FULL PAPER Immunology

Protection against Atypical *Aeromonas salmonicida* Infection in Carp (*Cyprinus carpio* L.) by Oral Administration of Humus Extract

Hiroshi KODAMA[1)], DENSO[1)] and Tsuyoshi NAKAGAWA[1)]

[1)]*Laboratory of Veterinary Immunology, Course of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai, Osaka 599–8531, Japan*

(Received 16 October 2006/Accepted 22 December 2006)

ABSTRACT. Humic substances are formed during the decomposition of organic matter in humus, and are found in many natural environments in which organic materials and microorganisms have been present. In the present study, oral administration of humus extract to common carp (*Cyprinus carpio* L.) induced effective protection against experimental atypical *Aeromonas salmonicida* infection. Mortality of fish and development of skin lesions such as hemorrhages and ulcers were significantly suppressed in carp treated with 10%, 5% or 1% humus extract adsorbed on dry feeding pellets. The median surviving days was also greater in fish treated with 10% or 5% humus extract than in untreated fish. Atypical *A. salmonicida* was isolated from ulcerative lesions of part of dead fish, but *Aeromonas hydrophila* and *Flavobacterium* sp. were also isolated from these fish, verifying bacterial population changes during the progression of skin lesions. These results clearly show that treatment of fish with humus extract is effective in preventing *A. salmonicida* disease.

KEY WORDS: atypical *Aeromonas salmonicida*, carp, humic substance, humus extract, ulcer.

J. Vet. Med. Sci. 69(4): 405–408, 2007

Humic substances are formed during the decomposition of organic matter in humus. They can be found in many natural environments in which organic materials and microorganisms are or have been present [14]. The chemical composition, structure and functional groups can vary greatly, according to their origin and age and the conditions of the humification process (humidity, aeration, temperature, mineral microenvironment, etc.). Humic substances can be divided into humic acids, fulvic acids and hymathomelanic acids according to their hydrophilic properties [7]. Natural humification products such as humus, peat, sapropel and mumie have been used to develop pharmacologic agents with diverse applications in medical practice [13, 15]. These have been successfully used as anti-inflammatory agents because they have local anti-inflammatory and analgesic properties [6, 11]. The use of coal-derived humic acid and fulvic acid as antimicrobials has also been investigated [10], and the anti-HIV activity of oxihumate [12] and synthetic humic acid analogues [8] has been reported. No measurable side effects have been observed [5, 11]. However, the potential of humic substances in the treatment of animal diseases has scarcely been investigated. Humic substances should be applicable to fish farming to reduce the incidence of various infectious diseases. Even when environmental conditions are favorable and the fish are healthy, mortality currently occurs if infectious agents are introduced into the farm.

Atypical *Aeromonas salmonicida* is a fish pathogen that causes several diseases in freshwater fish such as goldfish (*Carassius auratus* L.) [9] and eel (*Anguilla japonica*) [1], and also seawater fish [3]. Since 1996, *A. salmonicida* infection characterized by the formation of ulcers on the body surface and fins has become prevalent in colored carp (*Cyprinus carpio koi* L.) cultivation in Japan [2]. Vaccination is believed to be effective in principle against the disease, but no effective vaccine has yet been developed. We therefore investigated the effect of oral administration of humus extract on protection of carp from atypical *A. salmonicida* infection.

MATERIALS AND METHODS

Fish: Fry of the common carp (*Cyprinus carpio* L.) were kindly provided by the Agricultural, Food and Environmental Sciences Research Center of Osaka Prefecture, Neyagawa, Japan. They were grown in 170 l plastic aquaria filled with dechlorinated tap water (passing through once, at a flow-through rate of 40 *l*/hr; water temperature regulated to 20 to 25°C) and aerated. The fish were fed a commercial floating dry pellet twice daily. A daily regimen of 15 hr of light followed by 9 hr dark was maintained.

