Hydralysin, a Novel Animal Group-Selective Paralytic and Cytolytic Protein from a Noncnidocystic Origin in Hydra[†,‡]

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ABSTRACT: In Cnidaria, the production of neurotoxic polypeptides is attributed to the ectodermal stinging cells (cnidocytes), which are discharged for offensive (prey capture) and/or defensive purposes. In this study, a new paralysis-inducing (neurotoxic) protein from the green hydra *Chlorohydra viridissima* was purified, cloned, and expressed. This paralytic protein is unique in that it (1) is derived from a noncnidocystic origin, (2) reveals a clear animal group-selective toxicity, (3) possesses an uncommon primary structure, remindful of pore-forming toxins, and (4) has a fast cytotoxic effect on insect cells but not on the tested mammalian cells. The possible biological role of such a noncnidocystic toxin is discussed.

The Cnidaria (Coelenterata) comprise a worldwide phylum of aquatic radially symmetrical venomous predatory organisms (approximately 9000 species), representing one of the most primitive levels of multicellular organization. The Cnidaria are composed of two epithelial layers (ectoderm and endoderm), with a body plan consisting of a sac surrounding a gastrovascular cavity (coelenteron) with an oral opening, crowned by hunting tentacles.

Cnidarian somatic cells are subdivided into a majority of multifunctional cells and a limited number of highly specialized cell types, such as the ectodermal cnidocytes (stinging cells). Cnidocytes (nematocytes) represent one of the most elaborate mechanical and morphological structures in nature (*1*), and function as autonomous weapon systems capable of identifying, targeting, and delivering toxic chemistry toward prey and/or opponent organisms. This delivery is performed by a subcellular organelle, the dischargeable cnidocyst (*1*, *2*).

Like other animal venom systems (*3*), the toxic chemistry of cnidarians is mainly based on a wide array of polypeptides. These can be divided into three main categories: (1) high-molecular mass (5−80 kDa) cytolytic pore-forming substances (*4*), some of which possess hemolytic, cardiotoxic, and dermatonecrotic activities (*5*, *6*); (2) toxic phospholipases (*7*); and (3) neurotoxic polypeptides, which are modifiers of ion conductance, usually acting on the sodium (*8*) or potassium voltage-gated channels (*9*, *10*).

Because of their paralytic action, the storage and delivery sites of cnidarian toxins are axiomatically attributed to cnidocysts (*8*). However, Endean and Noble (*11*) have demonstrated that extracts of cnidocyst-deprived tentacles of *Chironex* medusae were lethal to mammals but differed in their cytolytic, neuronal, and muscular effects from the cnidocyst-derived venom. The above observations have demonstrated the occurrence of a cnidocyst-independent toxicity in a cnidarian.

With this background, this study is dedicated to a new and unique kind of cnidarian paralytic polypeptide, which is derived from noncnidocystic tissue, possesses an uncommon primary structure, and has a selective cytolytic activity.

MATERIALS AND METHODS

Biotechniques

Test Animals. Green hydra (*Chlorohydra viridissima*) were bred in glass trays in "M" medium as described previously (*12*) at 20 °C and fed newly hatched larvae of *Artemia salina*. For toxicity assays, we employed laboratory-bred blowfly larvae (*Sarcophaga faculata*), field-collected isopods (terrestrial crustaceans, *Hemilepistus* sp.), albino mice (Sabra strain, Hadassah Medical School, Jerusalem, Israel), and fish (*Gambusia affinis*).

Tissue Extracts. Two kinds of toxic extracts derived from hydra tissues were employed: body extract (BE) and cnidocyst extract (CE). The body extract was obtained by a mild manual homogenization (five to seven slow strokes in a Teflon glass mortar) of intact hydrae in distilled water, followed by centrifugation for 30 min at 14000*g*. The pellet was discarded, and the supernatant was designated as the BE. The CE was obtained by vigorous manual homogenization of cnidocysts isolated by fractionating centrifugation on a Percoll (Sigma) gradient according to the methods of ref *13*.

