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RAPID COMMUNICATION

Intestinal Transfer of Growth Hormone Into the Circulatory System of the Rainbow Trout, *Salmo gairdneri*: Interference by Granule Cells

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ABSTRACT To test if growth hormone, well known for its strong effect on fish growth, could be incorporated into the diet, bovine growth hormone (bGH) was introduced into the lumen of the digestive tract of trout yearling (*Salmo gairdneri*). Radioimmunoassay (RIA) and radioreceptor assay (RRA) were used to assess plasma concentration of bGH. In our experimental conditions, the concentration of bGH peaks after 1 hour, in treated fish only, attesting to the intestinal hormone transfer (coefficient of absorption is about 0.2%). Immunocytochemistry is used to follow the hormone transfer process. Most bGH molecules absorbed by enterocytes of the distal segment are found in the vacuoles (phagolysosomes) of the apical hyaloplasm. A small part escapes the intracellular degradation and is found in the intercellular space and in the interstitial space of the lamina propria. At this level, the granule cells trapped the antigenic hormone, as did immune cells.

The results of this study indicate that oral administration of GH can be potentially used in fish culture.

It has been known for a number of years that bovine growth hormone (bGH) can be used as growth promoting agent in teleost fish (see review by Donaldson et al., '79). Its administration leads to an increase in the growth rate (two- or three-fold), a reduction in adipose tissue, and a better transformation index. The treatment methods (injection, implant, dip), however include a number of inherent difficulties (intensive manipulation), and the likely side effects resulting from scale removal and frequent handling of the fish can create sites for bacterial infections. Two strategies are currently envisioned: one involves transferring growth hormone genes (Chourrout et al., '86; Dunham et al., '87); the other is derived from new data on protein absorption, especially in salmonids, consistent with the possibility of the incorporation of the hormone into fish diet, a suitable treatment in fish farming.

Proteins ingested by teleost fish can be transferred to the circulatory system, intact or partially hydrolyzed (Georgopoulou et al., '86; McLean and Ash, '86, '87; Suzuki et al., '88). Studies in rainbow trout have shown that transfer of ingested proteins into the plasma occurs in the posterior gut via enterocytes, which are structur-

ally differentiated for absorbing proteins and carrying out intracellular digestion (Georgopoulou et al., '88). Numerous macrophages and lymphoid cells are infiltrated between the epithelial cells in teleost fish (Zapata, '79; Davina et al., '80; Lamers, '85). A portion of the antigen molecules transferred enters into contact with macrophages, which can ingest them (Noaillac-Depeyre and Gas, '76; Rombout et al., '85; Georgopoulou and Vernier, '86). The analogy between the absorbing enterocyte in the posterior intestine of teleost fish and the M cell of the mammalian intestinal epithelium is extended by the demonstration of the existence of secretory immunoglobulin-forming cells in the same intestinal segment (Georgopoulou and Vernier, '86). Protein ingestion would thus be capable of triggering a local immune response.

A general feature of the digestive tract of salmonids is the presence of a continuous collagen

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layer in the lamina propria, called the stratum compactum, which is associated with eosinophilic granule cells. These two structural components are absent in young fish and appear in the course of development (Kimura and Kudo, '75; Ezeasor, '86). A continuous layer of granule cells, the stratum granulosum, is present in 1-year-old fish (Woodward and Bergeron, '84). The stratum compactum and granular cells are believed to form a composite defense system, mechanical and humoral, which develops in response to demands by the environment (Ezeasor and Stokoe, '80).

We have verified that bGH passes through the intestinal barrier of trout into the circulatory system as well as the possible involvement in this transfer of granule cells which colonize the lamina propria.

MATERIAL AND METHODS

Rainbow trouts, *Salmo gairdneri*, (26 weeks old) weighing 30–45 g and raised in the laboratory in running water with constant flow-rate and temperature ($12 \pm 1^\circ\text{C}$) were fasted for 3 days before beginning experiments.

Anesthetized animals (0.3 ml of phenoxy-2-ethanol/liter of water) received 500 μl of 0.7% NaCl containing 1 mg of bGH (B1 from the National Hormone and Pituitary Program, NHPP; MW: 22,000) into the posterior intestinal lumen, by the anal way, with the aid of a catheter. Controls received 500 μl of 0.7% NaCl alone.