Humus extract: Humus extract was prepared from humus (collected in Nagasaki Prefecture, Kyushu, Japan) using water. The humus was added 6 volumes of dechlorinated water (v/w), and the mixture was agitated every day for 30 days, then left to stand at 25 to 28°C for 4 months. Supernatant was collected and filtered using a membrane filter (pore size: 25 μm). The resulting humus extract has pH 2.8 and contains various minerals including Al, Ca, Mg, Na and Si. The extract contained 1,500 ppm of sulfate. No culturable bacteria were found in the extract. There were small amounts of protein and carbohydrate (0.7% of the total weight).

Administration of humus extract: Fish were divided in each group and acclimatized in 40 *l* aquaria (flow-through rate 20 *l*/hr, water temperature 18–19°C) which were aerated. Fish weighing 26 ± 4 g (first trial) and 19 ± 2 g (second trial) were used for the experiments. Humus extract was sprinkled on the dry feeding pellets to provide final concentrations of 20 to 0.2% of the dry weight, and was

adsorbed into the pellets which were then dried in an incubator at 25°C. The fish were fed pellets containing humus extract twice daily (total 2% of the fish body weight per day) for 30 days prior to challenge by *A. salmonicida*, and for 22 consecutive days immediately after bacterial challenge. Control fish were fed the dry pellet without humus extract.

Bacterial challenge: The fish were challenged with virulent atypical non-pigment producing *A. salmonicida*. Strain T1031 donated by Niigata Prefectural Inland Water Fisheries Experimental Station, Nagaoka, Japan was cultured in heart infusion broth (Nissui Pharmaceutical Co., Tokyo, Japan) at 23°C for 5 days, with shaking. The fish were immersed at 1×10^6 cfu/m*l* for 60 min in both experiments, and were then observed for 22 days to determine survival, and any formation of ulcers and hemorrhagic lesions on the skin. Bacterial isolation was performed by cultivation from hemorrhagic and ulcerative lesions, and from visceral organs of dead fish. This was also done in all surviving fish.

RESULTS

Fish mortality after A. salmonicida challenge: Challenge tests were performed two times individually under similar experimental conditions. Figure 1A shows that the administration to carp of humus extract (10% and 5%) induced effective protection against experimental *A. salmonicida* infection in the first experiment (10 fish in each group). Of the non-treated carp in the control group, following challenge with *A. salmonicida*, 8 carp died within 11 days. In contrast, the survival rates of fish treated with 10% and 5% of humus extract were 70% ($P<0.05$ compared to control fish by χ^2 test) and 90 % ($P<0.01$), respectively at 22 days after challenge. However, only 30% of the carp administered 20% extract survived (not significant). In the second experiment (8 fish in each group), 63% ($P<0.01$) and 50% ($P<0.05$) of fish administered 5% and 1% humus extract, respectively, survived at 22 days after challenge, whereas all control fish died within 15 days.

Development of skin lesions: Skin lesions were significantly suppressed in humus-treated carps (see Table 1). The control fish showed skin hemorrhages 4 days after challenge, and skin ulcers developed 5 days after challenge; Figure 2 shows fish died 8 days (note hemorrhage on the peduncle and abdomen, Fig. 2A) and 11 days (ulcer formation and hemorrhage, Fig. 2B) after challenge, respectively. Gross lesions observed in control fish group were much more severe than in 10%, 5% or 1% humus-treated fish. The median surviving days is also greater in fish treated with 10% or 5% humus extract ($P<0.001$, Student's *t*-test) than in untreated fish.

Bacterial reisolation: Atypical *A. salmonicida* was isolated from hemorrhagic and ulcerative lesions of part of dead fish, but *Aeromonas hydrophila* and *Flavobacterium* sp. were also isolated from these fish, verifying bacterial population changes during the progression of skin lesions. No *A. salmonicida*, *A. hydrophila* or *Flavobacterium* was isolated from survived fish treated with 10, 5 and 1% humus extract. The results of the present study clearly show that treatment of fish with humus extract is effective in preventing *A. salmonicida* disease.