Activity Assays. Toxicity was tested by injecting the compound into fly larvae, isopods, and mice as described previously (*14*). Fish were injected laterally at the base of

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the tail. The paralytic dose (PD_{50})[1] was determined from sampling and calculation according to the method of Reed and Muench (*15*) 30 min following injection. Paralysis was defined as any locomotory disturbance which prevents the animal from moving and changing its location. Hemolysis and proteolytic activity were assayed according to ref *16* and refs *17* and *18*, respectively.

Protein Chemistry

Column Chromatography. Hydralysin was purified by two successive steps of anion exchange chromatography. The first separation was carried out on a HiTrap Q column (Pharmacia), equilibrated with 20 mM Tris-HCl (pH 8.8) and eluted with a gradient of molarity of NaCl (0.5 M) in the same buffer in an HPLC system (Spectra-Physics). The active fraction was concentrated by ultrafiltration (Amicon) and charged on a Resource-Q ion exchange column (Pharmacia) equilibrated and eluted under identical conditions.

Electrophoresis. Analytical isoelectric focusing (IEF) (*19*) was performed on the 2117 Multiphor II electrophoresis system (Pharmacia-LKB). SDS−PAGE and blotting were performed on a mini-PROTEAN 2 system (Bio-Rad), and the immunoblots were detected with the aid of a BM-Chemiluminescence Western blotting kit (Boeringer-Mannheim).

Amino Acid Sequence. One hundred micrograms of purified toxin was blotted onto a polyvinylidine diflouride (PVDF) membrane after electrophoretical identification by SDS−PAGE. The PVDF membrane was eluted in 1 mL of 1 mM NaOH and 20% acetonitrile and sequenced by automated Edman degradation with an Applied Biosystems 457A gas-phase protein sequencing system at the Blatterman Laboratory for Macromolecules, Faculty of Medicine, Hebrew University.

Protein Determination. Proteins were quantified by the modified Bradford Coomassie assay (*20*) standardized by BSA.

Cloning and Sequence Analysis. Hydralysin was cloned by RT-PCR using degenerate primers based on the N-terminal amino acid sequence, with the aid of SMART technology (Clontech). Briefly, total RNA was extracted from hydra (*21*), and reverse transcribed using the SMART CDS primer and Powerscript RT (Clontech). PCR was performed using the degenerate upper primer TTY CAN AGR CAR TAY GG (based on the FTRQYG amino acid sequence) and the lower SMART PCR primer. The following PCR program was used: 2 min at 95 °C, followed by 32 rounds at 95 °C for 30 s, 45 °C for 45 s, and 72 °C for 1 min, and ending with a 7 min extension at 72 °C, in a Crocodile II thermal cycler (Appligene). The PCR product was reamplified, ligated into a pGEM-T-easy vector (Promega), and sequenced at the Genome Center, Hebrew University. 5′-RACE was performed by PCR using the specific lower primer GGT GAC TGG AAC CTC GAA GA and the SMART PCR primer as an upper primer under the same conditions except that the annealing temperature that was used was 50 °C. Sequence analysis was performed using the GCG package (University of Wisconsin, Madison, WI). Database searches were performed using BLAST (*22*) and PSI-BLAST (*23*). Secondary structure was predicted using PSI-PRED at the University College of London (London, England) (http://bioinf.cs.ucl.ac.uk/psipred) and Predict-Protein at Columbia University (New York , NY) [http://cubic.bioc.columbia,edu/predictprotein (*24*)].

Expression. PCR was performed with Bio-X-Act Taq (Bioline), using the primers AGT GGT AAA GAG CTT CTA ACC TTT AGT G and TTA CAA AGG CTC ACT TGG TCC GA with the addition of recombination sites (Gateway-Invitrogen). The PCR product was recombined into the pEntry vector, followed by recombination into pDEST 14, which was used for expression in BL-21(DE3)-SI bacteria (Invitrogen).

Antibody Preparation. Rabbits were immunized according to the method described in ref *25*, by two intramuscular injections of 100 μg portions of the toxin with TiterMax (CytRx) adjuvant (*26*). Purification of the antibody was achieved by Western blotting according to the method of Lotan *et al.* (*7*).