Blood and tissue samples were obtained 30 min, 1, 3, 4, 7, and 14 hr after bGH administration. For each time, three animals were used.

Plasma assays of bGH

Heparinized blood samples were taken from the caudal vasculature. After centrifugation, plasma were stored at -20°C until GH measurement by assays.

Reagents and bGH radioimmunoassay (RIA) procedure came from NHPP. Sensitivity was 0.2 ng/ml, and cross reactivity of salmonid GH was less than 1% (Le Bail et al., unpublished data).

bGH activity was tested using a mammalian receptor assay according to the technique of Gerasimo et al., ('79). bGH (USDA bGH I1) for standards and iodination came from NHPP. Sensitivity was 1 ng/ml (Le Bail et al., '89), and cross-reactivity of salmonid GH was about 10% (Nicoll et al., '87).

The coefficient of absorption of bGH was determined by applying the principle of occupation (Shimmins et al., '69). To determine the kinetics

of disappearance of bGH into the plasma, the dorsal aorta of two trouts was catheterized according to the technique of Zohar ('80). bGH (3 $\mu\text{g}/100 \mu\text{l}$ of NaCl 0.7%) was injected into the dorsal aorta, and 50 μl of blood was sampled every 30 min during 4 hr. Plasma was stored at -20°C before bGH radioimmunoassay. For calculations, total plasma volume was taken as 4% of animal body weight (Zohar, '82).

Immunoelectron microscopy techniques

Fragments of posterior intestine were fixed for 2 hr at ambient temperature with 0.5% glutaraldehyde in 0.1 M phosphate buffer (PBS), pH 7.4, then rinsed in the same buffer and embedded in Epikote 812. Ultrathin sections were deposited on copper grids coated with formvar. One drop of 1% of ovalbumin in PBS was deposited on each grid for 5 min. The sections were incubated 2 hr at ambient temperature with the anti-bGH (NHPP, diluted 1/500) and then rinsed with PBS. The protein A/colloidal gold (Sigma) complex was then deposited on each grid for 1 hr at ambient temperature. After rinsing with PBS and distilled water, the sections were counterstained with uranyl acetate for 15 min, then examined by electron microscopy.

RESULTS

The assay results (Fig. 1A,B) show that a fraction of bGH added to the posterior intestinal lumen reached the circulatory system. Transfer was rapid, since the plasma peak was situated between 30 and 180 min. Plasma load remained high until more than 7 hr after administration. Clearance appeared to be complete after 14 hr. The half-life of bGH, obtained from assay results (Fig. 1C) is about 44 min. The intubated bGH passed through the intestinal epithelium and entered into the general circulation is about 0.2%.

Immunocytochemistry was used to follow the bGH absorption process by the intestinal epithelium. The hormone could be visualized in the vacuoles of the apical hyaloplasm (Fig. 4), which were numerous at all stages of the observations (Fig. 2); visualization was difficult in the intercellular spaces between enterocytes (Fig. 4). The hormone was also present (Fig. 5) in vacuoles of various sizes in numerous macrophages infiltrated in this space (Figs. 2, 3). At the base of the epithelium, bGH crossed the basal membrane and could be seen disseminated in the interstitial space of the lamina propria. In all animals, the granule cells of the lamina propria, dispersed or

