-like peptides that are active against faster-growing microorganisms found there. Our data are also consistent with the idea that cecropins have evolved from an early *rpl1* gene in a prokaryote that passed from being an intracellular parasite to a symbiont, ending up as an organelle. When the *rpl1* gene moved from the organelle to the host nucleus, a duplicated sequence could have begun to evolve towards a specialized antimicrobial peptide.

Katrin Pütsep, Carl-Ivar Brändén, Hans G. Boman, Staffan Normark
Microbiology and Tumour Biology Centre, Karolinska Institutet, Box 280, SE-171 77 Stockholm, Sweden
e-mail: hans.g.boman@swipnet.se

1. Blaser, M. *Br. Med. J.* **316,** 1507–1510 (1998).
2. Mattson, A., Lönroth, H., Qiuding-Järbrink, M. & Svennerholm, A.-M. *J. Clin. Invest.* **102,** 51–56 (1998).
3. Hultmark, D. *et al. EMBO J.* **2,** 571–576 (1983).
4. Boman, H. G. *Scand. J. Immunol.* **48,** 15–25 (1998).
5. Sahl, H. G. & Bierbaum, G. *Annu. Rev. Microbiol.* **52,** 41–79 (1998).
6. Holak, T. A. *et al. Biochemistry* **27,** 7620–7629 (1988).
7. DeLucca, A. J., Jacks, T. J. & Brogden, K. A. *Mol. Cell Biochem.* **151,** 141–148 (1995).
8. Suda, Y. *et al. J. Biochem.* **121,** 1129–1133 (1997).
9. Phadnis, S. H. *et al. Infect. Immun.* **64,** 905–912 (1995).

Cleaner fish really do clean

The cleaning of client fish by cleaner fish is one of the most highly developed interspecific communication systems known. But even though it is a seemingly obvious mutualism[1,2], several quantitative studies[3–5] have failed to show any benefit for the clients, leading to the hypothesis that cleaner fish are 'behavioural parasites' that exploit the sensory system of the clients[6] to obtain food, rather than to increase the client's fitness. The cleaner fish *Labroides dimidiatus* eats parasitic gnathiid isopods, which decline in number on the client fish *Hemigymnus melapterus* daily between dawn and sunset[7,8]. I find that the cleaner fish reduces parasite abundance, resulting in a 4.5-fold difference within 12 hours, supporting the hypothesis that cleaning behaviour is mutualistic.

Various models have been proposed to explain cooperative interactions among unrelated individuals[9]. The Iterated Prisoner's Dilemma is the generally accepted model[10], although it has been criticized[2]. The behaviour of cleaner fish has been used to highlight the limitations of repeated games, and may be useful for developing alternative models, because if the client cheats by eating the cleaner, the game is over, so it is not a repeated game[2]. Understanding the benefits of cleaning (clients have fewer parasites) is essential for testing such models.

I compared the number of parasitic gnathiids on caged *H. melapterus* on reefs with and without cleaner fish. Measurements were taken after 12 days at sunset to examine the long-term effect of cleaner fish, and after 12 and 24 hours, at dawn and sunset, respectively, to determine whether the observed decline in gnathiid abundance[8] over the day was caused by cleaner fish.

Six cages, each containing three *H. melapterus*, were placed on each of five small coral reefs adjacent to Lizard Island, Great Barrier Reef (reefs 7, 8, 14, 15 and 16 on map in ref. 4), of which three reefs had all cleaner fish, *L. dimidiatus*, removed. Cages were put out at dawn on a different reef, randomly selected, each day, and prawns were fed to fish daily. Fish on reefs 7 and 8 were cleaned regularly after cages had been on the reefs for 9 and 8 days, respectively. In the first experiment, after 12 days, the fish were recovered at sunset and gnathiids counted. The effect of cleaner fish on gnathiid abundance was tested (Fig. 1).