Cytolytic Activity Tests. Insect SF-9 and human HEK293 cells were maintained as described previously [http://www.invitrogen.com/content/sfs/manuals/3910.pdf (*27*)]. Cells were used 1 day after being seeded onto 24-well plates, at approximately 100 and 80% confluence, respectively. The toxin [or Pardaxin (Alomone)] in 300 μL of a 1:1 mixture of the cell growth medium and PBS was added to the cells, which were incubated at the appropriate temperature. After 20 min, 300 μL of 4% trypan blue (Sigma) was added to the cells, followed by an additional 10 min incubation. The cells were washed with PBS, detached by gentle pipetation, and counted on a hemacytometer (Clay Adams). For microscopic observation, cells were grown to approximately 50% confluence in wells containing microscope cover glasses (Knittel Gläser) pretreated with sulfochromic acid. The cells were treated with either hydralysin or control as described, fixed in 4% paraformaldehyde, and viewed with an Axiovert S-100 inverted microscope (Zeiss) fitted with a DVC 1300 camera (DVC Co.).

RESULTS

The BE and CE Differ in Their Biological Activity. As shown in Figure 1A, a mild homogenization of hydra tissues yields a suspension of cellular fragments, symbiotic algae, and intact undischarged cnidocysts. The clear supernatant of this suspension, when injected into the test animals, induced obvious paralysis in the arthropods. The homogenate obtained by intensive homogenization of hydrae (60 manual strokes with a glass−Teflon homogenizer) revealed that 45 $\pm$ 2.2% of the cnidocysts were discharged ($n = 6$ microscope fields), and its supernatant yielded a PD_{50} value to blowfly larvae of 0.92 μg of protein/100 mg of body weight. On the other hand, mild homogenization (two strokes) yielded 19.6 $\pm$ 5.6% discharged cnidocysts ($n = 6$) with a PD_{50} value of 1.33 μg/100 mg of body weight. These data reveal that the body extract is contaminated by cnidocyst content, but its toxicity is not proportional or related to the cnidocyst discharge. This raised the possibility that the paralytic toxicity of the body extract is not derived from the cnidocysts.

To test the above hypothesis, the BE was compared with the CE, which was obtained from washed isolated cnidocysts

[1] Abbreviations: PBS, phosphate-buffered saline; BSA, bovine serum albumin; RT-PCR, reverse transcription polymerase chain reaction; PD_{50}, dose at which 50% of the test animals were paralyzed.

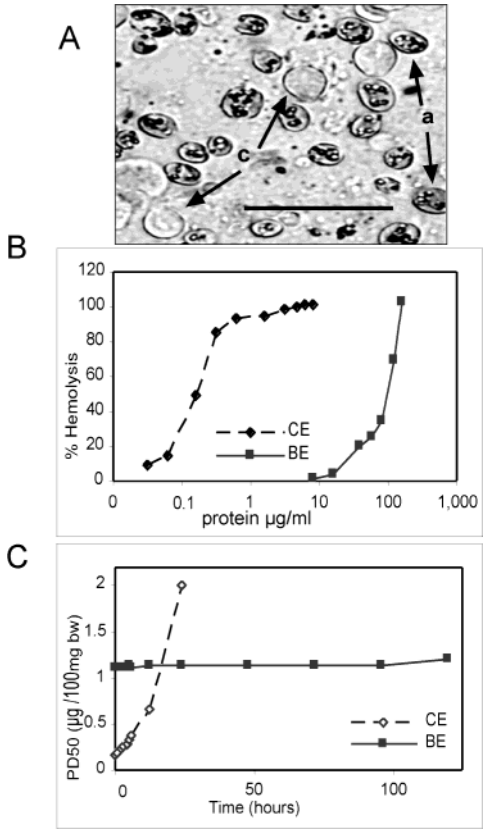

FIGURE 1: Noncnidocystic toxicity in hydra. (A) Microscopic examination of homogenized hydra revealing cellular debris, symbiotic algae (a), and undischarged cnidocysts (c). Bar = 20 μm. (B) Hemolytic activity of the CE and the BE, revealing that the BE is ~2−3 orders of magnitude less hemolytic than the CE. (C) Stability of the CE and BE. The extracts were incubated at room temperature, and the PD_{50} to fly larvae is noted.

