

Antimicrobials

Peptide antibiotics in mast cells of fish

Antimicrobial peptides are increasingly recognized as a critical first line of defence against many pathogens and have been isolated from epithelial tissues and blood cells of many vertebrates, as well as from prokaryotes, plants and invertebrates^{1,2}. Here we show that ‘piscidins’, a previously undiscovered family of peptide antibiotics isolated from fish, reside in mast cells, an immune cell of uncertain function that is present in all vertebrate classes^{3,4}. Until now, no peptide antibiotic has been isolated from the mast cells of any animal, and our discovery indicates that these cells may be critical in fighting many infectious diseases.

We isolated piscidins from the tissues of an important aquacultured fish, hybrid striped bass (*Morone saxatilis* × *M. chrysops*) using a previously described method⁵ which we modified to include a further purification step after weak cation-exchange chromatography using continuous acid-urea polyacrylamide-gel electrophoresis⁶. Piscidins are 22-amino-acid peptides with a highly conserved amino terminus that is rich in histidine and phenylalanine (Table 1). The peptides probably adopt an amphipathic α -helical conformation (Fig. 1a, b).

Piscidins are more haemolytic than magainin 2, a peptide antibiotic from the aquatic frog *Xenopus laevis*, but less haemolytic than mellitin, a peptide from bee venom that has relatively weak antibacterial activity (Fig. 1c). Piscidin 3 is the least haemolytic of the piscidins; its most significant structural distinction seems to be a glycine substituted for histidine at position 17, which would tend to disrupt the amphipathic α -helix⁷ and lower

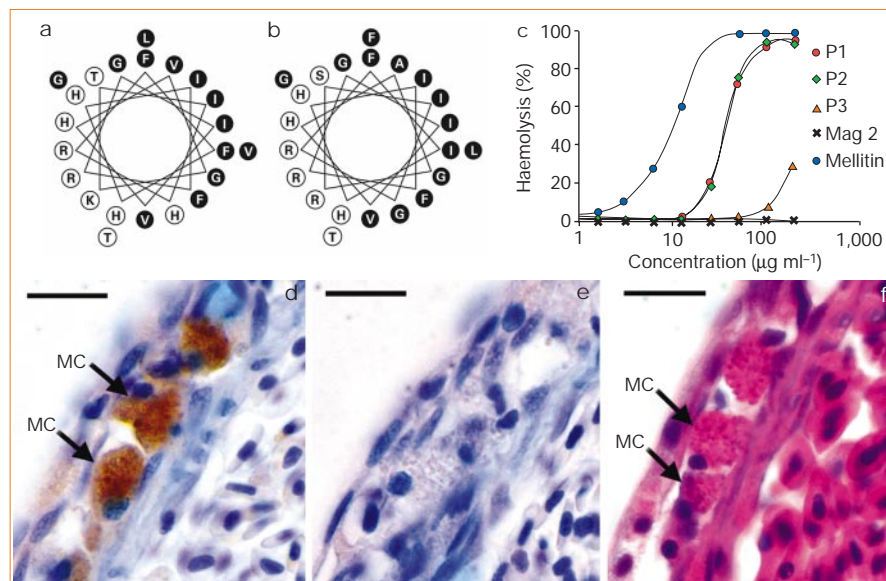


Figure 1 Characterization and localization of peptide antibiotics in hybrid striped bass. **a, b**, Helical wheels predicting amphipathic α -helical conformations for piscidins 1 (**a**) and 3 (**b**). Circles, hydrophilic residues; filled circles, hydrophobic residues. **c**, Haemolytic activity of piscidins 1–3 (P1, P2, P3), magainin 2 (Mag 2) and mellitin against human erythrocytes. **d**, Bass gill treated with anti-piscidin antibody. MC, mast cell. Affinity-purified anti-piscidin antibodies were prepared against a haemocyanin-conjugated peptide fragment (FFHHIFRGIVH)¹³. Deparaffinized tissue sections were blocked with goat serum, incubated with non-immune serum or specific antibody, and sequentially incubated with biotinylated goat anti-rabbit serum, streptavidin-conjugated peroxidase, and diaminobenzidine. **e**, Non-immune serum control of **d**. **f**, Serial section of **d**, showing mast cells with eosinophilic granules (haematoxylin and eosin stain). Scale bars: **d**, **e**, 25 μ m; **f**, 10 μ m.

