NAG INFECTION IN *Rana temporaria* AFTER SUPPRESSION OF THE NORMAL INTESTINAL MICROFLORA

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Frogs (*Rana temporaria*) with disturbed biocenosis of their intestinal microflora produced by a combination of tetracycline and hypothermia (+4°C) were used as the experimental model. The animals were infected perorally with a culture of NAG vibrios and then kept under clinical observation and tested bacteriologically, immunomorphologically, and by electron microscopy. The possible lymphohematogenous pathway of generalization of infection was demonstrated and some causes of the prolonged persistance of vibrios in the animals' bodies are discussed.

KEY WORDS: frog; NAG infection; vibrio carrier state; tetracycline; dysbacteriosis.

Present-day cholera and NAG infections are characterized by the predominance of atypical, abortive, and symptom-free forms of these diseases, which frequently terminate in a prolonged vibrio carrier state [2-6]. It is therefore interesting to study some of the possible causes of prolonged persistence of vibrios in the gastrointestinal tract of man and animals. The normal intestinal microflora is one of the factors in the protective and compensatory mechanisms of the host in intestinal infections. To study the character of relations between NAG vibrios and their animal hosts, it was decided to use experiments on frogs with a preassigned and controllable microflora. To suppress the normal intestinal microflora the broad-spectrum antibiotic tetracycline, which is used for the treatment and prevention of chlorea and NAG infections, was chosen

The aims of the investigation were: 1) to study the character of spread of vibrios in frogs at different stages of the infectious process with the aid of bacteriological, histological, immunomorphological, and electron-microscopic methods, and 2) to study changes characterizing interaction between the host and vibrios at the cellular level.

EXPERIMENTAL METHOD

For 20 days before the experiments, 180 male frogs (*Rana temporaria*) weighing 30-40 g were kept at a temperature of 4°C without food. The composition of the microflora was verified by seeding on Endo's and Levine's differential-diagnostic media and Hottinger's agar. The sensitivity of the grown cultures to tetracycline was determined. By means of a tube, the animals were given 250 μg tetracycline in 0.5 ml physiological saline perorally daily for 5 days. Two days after the end of administration of the antibiotic, bacteriological tests of the intestinal contents were repeated daily for 3 days. The frogs were infected with an enteropathogenic strain of NAG vibrio in a dose of $5 \cdot 10^8$ bacterial cells in 0.5 ml Hottinger's broth. The distribution of the animals by groups and the corresponding experimental condition are given in Table 1.

The investigations were carried out 30 min and 3, 24 and 48 h after infection, and thereafter at intervals of 3-8 days until the end of the period of observation (50 days). Cardiac blood, bile, homogenates of the liver and kidneys, and the contents of the stomach and small and large intestines of the experimental frogs were seeded on differential-diagnostic media (peptone water, Endo's and TCBS media). The stomach, small and large intestines, and kidneys of the experimental and control animals were studied histologically, immunomorphologically,

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TABLE 1. Experimental Condition

Groups	Number of animals	Peroral administration of	
Experimental 1 st	60	Tetracycline 250 μg	$5 \cdot 10^8$ NAG vibrios in 0.5 ml broth
Control:			
2-nd	30	The same	0.5 ml broth
3-rd	30	» »	—
4-th	30	—	0.5 ml broth
5-th	30	—	—

and by electron microscopy. Sections for microscopic observations were stained with hematoxylin-eosin, thionine, and the PAS reaction. For bacterioscopy preparations were treated with azure-II-eosin, thionine, and Leishman's stain. NAG vibrios were identified by the indirect Coons' method. A specific serum against a culture of NAG vibrios was prepared. The essential controls were set up for specificity of fluorescence. For the electron-microscopic investigations the material was fixed with a 1% solution of osmium tetroxide in acetate-veronal buffer (pH 7.4), dehydrated in acetones of increasing concentration, and embedded in a mixture of Epon with Araldite. For analysis of the material under the light microscope, semithin sections 1 μ thick were cut and stained with tyronine and toluidine blue by Weakley's method [5]. Ultrathin sections were examined in the JEM-100B electron microscope with an accelerating voltage of 80 kV.