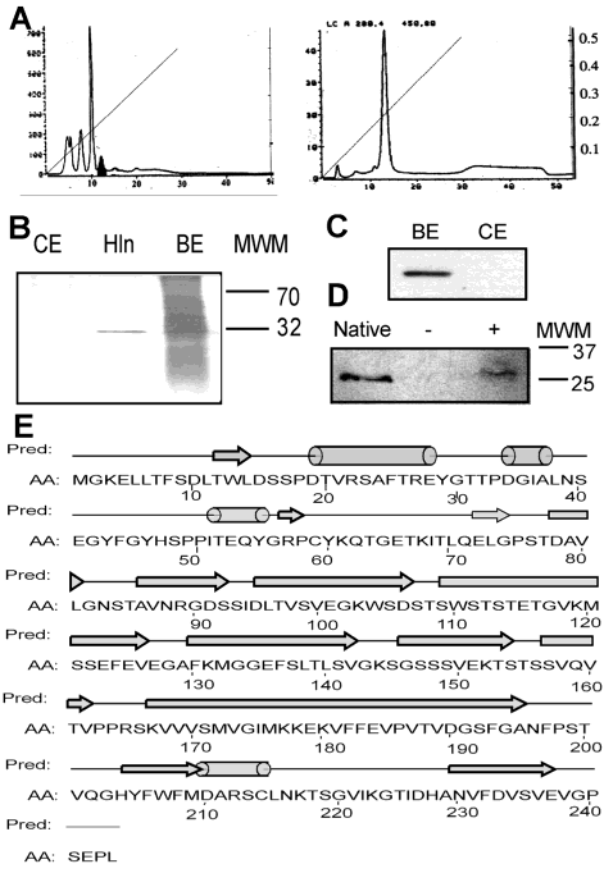

FIGURE 2: Purification and characterization of hydralysin. (A) Two successive anion exchange chromatograms resulting in the pure toxin. The first separation was carried out on a HiTrap Q column (Pharmacia, Uppsala, Sweden) which has been equilibrated with 20 mM Tris-HCl (pH 8.8) and eluted with a gradient of molarity of NaCl (0.5 M) in the same buffer in an HPLC system (Spectra-Physics). The active fraction (darkened) was concentrated by "Centricon" ultrafiltration (Amicon, Beverly, MA) and charged on a Resource-Q ion exchange column (Pharmacia) which had been equilibrated and eluted under identical conditions. (B) SDS−PAGE of the crude body extract (BE), cnidocyst extract (CE), and purified toxin (Hln). MWM is the molecular mass marker, and the lines and numbers represent the molecular mass marker that was used. The gel was silver-stained. (C) Western blot of the hydra BE and CE, using antibodies raised against hydralysin. (D) Western blot of natural and recombinant hydralysin: Native, native toxin purified from hydra; + and −, lysates of *E. coli* with and without the plasmid encoding the cloned hydralysin, respectively. The lines and numbers represent the molecular mass marker that was used. The apparent molecular masses are approximately 27 kDa for the native protein and 31 kDa for the recombinant one. (E) Deduced amino acid sequence of hydralysin, and its secondary structure as predicted by PSI-PRED. AA denotes the amino acid sequence. Pred denotes the predicted secondary structure. Arrows mark β-strands, and barrels mark α-helices.

and is therefore devoid of body contents. Several differences between the two were revealed. (a) The BE has a 7-fold lower toxicity to arthropods than CE (PD_{50} = 0.752 and 0.108 μg of protein/100 mg of body weight, respectively, to blowfly larvae). (b) The BE has a much lower (by ~3 orders of magnitude) hemolytic activity (Figure 1B). (c) The BE is highly stable at room temperature, whereas CE is unstable and rapidly loses its paralytic toxicity under the same conditions (Figure 1C). (d) The electrophoretic patterns of the BE and CE (Figure 2B), as well as the elution pattern and the location of the active peak in column chromatograms (data not shown), were different. Therefore, the toxicity found in the body extract cannot be attributed to cnidocyst contents.

Sarcophaga blowfly larvae exhibit various locomotory disturbances in response to envenomization, expressed as either contractions or extensions of their flexible integument and segmented body musculature (*14*). In the past, these locomotory disturbances were used to distinguish between three separate categories of neurotoxic−paralytic polypeptides derived from scorpion venom (*28*). The *Sarcophaga* larvae responded to the injection of hydra body extract and its purified fraction by an immediate fast contraction within a few seconds of the injection, followed by a flaccid, extended body paralysis reaction.

