

# Cytolytic action of *Vibrio vulnificus* haemolysin on mast cells from rat peritoneal cavity

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**Summary.** The mode of action of *Vibrio vulnificus* haemolysin (VVH) on mast cells from the peritoneal cavity of the rat was examined. VVH induced histamine release, and damage to the mast cells, in a dose-dependent fashion. When 1 µg of VVH was added to *c.* 10<sup>5</sup> mast cells at 37°C, histamine release was observed after a lag period of 5–10 s, and was complete within 5 min. The action was temperature-dependent, and was not induced at 4°C. Disodium cromoglycate, a membrane stabiliser for mast cells, inhibited the histamine release significantly, but the effect was not dose-dependent. Moreover, leakage of lactate dehydrogenase from VVH-treated mast cells was observed. These results suggest that VVH acts on the cell membrane of mast cells and is cytolytic.

## Introduction

*Vibrio vulnificus* is a halophilic marine vibrio,<sup>1,2</sup> associated with well-characterised disease<sup>3</sup> including wound infection, septicaemia and other infections.<sup>4,5</sup> Septicaemia is commonly induced in persons with pre-existing disease such as liver damage, immune deficiency syndromes, or haemochromatosis.<sup>6,7</sup> The infection caused by this bacterium has attracted special interest because of its high mortality.<sup>8</sup> Fever, chill, nausea, vomiting and severe skin lesions have been reported.<sup>9</sup> Erythema, bullae, necrotic ulcer, cellulitis and oedema may occur.<sup>10,11</sup>

We have proposed that a protease exotoxin produced by *V. vulnificus* may be related to the pathogenicity, including the production of skin lesions,<sup>12</sup> because the protease degrades collagen, elastin and casein<sup>13</sup> and enhances vascular permeability.<sup>14,15</sup>

On the other hand, it was shown that a 56-Kda cytotoxin (haemolysin), another exotoxin produced by this vibrio, enhanced vascular permeability, and had a cytopathic effect on Chinese hamster ovary cells, and lethal activity in mice;<sup>16</sup> and Gray and Kreger<sup>17</sup> have reported that it induced mouse skin damage. We<sup>18</sup> have isolated a *V. vulnificus* haemolysin (VVH), with the same biological activities, from strain B3547, although its molecular weight (50 Kda) was slightly less than that of the above

cytotoxin. The enhancement of vascular permeability by VVH in the dorsal skin of the rat was inhibited by the addition of diphenhydramine, an antihistamine (unpublished observation). Therefore, we suspected that VVH may affect mast cells, which contain much histamine; and we report here the effect of VVH on mast cells from the peritoneal cavity of the rat.

## Materials and methods

### Purification of *V. vulnificus* haemolysin (VVH)

VVH was purified from the culture supernate of a virulent strain (CDC B3547) of *V. vulnificus* as previously described.<sup>18</sup>

### Isolation of mast cells

Mast cells were collected from the peritoneal cavities of male Wistar rats (300–400 g) and purified by use of Percoll (Sigma).<sup>19</sup> The mast cells, *c.* (1–3) × 10<sup>5</sup>/ml, were suspended in phosphate-buffered saline (PBS) consisting of 154 mM NaCl, 2.7 mM KCl, 0.9 mM CaCl<sub>2</sub>, 6.7 mM KH<sub>2</sub>PO<sub>4</sub>–Na<sub>2</sub>HPO<sub>4</sub> (pH 7.2) and bovine serum albumin 0.01%. The purity of the mast cells thus obtained was more than 90%, as assessed by microscopy.

### Measurement of histamine release from mast cells treated with VVH

For histamine release, a suspension of *c.* 10<sup>5</sup> mast cells in 0.5 ml of PBS was incubated with various amounts of

VVH for 10 min at 37°C. The reaction was then terminated by addition of 1.5 ml of ice-cold PBS. The mixture was centrifuged at 1000 *g* for 10 min at 4°C. The histamine in the supernates and the precipitates was measured by the method of Shore *et al.*<sup>20</sup> Histamine release, in the supernate, was calculated as the percentage of the total histamine in the system. For study of the kinetics of histamine release, VVH was added to the cell suspension and incubated for 5 min at 37°C; the reaction was terminated after various periods by addition of ice-cold PBS.