EXPERIMENTAL RESULTS

Bacteriological investigation of the contents of the large intestine of intact frogs revealed *Escherichia coli* cells, enterococci, and solitary colonies of staphylococci. The microflora was sensitive to tetracycline in concentrations of between 65 and 250 μg/ml. Five days after the end of tetracycline administration, in a very few cases single colonies of *E. coli* were seeded from the contents of the large intestine, evidence of sharp inhibition of the normal microflora. After 3, 24 and 72 h, bacteremia was observed in nine frogs, and vibrios were isolated from all the organs. In the remaining experimental frogs, mainly vibrios were isolated from the gastrointestinal tract until the 10th day, but their number subsequently decreased and the number of *E. coli* cells increased correspondingly. By the 30th-50th day the normal intestinal microflora was restored, but throughout the period of investigation vibrios continued to be seeded from the stomach and the small and large intestines, and in some frogs from the gall bladder and liver also. Colonies of NAG vibrios, isolated at different times after infection, were indistinguishable from the original culture in morphology, enzyme activity, and serologic properties. Serum against the culture of NAG vibrios did not give a crossed agglutination reaction or antigen—antibody reaction in Coons' method with *E. coli* or with other microorganisms isolated from the frogs. At autopsy on the animals 24-28 h after infection, a dilated intestine with watery contents and static congestion of the parenchymatous internal organs were observed. On the 3rd-4th days the visible clinical manifestation disappeared. In all the control frogs there were no visible changes in the internal organs.

The reaction of the organelles of the intestinal epitheliocytes with a blackened border in the experimental frogs was much weaker than that in the control animals infected with NAG vibrios without preliminary tetracycline treatment. No large dilated cavities of the agranular and granular endoplasmic reticulum of vacuolation of the cytoplasm such as were present in the animals of this group [1] could be observed. Meanwhile the frequency of discovery of lysosome-like structures and phagosomes in the epitheliocytes of the small intestine increased considerably during the first 24 h after infection. After 48 h, lysosomes also were found in individual endotheliocytes of the blood capillaries. An increase in the number of free monosomes and polysomes of micropinocytotic vesicles, and also of polymorphic microcytoplasmic processes reflecting an increased intensity of the process of microplasmatosis, was observed in the cytoplasm of these cells (Fig. 1). In the first week of the disease NAG vibrios caused considerable engorgement of the capillaries in the mucosa and submucosa of the gastrointestinal tract, and in the kidneys and liver, as well as edema of the mucosa and submucosa of the small and large intestines. Fluorescent antibody investigation 30 min after infection revealed intensive fluorescence of vibrios in the lumen and on the surface of the mucosa of the stomach and small intestine in all the experimental frogs, and this was confirmed by bac-