A Novel Neurotoxic Polypeptide, Hydralysin, Is Responsible for the Body-Derived Toxicity. The toxic factor was isolated by two steps of anion exchange chromatography (Figure 2A) monitored by the blowfly paralysis assay, resulting in 70−80% recovery of the paralytic activity (Table 1). The toxicity of the body extract and the isolated fractions was lost after incubation with the proteolytic enzyme proteinase K or Pronase (5% E/S, 37 °C, 60 min), indicating the active component is a polypeptide. The purified component is a 27 kDa protein (Figure 2B) with an isoelectric point of ~7.2, which we named hydralysin. Western blotting using antibodies raised against hydralysin revealed its presence in the BE but not in the CE (Figure 2C), providing further proof of the noncnidocystic origin of the toxin. Approximately 100 μg of the purified toxin was obtained

Table 1: Recovery of the Body-Derived Toxicity

	Sarcophaga fly larvae			isopods			mice
	PD_{50}[a]	SA[b]	recovery (%)	PD_{50}[a]	SA[b]	recovery (%)	PD_{50}
body extract	0.752 ± 0.27[c]	587	100	0.407 ± 0.23[c]	1761	100	7.27
first separation	0.156	6745	87.5	0.057	24023	95	>0.236
pure toxin	0.036 ± 0.011[c]	27636	80	0.15 ± 0.003[c]	59090	72	

[a] The units of PD_{50} are micrograms per 100 mg of body weight. [b] SA is the specific activity (number of PD_{50}s per 100 μg of protein). [c] Standard deviation given ($n = 5$).

from 2000 hydra polyps, corresponding to 1.8 g of wet weight, of which 1−2% (18−36 mg) is dry weight.

Animal Group-Selective Toxicity. As described above, the crude hydra extract and the purified toxin exhibited a rapid paralytic effect to arthropods. In comparison, the effect of both the crude extract and the purified fraction on mice was much weaker, the PD_{50} of the crude extract being lower by ~1 order of magnitude (Table 1). Similar results, with an even more marked difference in toxicity, were obtained in a separate set of experiments with fish ($PD_{50} = 1.72 \pm 0.043$ and 128 ± 15 μg/100 mg of body weight to blowfly larvae and *G. affinis* fish, respectively).

Cloning and Expression. The N′-terminal amino acid sequence of hydralysin was sequenced by Edman degradation, and the full coding sequence of hydralysin was cloned using an RT-PCR approach with degenerate primers based on the amino acid sequence (GenBank entry AY178276). The deduced amino acid sequence of hydralysin is presented in Figure 2E. The cloned toxin was recombinantly expressed in *Escherichia coli*, and purified by ion exchange chromatography followed by gel filtration. Western blotting (Figure 2D) reveals that the recombinant toxin migrates more slowly on SDS−PAGE gels, raising the possibility that the native toxin undergoes post-translational modification. Nevertheless, the effects of the recombinant toxin mimic those of the native toxin, revealing similar toxicity ($PD_{50} = 36$ and 22 ng/100 mg of body weight for the native and recombinant toxins, respectively) and low hemolytic activity (50% hemolysis at 93 μg/mL). In addition, the recombinant toxin, like the native one, was highly stable, and no loss of activity was perceived after 96 h at room temperature. *In silico* structural modeling suggests that hydralysin is a globular protein comprised mainly (more than 50%) of β-strands (Figure 2E). A search of the current databases reveals no significant homologues either at the level of the DNA or at the protein level. The protein does not include a conserved nine-amino acid propeptide found in cnidarian nematocyst toxins and collagens (*29*). However, a position specific iterative search using PSI-BLAST (*23*) reveals several proteins putatively homologous to hydralysin, among them several toxins (Figure 3). Common to these toxins is the creation of pores (usually cation-selective) in the target membranes (*30*, *31*), as well as a secondary structure rich in β-sheets (*32*, *33*).