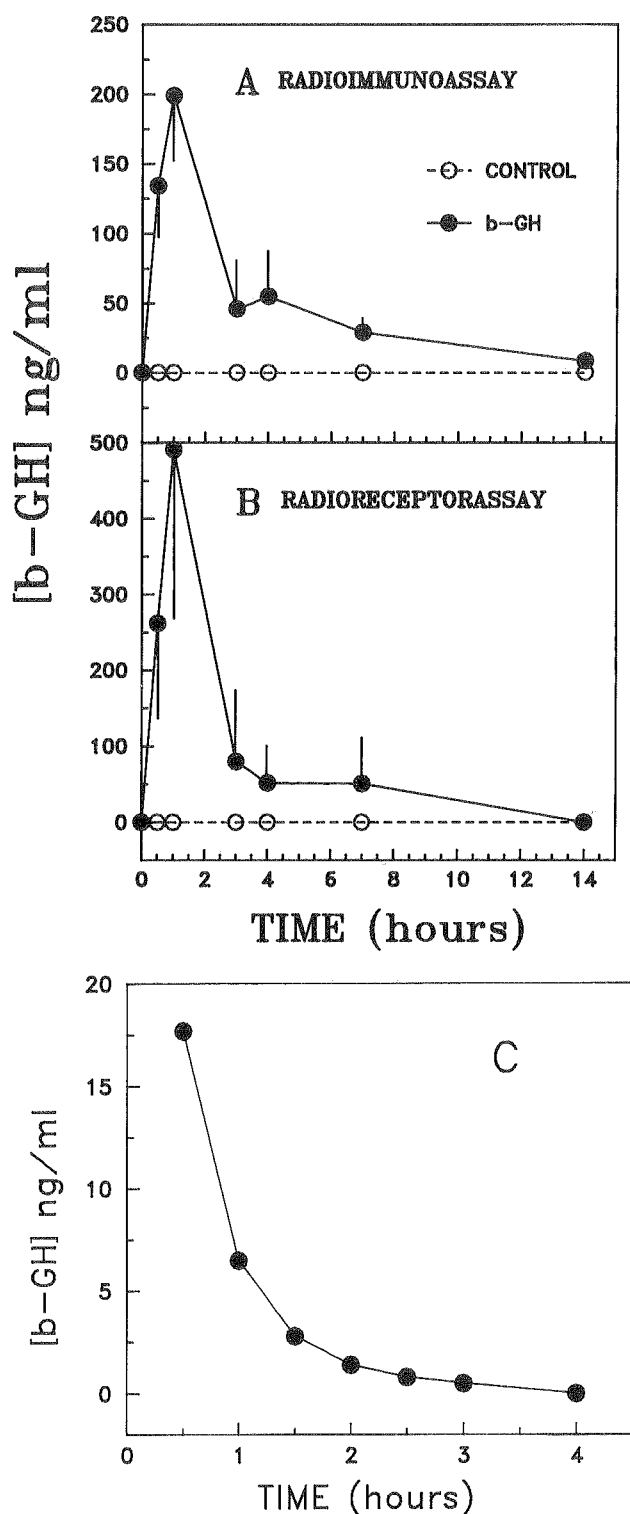


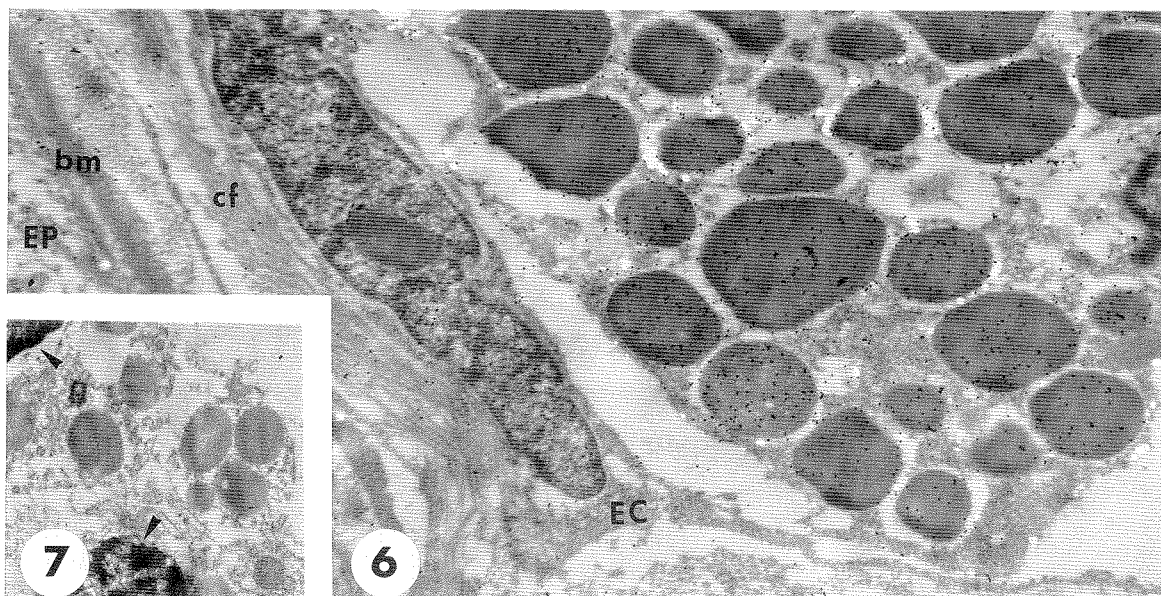
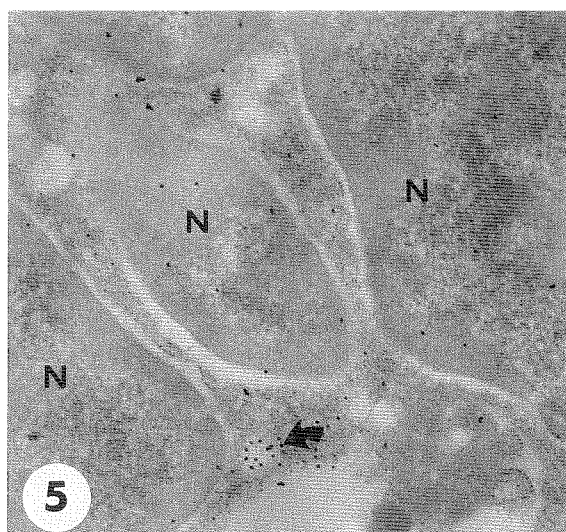
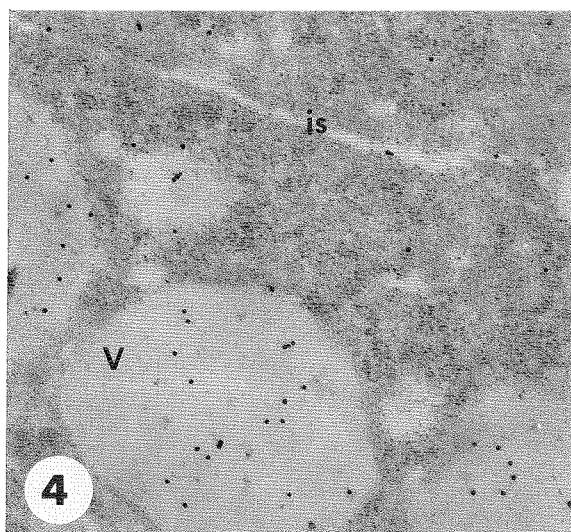
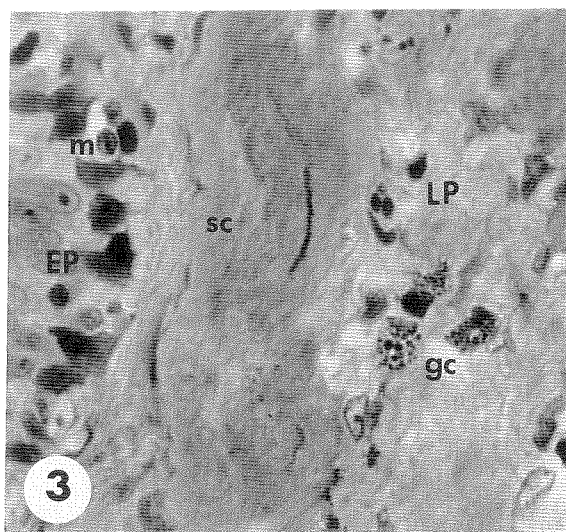
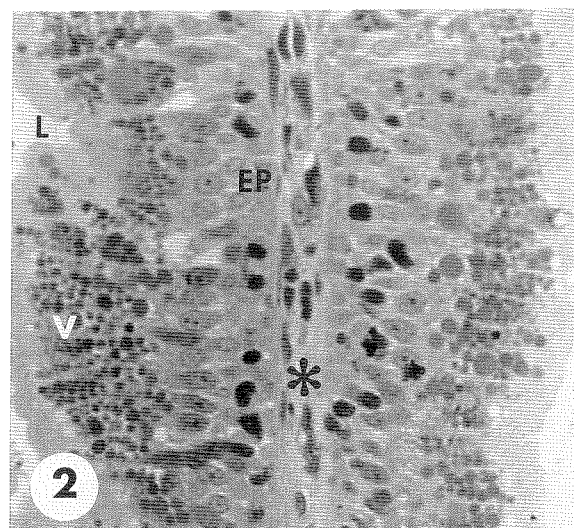
Fig. 1. A,B: Kinetics of appearance of bGH in the plasma after anal administration of bGH (1 mg/500 μ l NaCl 0.7%). Each sample corresponds to three animals. Vertical bars: standard error of the mean. A: RIA assay. B: RRA Assay. C: Kinetics of disappearance of bGH in trout plasma after intravenous administration of bGH (3 μ g/100 μ l NaCl 0.7%).