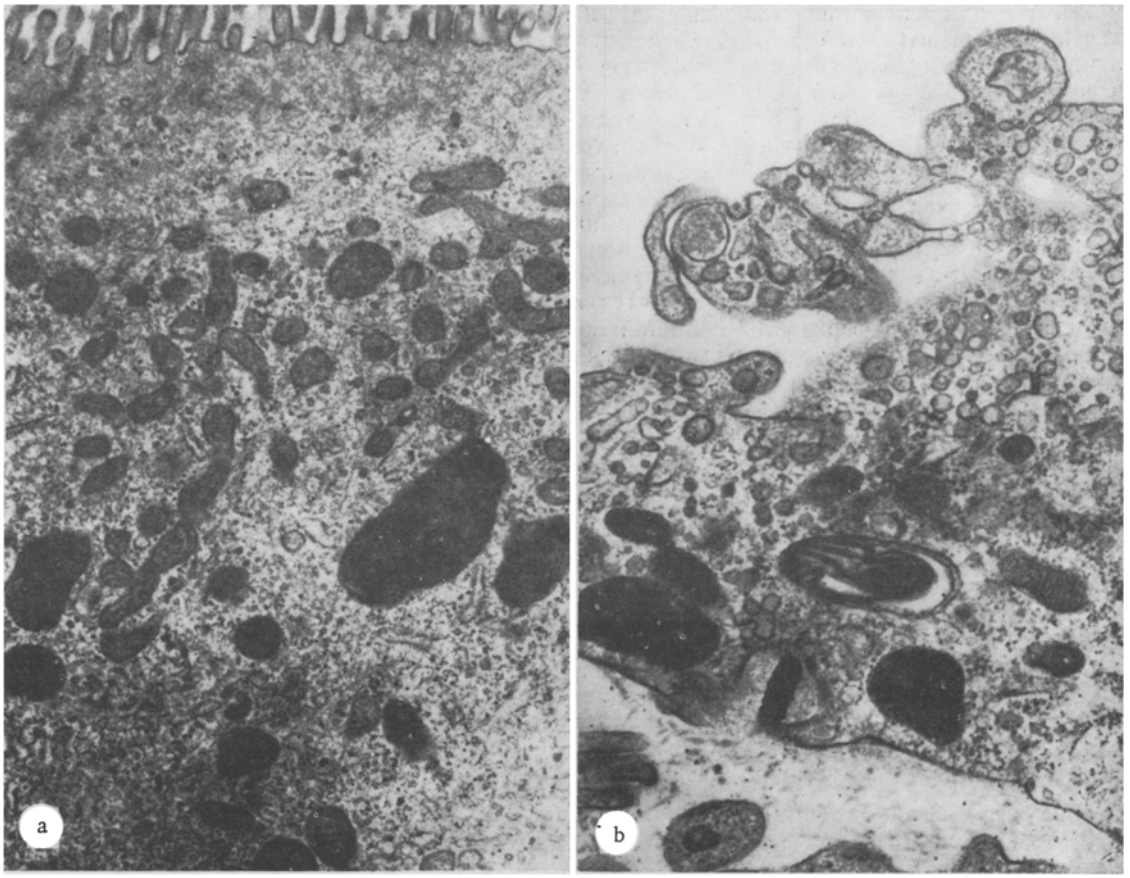

Fig. 1. Photomicrograph of small intestine of *R. temporaria* after suppression of normal intestinal microflora and exposure to hypothermia followed by NAG sections: a) epitheliocyte with blackened border (24 h after infection), 16,000×; b) endotheliocyte of blood capillary (48 h after infection), 30,000×.

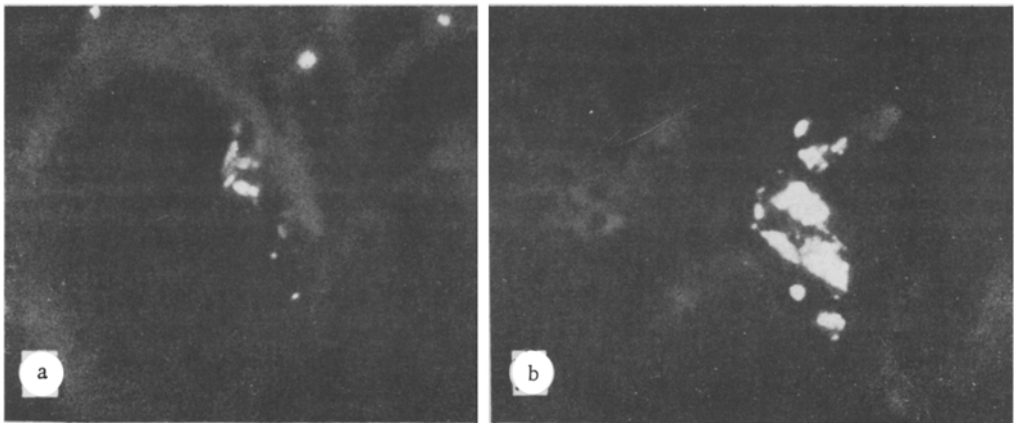

Fig. 2. Photomicrograph of colonies of NAG vibrios: a) in lymphatics of stomach on 7th day after infection, 630×; b) in bile ducts of liver and sites of extrasavation of bile (first day after infection), 630×.

terioscopic and electron-microscopic methods. Frogs in which the infection was generalized in character were of the greatest interest. In individual frogs vibrios were clearly visible after 3 h on the surface of the mucosa, in the intercellular spaces, and in the lymphatic capillaries of the stomach and duodenum where they could be detected until the 7th day (Fig. 2a). After 24 h solitary vibrios were found in lymphatic capillaries and lymphatics of these organs and also in the large intestine. In the liver, after 24 h groups of vibrios were seen in the form of microcolonies in the bile ducts and at sites of extravasation of bile (Fig. 2b). Vibrios were found in the kidneys after 48 h but only in the lumen of the tubules, where they lay on the surface of the epithelial cells.

The investigations showed that tetracycline and hypothermia reduce the natural resistance of animals and contribute to the development of infection followed by a prolonged vibrio carrier state. Electron-microscopic and histological methods showed that under these conditions intracellular destructive processes are considerably intensified both in the epitheliocytes of the small intestine and in the endotheliocytes of the blood capillaries. Vibrios penetrated through the lymphatic capillaries of the gastrointestinal tract into the lymphatic and blood vascular system, giving rise to generalized infection in some animals. Vibrios localized in the liver and gall bladder can persist for a long time in these organs and reappear periodically in the intestine, maintaining a prolonged or sometimes transient vibrio-carrier state.

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