Selective Cytolytic Activity of Hydralysin. In view of the putative similarity to pore-forming toxins, several assays were used which test for loss of membrane integrity. As noted previously (Figure 1B and text above), both native and recombinant Hydralysin have very weak hemolytic activity. However, a nanomolar concentration of recombinant hydralysin had a dose-dependent cytotoxic effect on insect sf9 cells, as measured by trypan blue staining (Figure 4A,B,E). When compared to pardaxin, a well-characterized

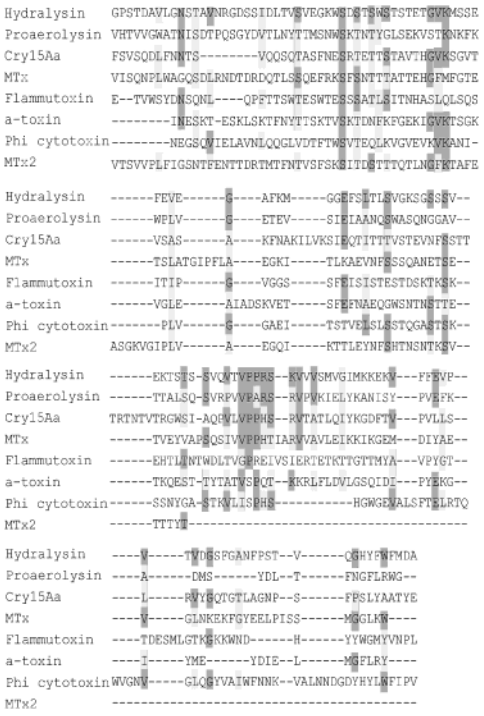

FIGURE 3: Hydralysin resembles pore-forming toxins. Sequences are aligned according to the PSI-BLAST search, using Jalview at EBI. Shades of gray represent amino acid types. Proaerolysin is from *Aeromonas trota* (GenBank entry 3283358) and Cry15Aa from *Bacillus thuringiensis* (GenBank entry 8928022). MTx and MTx2 are from *Bacillus sphaericus* (GenBank entries 1302632 and 1378030, respectively). Flammutoxin is from *Flammulina velutipes* (a mushroom; GenBank enrty 3551186). a-Toxin is from *Clostridium septicum* (GenBank entry 452163). Phi cytotoxin is from a bacteriophage (GenBank entry 17313218).

pore-forming toxin (*34*), hydralysin revealed a cytotoxic effect at much lower concentrations (50% cytotoxicity to sf9 cells was observed at 50 and 16 000 nM for hydralysin and pardaxin, respectively). The cells treated with hydralysin revealed general morphological changes within 5 min of the treatment, including vacuolization and loss of a well-defined plasma membrane (Figure 4C,D). In contrast to the insect sf9 cells, human HEK293 cells were not stained with trypan blue after treatment with high concentrations of hydralysin (Figure 4E), and seemed morphologically unchanged compared to control cells (not shown).

DISCUSSION

Paradox of a Noncnidocystic Toxin in Hydra. In this work, we reveal the occurrence of a novel, noncnidocystic toxin in hydra, which is weakly homologous with known pore-forming proteins. This toxin reveals selectivity in its action, illustrated by its paralytic activity toward arthropods as opposed to vertebrates and its differential toxicity toward