clustered immediately under the epithelium or in contact with the stratum compactum (Fig. 3), exhibited a spectacular and specific labeling of their grains (Fig. 6). This label was absent in the granule cells of control animals (Fig. 7).

DISCUSSION

Both zootechnical and ontogenic considerations were used for the choice of the animals, juveniles 26 weeks posthatching, bodyweight 30 to 45 g. On the one hand, it was economically advantageous to modify the growth curve early in the course of development. It has been found that fish undergoing growth gains early in development remain larger than their peers (Donaldson et al., '79). Agellon et al. ('88b) showed the efficacy of a recombinant growth hormone by the dip method in rainbow trout fries (1.32 ± 0.6 g) and by the intraperitoneal injection in yearling (77 ± 13 g) of the same species. Kawauchi et al. ('86) also obtained positive results with the latter method in 10 g salmon yearlings. In addition, administration at an early stage reduces the quantities of hormone required and consequently the cost of the experiments. On the other hand, results published by Bergeron and Woodward ('82) on the ontogenesis of the stratum granulosum of the rainbow trout intestine showed that after this stage, all the animals possess granule cells in the lamina propria. Although ontogenesis exhibits considerable interindividual variability, its steps are always the same. The cells are at first dispersed, subsequently forming agglomerates of four to five cells. Finally, they form a practically continuous cell layer, the stratum granulosum, in contact with the stratum compactum. At 12–13°C, this development occurs between weeks 24 and 34. At this time, the stratum granulosum is in place in all animals. We thus chose animals that could correspond to either of these steps and thus be able to test the hypothesis of the installation of a local immune system (Ezeasor and Stokoe, '80; Ezeasor, '86).

The results of plasma assays show the bGH transfer actually occurs. Up to more than 7 hr after administration, concentrations of bGH obtained by RIA can be considered as very high if they are compared to the level of endogenous GH (2 ng/ml: unpublished results of several hundred assays in trout of different sizes) and high when taking into account data on coho salmon yearlings in which the mean plasma GH concentrations are 32 and 41 ng/ml, respectively, in animals raised in sea water or fresh water, weighing



Figures 2-7

78 and 87 g (Bolton et al., '87). The higher levels of bGH obtained with RRA than with RIA are the usual ones found (Nicoll, '75). During bGH transfer, the binding sites with receptors are preserved, consistent with the hormone's remaining potentially active. Moore et al. ('86) showed that rectal administration of bGH enhances growth of hypophysectomized rats. The anal introduction of the hormone, in direct contact with cells that can absorb it, results in its rapid transfer. A pronounced plasma peak was observed as soon as the first hour. By analogy with results with horseradish peroxidase (HRP) (Georgopoulou et al., '86, '88; Sire et al., '88), it is possible that the oral administration of bGH leads to a slower and more prolonged transfer of bGH, with a less pronounced plasma peak. The transport of orally ingested salmon GtH through the digestive epithelium in goldfish is reflected by the ensuing high levels of salmon GtH in the plasma, the increases reaching maximal levels at 6 to 12 hr (Suzuki et al., '88).