### Mode of action of VVH on mast cells

Activity of lactate dehydrogenase (LDH), which is contained in the cytosol of mast cells, was determined by the fluorimetric method of Roy *et al.*,<sup>21</sup> to study whether VVH would induce cytolysis. If it does, LDH release is detectable in the reaction mixture; and this was estimated in relation to the fluorescence intensity obtained by addition of Triton X-100 0.01% to  $(4-6) \times 10^5$  mast cells. To determine the effect of membrane stability in the histamine release, disodium cromoglycate (Sigma; 2–50  $\mu$ M) was used. Also, the morphological changes of mast cells treated with VVH were observed by phase-contrast microscopy. The action of compound 48/80 (Sigma), a chemical agent which induces a typical degranulation of mast cells, was compared with that of VVH.

All experiments were performed in duplicate or triplicate; mean values are shown.

## Results

### Histamine release from mast cells treated with VVH

VVH induced histamine release from isolated mast cells in a dose-dependent manner. The amount of VVH that induced 50% release from  $c. 10^5$  cells was  $c. 0.2 \mu$ g (fig. 1). After VVH had been heated at 60°C for 10 min, its haemolytic activity disappeared, and histamine release was not observed.

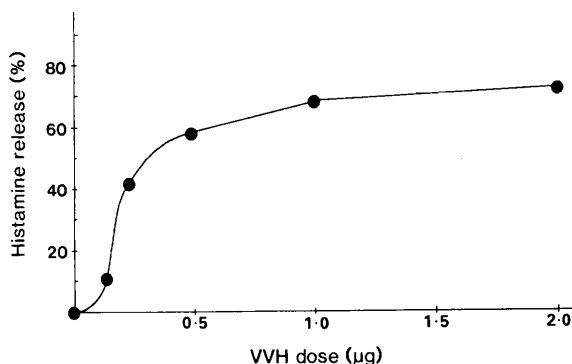


Fig. 1. Histamine release from  $c. 10^5$  mast cells treated with various doses of VVH and incubated for 10 min at 37°C.

### Duration of histamine release

The release of histamine from mast cells treated with VVH was induced rapidly at 37°C after a short lag-period of 5–10 s (fig. 2), and the maximal release was reached within 5 min.

### Influence of temperature on the action of VVH

Histamine release from mast cells treated with VVH was temperature dependent (fig. 3). No release was detected at 4°C; action was induced at 15°C, was greater at 37°C, but diminished at 45°C.

### Mode of action of VVH on mast cells

A previous report<sup>21</sup> suggested that histamine release by a small amount of compound 48/80 was not accompanied by the leakage of LDH contained in the cytosol of mast cells, and there was no cytolysis.

Histamine release induced by VVH was accompanied by the leakage of LDH (fig. 4). Moreover, it

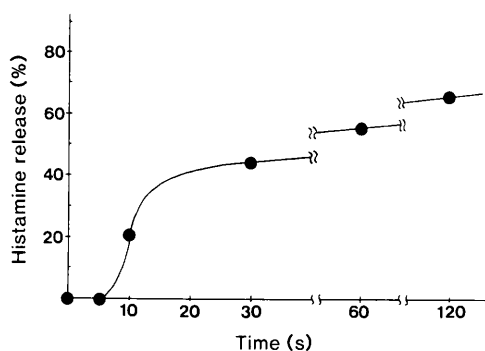


Fig. 2. Duration of histamine release from  $c. 10^5$  mast cells, induced by 1  $\mu$ g of VVH at 37°C.