DISCUSSION

We infer that humus extract protected carp against *A. salmonicida* challenge as shown by reduced mortality, extended survival, and only mild skin lesion formation. To investigate the mechanism of protection by the humus extract we tested lysozyme activity, which exhibits antibacterial activity against gram-positive bacteria and is an indicator of fish health condition, both in skin mucus and serum collected from humus-treated and control carp. No measurable increase in activity was detected when *Micrococcus*

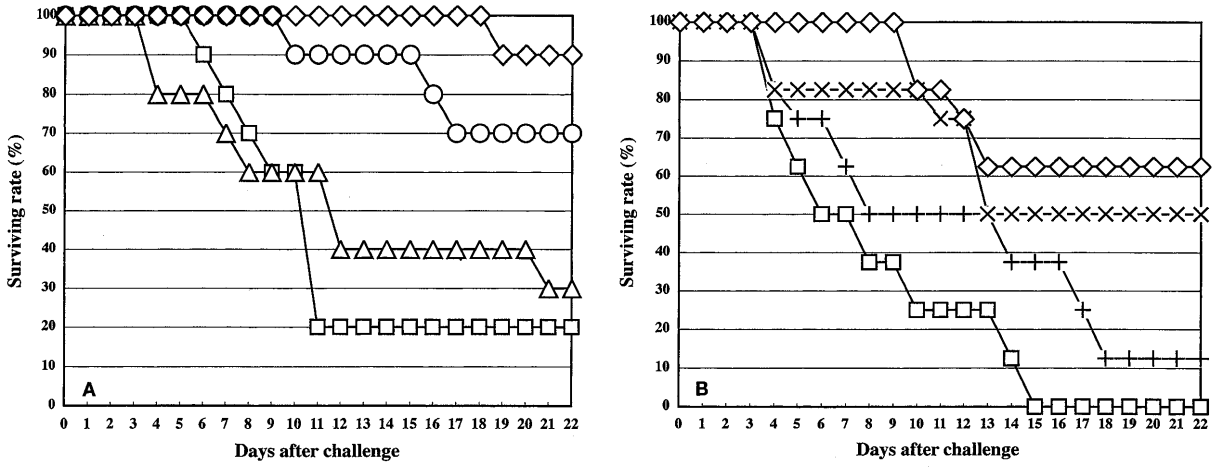

Fig. 1. Mortality of carp challenged with T1031 strain of atypical *Aeromonas salmonicida*, after treatment of 20% (△), 10% (○), or 5% (◇) humus extract (A). The extract was administered for 30 days orally as a dry feeding pellet (total 2% of body weight per day) containing or not containing (□) extract. Similar experiment was repeated in which humus extract was administered with concentrations of 5% (◇), 1% (×), and 0.2% (+) for the same period (B).

Table 1. Assessment of skin lesions of carp 22 days after atypical *Aeromonas salmonicida* challenge

Humus extract		Degree of skin lesion (%)					Median surviving days
		no lesion	slight (hemorrhage)	weak (ulcer)	severe (severe ulcer)	dead	
Treated	20%	0	30	0	0	70 (NS)	9 (NS)
	10%	20	40	10	0	30 ($P<0.001$)*	45 ($P<0.001$)**
	5%	22	28	17	11	22 ($P<0.001$)	57 ($P<0.001$)
	1%	0	38	13	0	50 ($P<0.05$)	16 (NS)
	0.2%	0	0	13	0	88 (NS)	9 (NS)
Non-treated		0	6	0	6	89	8

Combined data from two individual challenge tests.
* Significant difference in mortalities between group receiving humus extract and non-treated group estimated by χ^2 test.
** Significant difference between group receiving humus extract and non-treated group estimated by Student's *t*-test.
NS: not significant.