its amphipathicity — this often correlates with reduced haemolytic activity⁸. Piscidins have potent, broad-spectrum antibacterial activity against fish pathogens, which is comparable to that of many peptides from aquatic animals⁹. This antibacterial activity seems to be correlated with haemolytic activity.

We immunolocalized piscidins to mast cells (Fig. 1d–f), the principal tissue granulocytes of vertebrates³; mast cells in gill, skin and gut, and those lining blood vessels in the viscera, were all positive. Pre-incubating anti-piscidin antibody with piscidin 1 for 30 min abrogated the signal, whereas

pre-incubation with diluent (phosphate-buffered saline) or an unrelated peptide (magainin 2) had no effect (results not shown). Not all mast cells were positive for piscidins, indicating that piscidin-negative mast cells may be at a different stage of development or may have differentiated independently of the piscidin-positive lineage. Phenotypic heterogeneity in mast cells has been reported in fish³ and mammals¹⁰.

Mast cells of members of the families Moronidae (*Morone*; Fig. 1d–f) and Sciaenidae (spot, *Leiostomus xanthurus*; croaker, *Micropogonias undulatus*; results not shown) are piscidin-positive. Both families are in the suborder Percoidei, indicating that piscidins may be evolutionarily conserved in this group, which is the largest suborder in the Perciformes, in turn the largest order of living vertebrates. The mast cells of fish species outside Percoidei are not immunohistochemically positive for piscidins.

Fish mast cells (also known as eosinophilic granule cells) are morphologically and functionally similar to their mammalian counterparts, but whether they are derived from the same lineage as mammalian mast cells is unclear. Peptide antibiotics may also be present in the mast cells of other vertebrates. Mast cells have been associated with defence against parasites for some time^{3,10}, and recent evidence suggests that they have an important role in fighting bacterial infection¹¹. However, their primary function has been thought to be in orchestrating the activation of other effector cells, such as neutrophils, to kill pathogens¹². Our results indicate that mast cells may also

Table 1 Antibacterial activity of piscidins

Bacteria	Minimum inhibitory concentration		Minimum bactericidal concentration	
	Piscidin 1	Piscidin 3	Piscidin 1	Piscidin 3
Fish pathogens				
<i>Streptococcus iniae</i> (+)	3.1	3.1	3.1	3.1
<i>Lactococcus garviae</i> * (+)	3.1	6.3	3.1	12.5
<i>L. garviae</i> † (+)	3.1	3.1	3.1	6.3
<i>Aeromonas salmonicida</i> (–)	3.1	12.5	3.1	25.0
<i>Aeromonas hydrophila</i> (–)	0.8	1.6	0.8	1.6
<i>Vibrio alginolyticus</i> (–)	3.1	6.3	3.1	6.3
Human pathogens				
<i>Staphylococcus aureus</i> (+)	3.1	3.1	3.1	3.1
<i>Streptococcus faecalis</i> (+)	3.1	12.5	3.1	12.5
<i>Escherichia coli</i> (–)	3.1	3.1	3.1	3.1
<i>Klebsiella pneumoniae</i> (–)	3.1	6.3	3.1	6.3
<i>Shigella flexneri</i> (–)	3.1	6.3	3.1	6.3
<i>Pseudomonas aeruginosa</i> (–)	12.5	25.0	12.5	25.0