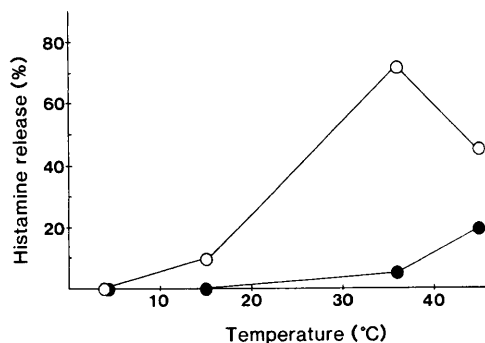


Fig. 3. Influence of temperature on histamine release from  $c. 10^5$  mast cells pre-incubated at various temperatures for 5 min, and incubated for a further 10 min with 1  $\mu$ g of VVH (○) or without VVH (●).

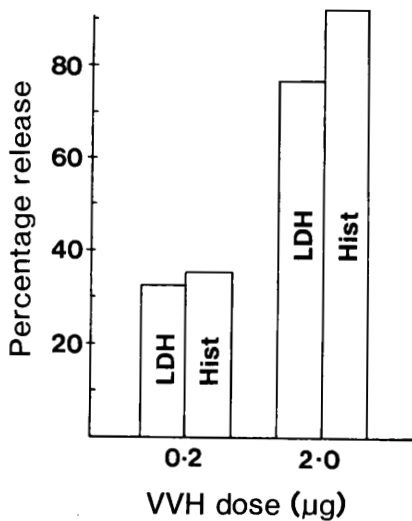


Fig. 4. Release of histamine (Hist.) and lactate dehydrogenase (LDH) from  $c. 5 \times 10^5$  mast cells incubated with VVH at 37°C for 10 min.

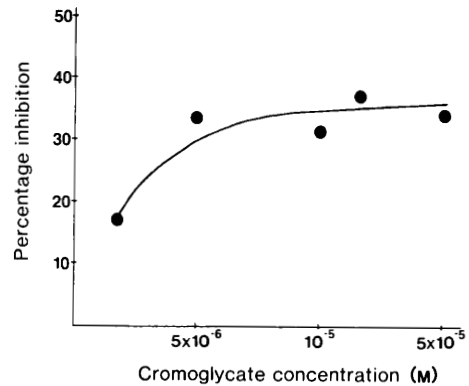


Fig. 5. Effect of pre-incubation with various doses of disodium cromoglycate on the action of 1 µg of VVH on  $c. 10^5$  mast cells. The maximal inhibition of histamine release was only  $c. 35\%$ .