In the 24-hour experiment, the above fish were returned to the holding tanks until the following sunset, when they were placed on the same reef; fish from the first experiment that were missing or dead were substituted with other fish from the laboratory. Half the cages on each reef were recovered the following dawn, and the rest were recovered the next sunset, and gnathiids were counted again. Gnathiid abundance was tested for the effects of cleaner fish and sampling time (Fig. 1). The effect of replacing the missing fish on gnathiid abundance was also tested and was not significant ($F_{1,52}=1.97$, $P=0.166$). Variability in gnathiid abundance between reefs from dawn to sunset, within a treatment, was found to change significantly (analysis of covariance, $F_{3,19}=4.10$, $P=0.021$), probably because there were high gnathiid loads at both dawn and sunset at reef 16, so I explored the effect of time on gnathiid abundance by reanalysing each time separately.

Cleaner fish had a clear effect on the abundance of parasites. After 12 days and at sunset, fish on reefs without cleaner fish had on average 3.8 times more gnathiids than fish on reefs with cleaners (Fig. 1a). In the 24-hour experiment, gnathiid abundance did not differ between treatments at dawn (Fig. 1b); in contrast, gnathiids on reefs without cleaner fish and sampled at sunset had 4.5 times more gnathiids than fish on reefs with cleaner fish (Fig. 1c).

Cleaner fish eat 1,200 parasites per day (mostly gnathiids) and feed only during the day[7], whereas gnathiids infest fish during both day and night[8]. The rapid reduction in gnathiid abundance between dawn and sunset indicates that the daily decline in gnathiids is probably due to cleaner fish. Similar quantitative studies, in which the cleaner fish were removed for periods from one month to two years, found no effect of cleaners on parasite abundance[3–5]. This may be due to the different client and cleaner fish species used, the presence of other unidentified cleaners, movement between reefs by clients, and spatial or temporal variation in parasite loads.

My results show that cleaner fish have an effect both within 12 days and within 12 hours. Cleaner fish are known to benefit from cleaning[7], but my data show that they greatly reduce the abundance of gnathiids, which can be deleterious to fish[11]. This is consistent with the hypothesis that cleaning behaviour is mutually beneficial to both participants, and paves the way to using

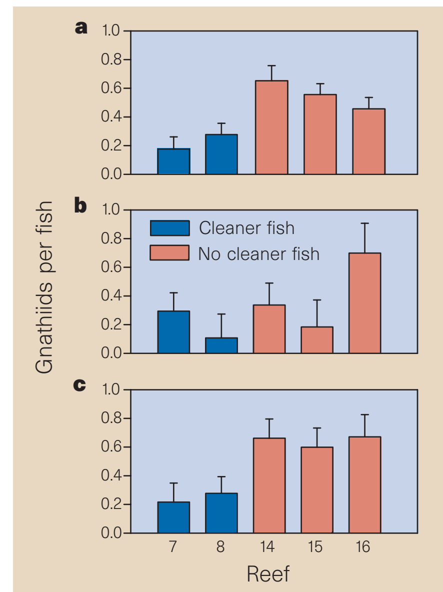

Figure 1 Gnathiids on caged fish on reefs with and without cleaner fish. Data are least-square means and standard errors for fish sampled at the following times: **a,** at sunset after 12 days ($F_{13}=17.64$, $P=0.0246$); **b,** at dawn after 12 hours ($F_{19}=1.80$, $P=0.213$; reef (treatment), $F_{3,9}=2.15$, $P=0.164$.); **c,** at sunset after 24 hours ($F_{13}=11.56$, $P=0.042$; reef (treatment), $F_{3,3}=0.11$, $P=0.950$). Variation in gnathiid abundance in **a** was tested with a nested analysis of covariance (ANCOVA) with presence of cleaners as the main effect and reefs nested within treatments; the error term testing for an effect of treatment was the type III mean square for reef (treatment). In the 24-hour experiment, a multifactor nested ANCOVA was used; the effect of using replaced fish was tested by including it as a factor; the error term used to test the main effects and interactions was the type III means square for cage (treatment × time × reef). Because there was a significant interaction of time × reef (treatment), separate ANCOVAs were used for each time with fish size as the covariate. Gnathiid abundance and fish size were $\log_{10}(x+1)$ and $\log_{10}(x)$ transformed, respectively. Least-square means were calculated to show gnathiid abundance while accounting for fish size.

© 1999 Macmillan Magazines Ltd