Antibacterial activity of piscidin 1 (amino-acid sequence FFHHIFRGIVHVGKTHRLVITG) and piscidin 3 (FFHHIFRGIVHAGRSGRFLIG); conserved residues are underlined. Piscidin 2 (P2) is the same as piscidin 1, except that K is substituted for R at position 18. Mass spectrometry shows that natural piscidins sometimes have an amidated carboxy terminus. Minimum inhibitory concentration and minimum bactericidal concentration were determined using the modified Hancock method for measuring antibacterial activity of peptide antibiotics (see <http://www.cmdr.ubc.ca/bobh/MIC.htm>). Details of the methods are available at http://www.cvm.ncsu.edu/cbs/noga_ed.htm

*Resistant to oxytetracycline, kanamycin, benzylpenicillin, florfenicol, erythromycin, enrofloxacin and novobiocin.

†Resistant to oxytetracycline, erythromycin, lincomycin and doxycycline (four isolates).

participate directly in killing microbes.
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Pathogenesis

HLA-DQ7 antigen and resistance to variant CJD

Variant Creutzfeldt–Jakob disease (vCJD) in humans is caused by a bovine spongiform encephalopathy (BSE)-like prion strain, and so far about 100 of the many people exposed to BSE prions have developed the condition. Here we show that there is a significantly reduced frequency of the human leukocyte antigen (HLA) class-II type DQ7 in patients with vCJD, but not in those with classical CJD. This apparently protective genetic factor should aid differential diagnosis and may have important implications for understanding host susceptibility to infection by BSE prions, and the distinctive pathogenesis of vCJD, as well as in the identification of targets for prevention and therapy of vCJD.

Prion diseases are characterized by deposition of an abnormal conformation of host-encoded prion protein, PrP^{Sc}. Human prion diseases exist in inherited, sporadic and acquired forms. The idea that vCJD, which was identified in 1996, arose as a result of dietary or other exposure to the BSE agent is supported by molecular and biological strain-typing studies — it is possible that many individuals have been infected^{1–4}. The pathogenesis of vCJD is distinct from that of other forms of CJD, with a marked accumulation of PrP^{Sc} in the lymphoreticular system^{5,6}. This distinctive feature may relate to the route of infection, to host genetic factors, or to a prion-strain-specific effect⁴.

Table 1 HLA-DQ typing of CJD patients and controls

DQ type	Frequency in vCJD (50 patients)	Frequency in sporadic CJD (26 patients)	Consecutive cadaveric controls (n=197)
DQB1*02 (DQ2)	42% (21)	30.8% (8)	40.6% (80)
DQB1*04 (DQ4)	4% (2)	3.8% (1)	2.5% (5)
DQB1*05 (DQ5)	32% (16)	30.8% (8)	26.9% (53)
DQB1*06 (DQ6)	58% (29)	38.5% (10)	41.6% (82)
DQB1*0301/4/9 (DQ7)	12% (6)†	46.2% (12)	35.5% (70)
DQB1*0302/5/7/8 (DQ8)	18% (9)	15.4% (4)	19.8% (39)
DQB1*0303/6 (DQ9)	18% (9)	15.4% (4)	12.7% (25)

†P=0.001; uncorrected from a two-sided Fisher's exact test.

The lymphoreticular system is also involved in sheep scrapie and in murine models of scrapie in which follicular dendritic cells accumulate PrP^{Sc} early in the incubation period, well before the onset of neurological signs. Prion replication in the lymphoreticular system may be a prerequisite for neuro-invasion after peripheral inoculation with low doses of infective agent⁷.

The aetiology of sporadic CJD, currently the main differential diagnosis of vCJD, remains unclear, but its random worldwide distribution and lack of clustering or association with local scrapie prevalence argues against an acquired aetiology in the majority of patients. Somatic mutation of the prion-protein gene (*PRNP*), or spontaneous formation of PrP^{Sc}, are thought to be more likely causes. A common *PRNP* polymorphism, in which either methionine or valine is encoded at codon 129, is a key determinant of genetic susceptibility to sporadic and acquired prion diseases, with heterozygosity being highly protective^{8,9}. All victims of vCJD studied so far have been methionine homozygotes at this locus⁴; about 38% of the UK population has this genotype.