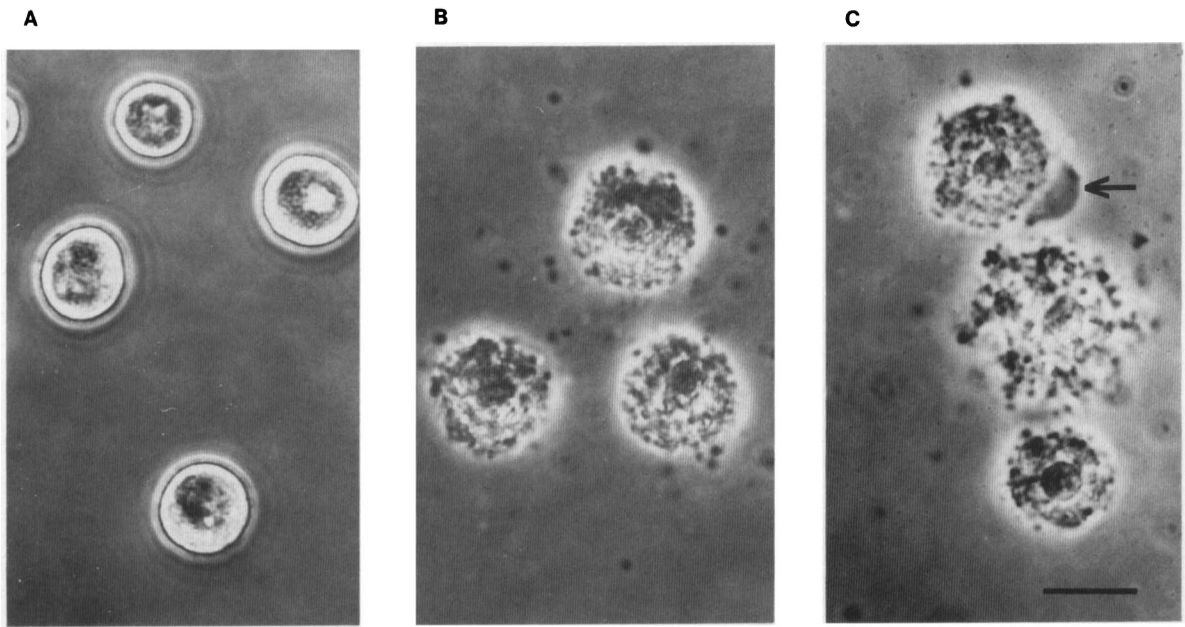


Fig. 6. Phase-contrast micrographs of mast cells (A) before treatment, (B) after treatment with 0.5 µg of compound 48/80, (C) after treatment with 2 µg of VVH. Blisters were often observed from the action of VVH (arrow). Bar = 20 µm.

was inhibited to some extent by prior administration of disodium cromoglycate, thought to be a membrane stabiliser of mast cells. But the inhibitory effect was not related directly to the drug concentration (fig. 5), and reached a maximum of only  $c. 35\%$  inhibition. The morphological changes of mast cells treated with VVH are shown in fig. 6. The cytolytic effect of VVH was compared with the action of compound 48/80 which induced a typical degranu-

lation after exocytosis of the cells. Furthermore, the formation of blisters was observed in VVH action, although the meaning of this observation is not clear.

### Discussion

It has been shown that VVH induces histamine release from isolated peritoneal mast cells of the

rat, concurrently with cytolytic action. It is thought that VVH has direct action on mast cells; heat-denatured VVH did not induce histamine release. The response to VVH was temperature dependent; it was not detected at 4°C, it increased up to 37°C, but was reduced at 45°C—perhaps because of the stabilising effect on mast cells of temperatures of 40–50°C.<sup>22</sup> A lag-period was observed in the initial response of mast cells to VVH. In haemolysis by VVH also, a lag-period is observed in K<sup>+</sup> release from erythrocytes.<sup>18</sup> We suppose that time may be needed for binding of VVH to the erythrocyte membrane, to form lesions, and likewise in the action of VVH on mast cells before histamine release. Maximal histamine release by compound 48/80<sup>23</sup> or dynorphin<sup>22</sup> (with which degranulation leads to histamine release) is reached within 10 s and a lag-period is not detected. Therefore, we suggested that the mechanism of histamine release by VVH may be different.

Histamine release by VVH was accompanied by the leakage of LDH, indicating that VVH induced cytolysis; and this suggestion was supported by the microscopic observations. Disodium cromoglycate has been shown to inhibit the exocytosis of mast cells stimulated with various agents such as anti-

gen,<sup>24</sup> compound 48/80,<sup>23,25,26</sup> dextran,<sup>27</sup> neurotensin<sup>28</sup> and dynorphin.<sup>22</sup> The histamine release by VVH was slightly inhibited by disodium cromoglycate, but this inhibition was not dose-dependent, and the maximal effect was 35% reduction. Disodium cromoglycate may have a stabilising effect on the mast cell membrane,<sup>19,23,25</sup> and it may affect VVH action because the latter acts directly on the target membrane.

It has been reported that many other bacterial haemolysins act not only on various erythrocytes but also on other mammalian cells.<sup>29–32</sup> The present findings suggest that VVH degrades mast cells and induces histamine release. Therefore, it seems that VVH, as well as *V. vulnificus* protease, is involved in oedema formation, haemoconcentration and other skin manifestations in *V. vulnificus* infection. Furthermore, it was observed that VVH possessed cytolytic activity on leucocytes (data not shown), as described by Gray and Kreger;<sup>17</sup> therefore, it may have an important role in *V. vulnificus* infection.

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