The transepithelial passage of bGH occurs at the level of the absorbing enterocytes. Most bGH molecules that penetrate the cells are degraded: the vacuoles of the apical hyaloplasm where the hormone can be localized are phagolysosomes

(Georgopoulou et al., '85, '86). Molecules escaping degradation follow the same path as that described for HRP (Georgopoulou et al., '88; Sire et al., '88), i.e., intercellular space separating the enterocytes, interstitial space of the lamina propria, capillaries whose ultrastructure is the same as that of mammalian lymphatic capillaries. The posterior intestine of the trout is thus structurally and functionally comparable to the ileum of suckling rodents. In these animals, milk proteins are absorbed and degraded by the enterocytes (Wilson et al., '87). Furthermore protein fragments resulting from intracellular degradation, such as epidermal growth factor (Gonella et al., '87), as well as intact peptides, such as nerve growth factor (Siminoski et al., '86), may reach the lamina propria. The immunoelectron microscopy results of this study have for the first time shown the interference of granule cells of the lamina propria with the transepithelial transport of a protein. Regardless of the animal and the developmental stage of these cells (dispersed, clustered, arranged in a continuous layer), the administration of bGH results in a slight labeling of their hyaloplasm and very intense labeling of the granules; label is absent in the cells of control animals. The role of the stratum granulosum of the lamina propria has until the present remained unknown. Bergeron and Woodward ('82) showed that granule cells did not participate in the differentiation of the stratum compactum. Until now, no finding has supported the second hypothesis often advanced of their belonging to a local immune system (Ezeasor and Stokoe, '80; Ezeasor, '86) composed of mobile cells transported by the circulatory system (Bergeron, '82; Bergeron and Woodward, '83). The present results show that proteins, bGH in this case, are trapped by granule cells, which thus behave as immune cells. It is to be remembered that the transepithelial passage of a protein triggers an immune reaction in the posterior intestine (Georgopoulou and Vernier, '86); in addition, granule cells are observed in salmonid gills, skin, and nostrils (Roberts et al., '73).

The induction of growth in the rainbow trout by the oral administration of bGH should be performed in young subjects before the definitive installation of the stratum granulosum (week 34 at 12–13°C). As a result of biotechnology, the possibility of using salmon GH (Sekine et al., '85; Nicoll et al., '87) or even better that of rainbow trout (Agellon and Chen, '86; Agellon et al., '88 a,b) should lead to improved efficacy of oral administration of the hormone, avoiding interference by granule cells and enabling the treatment

Figs. 2–6. Sections of the posterior intestine of a 32 g troutlet, 1 hr after administering bGH. Figs. 2 and 3. Semithin sections stained with toluidine blue.

Fig. 2. Section at the level of a fold. Above the lamina propria (*), tall and thin enterocytes (EP) with a highly vacuolized hyaloplasm (V). The nuclei are in basal position. L, lumen ($\times 624$).

Fig. 3. At the base of the epithelium (EP), note the presence of numerous infiltrated lymphoid cells, particularly macrophages (m). Granule cells (gc), characteristic element of the digestive tract of salmonids, are present under the stratum compactum (sc) already well differentiated. LP, lamina propria ($\times 770$).

Figs. 4–7. Ultrathin sections. Protein A-colloidal gold. Fig. 4. Gold grains showing the presence of bGH are seen in the vacuoles (V) of the apical hyaloplasm of the epithelial cells and in the intercellular space (is) ($\times 30,000$).

Fig. 5. Fragment of the macrophage infiltrated between epithelial cells above the basal membrane, before uptake of bGH, sequestered in the vesicles (arrow). N, lobes of nucleus ($\times 18,000$).

Fig. 6. Granule cells situated just below the basal membrane (bm) having taken up bGH. This cell is surrounded by cytoplasmic projections of a fibroblast-type "enveloping cell" (EC). EP, epithelial cell; cf, collagen fibers ($\times 16,000$).

Fig. 7. Control animal. Two granule cells lacking specific label. Arrowheads; nuclei ($\times 8,000$).

to be prolonged beyond the installation of the stratum granulosum, or even to be applied at a later developmental stage.

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