In view of the distinctive pathogenesis and well-known associations between HLA and susceptibility to other infectious diseases, we compared the HLA type of vCJD patients with normal controls and with those with sporadic CJD. We carried out an initial study on 32 vCJD and 12 sporadic CJD DNA samples. Tissue samples were obtained at autopsy with the consent of relatives. These samples were HLA-typed for HLA-A, B, C, DRB and DQB1 at low-to-medium resolution using a sequence-specific-primer polymerase chain reaction (PCR-SSP) method¹⁰. All patients included were British caucasoids. HLA frequencies from a panel of 197 consecutive cadaveric British caucasoid organ donors were used as control values.

The only statistically significant deviation from control frequencies was seen at the *DQB1* locus — only 2 out of the 32 vCJD patients were positive for *DQB1**0301/4/9 (DQ7), compared with 70 out of 197 controls (35.5%), giving an uncorrected P value of 0.0004 (two-sided Fisher's exact test). In the case of sporadic CJD, 50% of patients were DQ7 (6 of 12), which was not significantly different from the controls (P=0.36).

We then typed a further 18 vCJD and 14

sporadic CJD patients for only HLA-DQB1, using a commercial PCR-SSP kit (Reli; Dynal, UK). Table 1 shows the frequency of HLA-DQ types in 50 vCJD patients, 26 sporadic CJD patients and 197 controls. HLA-DQ types were assigned by considering the DQB1 alleles of each subject. HLA-DQ7 remained significantly under-represented in vCJD patients (Table 1). HLA-DQ frequencies in the sporadic CJD group were comparable to those of controls.

Our results suggest that the presence of DQ7 protects against vCJD, with a relative risk factor for individuals negative for DQ7 of 3.3. DQ7 typing of suspected vCJD patients, in conjunction with *PRNP* genotyping, could prove to be useful in diagnosis. Although we have typed around 50% of all reported vCJD patients, our sample size is necessarily small. As with a more prevalent disease, these data should be confirmed on a much larger group of patients, although this is not possible for vCJD at present.

A firm tissue-based diagnosis of vCJD can be achieved by tonsil biopsy⁶, but blood-based diagnostics would be preferable. Although no such test has yet been reported, combinations of blood-based markers may have strong predictive value, with DQB typing being useful for differential diagnosis and identification of high-risk groups.

The molecular basis of the protective association of DQ7 and vCJD is unknown. Class II molecules of the major histocompatibility complex (MHC) may have a direct role in disease pathogenesis, or a gene linked to the *DQB* locus may be involved. MHC class II could help in the fortuitous carriage of PrP^{Sc} around the body, or from the gut into the lymphoreticular system — with the DQ7 molecule being less efficient in this role. Alternatively, HLA-DQ7 molecules may be more effective at presenting a putative pathogenic peptide and thereby initiating an immune response. Further investigation should reveal the role of MHC class II molecules in disease pathogenesis and aid the development of secondary prophylaxis or therapeutic intervention.

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differential gametic imprinting, as well as on the amount of gene product needed for biological function.

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Vielle-Calzada et al. reply — Our results, based on a study of 20 loci, indicate that the contributions by the maternal and paternal genome to early seed development in *Arabidopsis* are not equivalent, as evidenced by a lack of detectable paternal gene activity during the first few divisions after fertilization. As these loci are distributed throughout the genome, we inferred that early embryo and endosperm development are mainly under maternal control, but this may not be true for every locus and, as in X-chromosome inactivation¹, we would expect some loci to escape this silencing mechanism. We did not claim that maternal control is complete, but suggested that the activity of many genes during early embryo and endosperm formation could depend solely on transcription of the maternally inherited allele before and/or after fertilization.