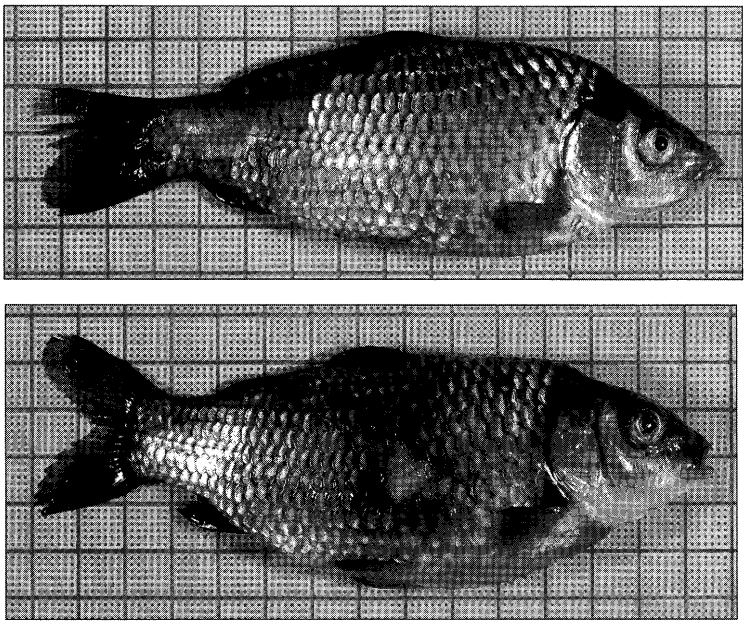

Fig. 2. Gross regions in fish died after atypical *A. salmonicida* challenge, showing hemorrhage on the peduncle and abdomen (fish in the control group died 8 days after the challenge; A) and ulcer formation (control fish died 11 days after the challenge; B).

luteus was used for indicator bacteria (data not shown). The mechanism by which humus extract protects against *A. salmonicida* infection is not clear at present, but it is likely that enhancement of innate protective responses would confer protection. Schepetkin *et al.* [7] reported that mumie, a semihard black resin formed by long-term humification, enhances the production of reactive oxygen species and nitric oxide in mouse peritoneal macrophages and enhances [^{3}H]thymidine uptake by splenic lymphocytes. A peat-based preparation enhanced the proliferative capability of murine thymocytes stimulated by mitogens, and prevented the immuno-suppressive effect of hydrocortisone [4]. These results indicate that humic substances enhance host immune responses nonspecifically. The protective effect was not due to specific antigenic stimulation of carp by humus extract, since the extract used in the present study contained no antigenic substances which react specifically with anti-*A. salmonicida* antibody. Further studies are required to determine how innate immune responses are induced in carp after treatment with humus extract.

The protective effect of the extract was not due to direct antimicrobial or antibiotic activity of the extract. *Aeromonas salmonicida* and six other species of fish pathogenic bacteria (*A. hydrophila, Edwardsiella tarda, Lactococcus garvieae, Photobacterium damsela, Pseudomonas plecoglossicida* and *Vibrio anguillarum*) were found not to be killed when the survival of the bacteria was tested by incubating them in humus extract solution (data not shown).

Van Rensburg *et al.* [10] reported *in vitro* antimicrobial activity of fulvic acid. Seven bacterial strains from the Genus *Streptococcus, Pseudomonas, Escherichia, Klebsiella* and *Proteus* were sensitive to fulvic acid at high concentration (eg; 15 mg/m*l*). It appears that the antibacterial activity was due to the low pH of the reaction mixture given the high concentration of fulvic acid. It is reasonable to assume that humic substances absorbed via the intestinal tract of the carp affect host physiological conditions, such as innate immune responses, conferring protection against microbial infection.

No toxicity has been reported in humic substances [5, 11], since these are mostly carboxylic acids and ordinary physiological metabolites. Humic substances and/or humus extract can therefore be used in fish as food additives and for immunopotentiating materials in aquaculture. Further analysis of protective mechanisms activated by humus extract, and separation of biologically active components in humus, are now in progress.