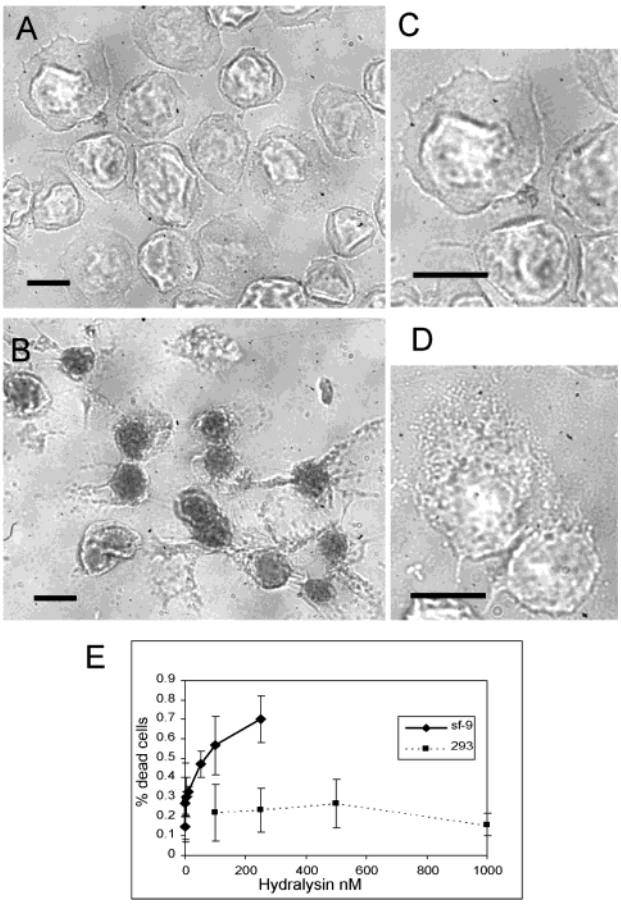

FIGURE 4: Hydralysin causes rapid lysis of insect sf9 cells but not of human HEK293 cells. (A and B) General view of sf9 cells treated with either PBS (A) or 160 nM hydralysin (B) for 30 min and stained with trypan blue. Cells stained with trypan blue appear darker and are nonviable. Bar = 10 μm. (C and D) Morphology of an sf9 cell treated with PBS (C, enlarged from panel A) or hydralysin (D). The cell in panel D was not treated with trypan blue; note the loss of both plasma membrane and nuclear integrity in the treated cell. Bar = 10 μm. (E) Hydralysin causes a dose-dependent cytolysis of insect sf9 cells but not of human HEK293 cells. Cells were treated with different concentrations of hydralysin, stained with trypan blue, and counted on a hemocytometer. Data points represent averages and standard deviations of four to eight counted fields.

cultured cells from the two phyla. Although noncnidocystic toxicities have been demonstrated previously (*11*, *35*, *36*), the large amount of of hydralysin found in the hydra's body is surprising and maybe even paradoxical, raising several intriguing questions. First, what is the purpose of producing a fast paralytic (presumably neurotoxic) protein without providing it with a mechanical, injection-like delivery device, as occurs in animal venom systems in general and particularly elegantly in the cnidarian cnidocysts? Second, why does an organism spend a significant metabolic effort in producing a secretory protein in amounts which correspond to 0.2−0.3% of its dry body weight? This question is amplified by the fact that we are dealing with a protein which is genetically controlled for a given preprogrammed purpose and is not a "secondary metabolite" or a metabolic byproduct.

Functional Significance of the Noncnidocystic Toxin. Several hypotheses can be raised which explain both hydralysin's noncnidocystic origin and its high concentration in the hydra. First, it may be found in the hydra's tissue as a defensive alomone, acting in combination with the stinging cells to deter possible predators. Such a role, for example, is performed by the neurotoxin tetrodotoxin in pufferfish and other animals (*37*).

The second possibility is that hydralysin fulfills an alomonal role in prolonging the paralysis of the prey after being ingested. This hypothesis is supported by our observations that *Artemia nauplii* caught by hydrae are not necessarily killed outright, and may be seen wiggling, partially paralyzed, as they are being engulfed by the hydra. The cnidocytes on the mesenterial filaments of acontiate sea anemones may perform this paralysis-prolonging task. In addition, Meinardi *et al*. (*38*, *39*) have previously isolated a cytolytic protein from the coelenteric fluid of sea anemones, which differs from the nematocyst toxins.

Common to both these hypotheses is the need of a protein toxin to cause a paralytic−neurotoxic effect without being delivered by a stinging−piercing delivery system. A pore-forming protein has the capacity to affect membrane barriers directly, both destroying membrane integrity and causing a neurotoxic effect, as has been demonstrated for pardaxin (*40*). This is the case with many defensive poisonous secretions, which are composed essentially of either lipophilic aromatic substances (*41*) or amphipathic cytolytic (*5*) pore-forming (*42*) proteins. Large amounts of such a protein would need to be secreted into the coelenteric fluid to reach a critical concentration, and could fulfill a role in keeping the prey paralyzed (based on depolarizing capability) and possibly also in extracellular digestion.

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