Previously, early seed formation was thought to involve transcription from both parental copies immediately following fertilization, and maternal effects were considered rare or non-existent². The time at which paternal activity can first be detected, however, is likely to vary from embryo to embryo and from gene to gene in different nuclei, as in *Drosophila*³. Weijers *et al.* report paternal expression of *AtRPS5A::GUS* as early as the two-cell stage, confirming that transcription in the zygote is not the rule for paternally inherited alleles, whereas transcription from maternal alleles has been demonstrated immediately after fertilization of the central cell⁴. We do not know what percentage of embryos show early *AtRPS5A::GUS* expression, nor the relative paternal and maternal activity, but there may also be less pronounced parent-of-origin differences.

New evidence supporting the non-equivalence of maternal and paternal genomes during early seed development is based on experiments with reporter genes^{5–8} and genetic assays revealing maternal effects of genes thought to act purely zygotically⁹ (S. Gilmore and C. Somerville, personal communication; J. Moore and U. G., unpublished results). Whether and at what stage expression of the paternal allele is sufficient for normal development will depend on the level of activity required for gene function. In a two-component transactivation system, no paternal activity was found during early seed development using *pOp::GUS* reporter lines with several activator lines⁸. Some early defects were evident with a *pOp::BARNASE* reporter, however, suggesting that paternal transcription is very low but is sufficient to cause *BARNASE*-induced defects in some embryos⁸. These results confirm the non-equivalence of maternal and paternal contributions to early seed development. Like imprinted genes in mammals, this difference is probably not absolute and may be due to different levels of maternal and paternal transcripts.

Our titration experiments indicated a difference in transcript levels of at least 80-fold for genes we tested by PCR. Weijers *et al.* report an expression difference in reciprocal crosses with *UAS::GUS* at the heart to torpedo stage (Fig. 1d), when we showed that both parental alleles are active at other loci we tested; indeed, this differential expression translates into an absence of detectable paternal activity at earlier stages using the *pOp::GUS* reporter system⁸. For some genes, such as *KEULE* or *KNOLLE*, low paternal expression may be sufficient for normal development, although very early defects (such as developmental delay) that are rescued by a paternal wild-type allele may be difficult to detect by scoring multinucleate

corrections

Night-time predation by Steller sea lions

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Nature **411**, 1013 (2001)

We stated that our acoustic surveys in Prince William Sound since 1993 and infrared surveys since 2000 suggested that these sea lions “feed exclusively” on herring. However, it has been drawn to our attention that this statement is misleading. In clarification, the sea lions were selectively targeting the relatively shallow (0–50-m depth) schools of Pacific herring (*Clupea pallasii*) at night as a source of winter forage to the exclusion of relatively larger and deeper (150–250 m) concentrations of walleye pollock.

Transatlantic robot-assisted telesurgery

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Nature **413**, 379–380 (2001)

embryos. Moreover, rescue of an early embryonic phenotype by a paternal wild-type allele provides no evidence against differences in parental transcript levels.

Although the exact time of paternal activation was not central to our report, most evidence so far suggests that no consistent paternal gene activity can be detected in the embryo or endosperm for several cell divisions. The results of Weijers *et al.* do not contradict our findings, but instead represent possible exceptions to a general rule. Specific genes that are important during early development (for example, those involved in cytokinesis that are distinctly regulated in the female gametophyte and the zygote⁹) may be under selection for earlier expression and be specifically activated early in development. Further investigation is required into how common early-expressing paternal genes are, and how maternal and paternal expression differs quantitatively.

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Peptide antibiotics in mast cells of fish

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Nature **414**, 268–269 (2001)

The concentrations listed in Table 1 are in $\mu\text{g ml}^{-1}$.

erratum

Nitrate flux in the Mississippi River

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Nature **414**, 166–167 (2001).

In Fig. 1 of this communication, the line referred to as “black” is in fact blue; also, in the fourth line of the third column, *P* should be greater than 0.05.