ACKNOWLEDGMENTS. The authors are grateful to Professor Tohru Miyajima (Faculty of Science and Engineering, Saga University, Saga, Japan) and Dr. Hisanori Mayumi (Souju Memorial Hospital, Iwatsuki, Japan) for helpful suggestions. This work was supported by a Grant-in-Aid from the Japanese Society for the Promotion of Science (No. 18580311). This work was also funded by Marinex Co., Sakai, Japan.

REFERENCES

1. Kitao, T., Yoshida, T., Aoki, T. and Fukudome, M. 1984. Atypical *Aeromonas salmonicida*, the causative agent of an ulcer disease of eel occurred in Kagoshima Prefecture. *Fish Pathol.* **19**: 113–117.
2. Matoyama, H., Hoshino, M. and Hosoya, H. 1999. Pathogenicity of atypical *Aeromonas salmonicida* isolated from colored carp *Cyprinus carpio* suffering from a new ulcerative disease. *Fish Pathol.* **34**: 189–193.
3. Nakatsugawa, T. 1994. Atypical *Aeromonas salmonicida* isolated from cultured shotted halibut. *Fish Pathol.* **29**: 193–198.
4. Obmiñska-Domoradzka, B. and Stefañska-Joñca, M. 2001. The effect of a peat-based preparation on mitogen-induced proliferation on thymocytes in non-treated and hydrocortisone-suppressed mice. *Phytomedicine* **8**: 184–194.
5. Reichert, B. 1966. Huminsäuren und ihre Derivate in der modernen Therapie. *Deut. Apotheker* **18**: 204–206.
6. Salz, H. 1974. Salhumin gel, a local therapeutic agent with hyperemic antiphlogistic and analgesic effect. *Med. Monat.* **28**: 548–550.
7. Schepetkin, I. A., Khlebnikov, A. I., Ah, S. Y., Woo, S. B., Jeong, C.-S., Klubachuk, O. N. and Kwon, B. S. 2003. Characterization and biological activities of humic substances from mumie. *J. Agric. Food Chem.* **51**: 5245–5254.
8. Schneider, J., Weis, R., Manner, C., Kary, B., Werner, A., Seubert, B. J. and Riede, U. N. 1996. Inhibition of HIV-1 in cell culture by synthetic humate analogues derived from hydroquinone: Mechanism of inhibition. *Virology* **218:** 389–395.
9. Shotts, E. B., Talkington, F. D., Elliott, D. G. and McCarthy, D. H. 1980. Aetiology of an ulcerative disease in goldfish, *Carassius autarus* (L.): Characterization of the causative agent. *J. Fish Dis.* **3**: 181–186.
10. Van Rensburg, C. E. J., Van Straten, A. and Dekker, J. 2000. An *in vitro* investigation of the antimicrobial activity of oxifulvic acid. *J. Antimicrob. Chemother.* **46:** 853–854.
11. Van Rensburg, C. E. J., Malfield, S. C. K. and Dekker, J. 2001. Topical application of oxifulvic acid suppresses the cutaneous immune response in mice. *Drug Dev. Res.* **53**: 29–32.
12. Van Rensburg, C. E. J., Dekker, J., Weis, R., Smith, T.-L., Van Rensburg, E. J. and Schneider, J. 2002. Investigation on the anti-HIV properties of oxihumate. *Chemotherapy* **48**: 138–143.
13. Vengerovskii, A. I., Golovina, E. L., Burkova, V. N. and Saratikov, A. S. 2001. Enteric sorbents potentiate hepatoprotective effect of Eplir in experimental toxic hepatitis. *Eksp. Klinich. Farmakol.* **64**: 46–48.
14. Visser, S. A. 1973. Some biological effects of humic acids in the rat. *Acta Biol. Med. Germ.* **31**: 569–581.
15. Yudina, N. V., Pisareva, S. I. and Saratikov, A. S. 1998. Antiulcerogenic activity of phenol compounds of peat. *Khim. Rastit Sirya* **4**: 29–32.