The Uptake of Fluorescent Microspheres into the Skin, Fins and Gills of Rainbow Trout During Immersion

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ABSTRACT — To elucidate the process of the uptake of suspended particles into the skin, fins and gills of rainbow trout Oncorhynchus mykiss, 100 fingerlings were immersed in a suspension of 1 μm diameter fluorescent microspheres and rinsed with water. The fate of microspheres adhering to body surface was traced for elapsed times ranging from 1 min to 24 h. Although most microspheres were removed in a 2 h-rinse with water, a few were observed by light microscopy to be located in both epidermal and dermal tissues which had microscopic injuries. As observed by electron microscopy, microspheres were taken up by migrating epithelial cells, or embedded in the dermis covered with these cells. Some of those microspheres embedded in dermis were endocytosed by macrophages. Such features were confirmed in artificial wound experiments. We conclude that microscopic injuries to trout could be an important route for the uptake of antigen administered by immersion, 2 to 24 h after fish suffer from injuries.

Key word: migrating epithelial cell, uptake, fluorescent microsphere, immersion, rainbow trout, macrophage, injury

In fishes, there seems no doubt that environmental particles, including particulate antigens, are taken into the body surface tissue (Goldes et al., 1986; Zapata et al., 1987; Moore et al., 1998). Moore et al. (1998) indicated that the skin, rather than gills, plays a major role in the uptake of particulate antigens, and that the antigens are internalized primarily within epithelial cells and underlying phagocytes. We have found that fluorescent microspheres adhere to the superficial layer of microscopic injuries, which consists of the swollen epithelial cells and edematous collagenous matrices, and such injuries are undoubtedly induced frequently in the skin and fins during fish culture (Kiryu and Wakabayashi, 1999). We have, however, not yet observed if environmental microspheres are taken up into the body or not. This study aimed to trace the fate of fluorescent microspheres adhering to the body surface of trout by quantitative morphological analyses.

Materials and Methods

Materials

One hundred rainbow trout fingerlings (Oncorhynchus mykiss), weighing 1 to 5 g, were raised in conditions very similar to those in the previous report (Kiryu and Wakabayashi, 1999). The model particles, one-micron carboxylated polystyrene yellow-green fluorescent microspheres (Polyscience, Inc., U.S.A.) were used. They were suspended in 2 L of dechlorinated tap water in a 5 L plastic beaker with aeration at a final concentration of 1.0 × 10⁷ particles/mL, for immersion experiments.

Experimental design

The fish were divided into 5 groups, each of 20 fish, for 5 different immersion experiments as follows. In experiment I (Ex. I), quantitative analysis of microspheres in the skin and fins was carried out as described
later. For convenience, the word of fins was used to be separated from the skin in this study. In Ex. II, microspheres in the body surface tissues were observed by glycerol-gelatin mounted specimens, also as described later. Light microscopy of artificial wounds (Ex. III), light microscopy of spontaneous injuries in the integument and of normal gill tissues (Ex. IV), and electron microscopy (Ex. V) were performed in almost the same manner as those of a preceding article (Kiryu and Wakabayashi, 1999). For artificial wounds in Ex. III, 20 fish were wounded and divided into three subgroups consisting of 6 or 7 fish. Each of the subgroups was immersed in the suspension for 5 min, 8 h or 24 h, and all fish were examined histologically. For spontaneous injuries and gills in Ex. IV, 20 fish were divided into 2 subgroups: one was immersed in the suspension for 4 h, and the other for 24 h. The tissue sections from these fish were stained with toluidine blue or hematoxylin and eosin. For electron microscopy in Ex. V, 5 of the 20 fish were randomly sampled after 4 h- and 24 h-immersion, respectively.

Quantitative analysis of microspheres in the skin and fins

Twenty fish (2.4 to 3.1 g body weight) immersed in the suspension for 5 min were placed in an aquarium with running water to rinse off the microspheres adhering to the body surface. For quantitative analysis, we used eighteen of twenty fish randomly selected. The 18 were further divided into 3 subgroups each consisting of 6 fish. Fish of each subgroup were sampled 1 min, 2 and 24 h after the fish were rinsed with water, respectively. To quantify microspheres in the integument of both the skin and fins, the technique of Moore et al. (1998) was adopted. The tissues were dissolved in alkaline solution, and the released microspheres in this solution were counted under a fluorescence microscope (BX-60, Olympus Co., Tokyo).

F tests and t-tests were undertaken to detect significant differences between the means of the 2 subgroups: cases of 1 min and 2 h rinsing, and 2 and 24 h rinsing, respectively.

Processing of glycerol-gelatin mounted specimens

Twenty fish immersed in the suspension for 24 h were placed in an aquarium with running water for 24 h to rinse away the adhering microspheres. These fish were killed with anesthetic and fixed in a 4% paraformaldehyde (TAAB Laboratories Co., U. K.) solution in 0.1M phosphate buffer (pH 7.3) for 5 days. The skin of the body was then peeled off, and the fins and two gill arches were cut off. Each of the tissues was mounted on glass slides in glycerol-gelatin mounting medium (Muto Pure Chemicals Co., Tokyo). The distribution of microspheres in the tissues was examined under a fluorescence microscope.

Results

Quantification of microspheres in the skin and fins

In Fig. 1 the number of microspheres present in the integument (the skin and fins) is expressed as mean ± standard deviation. The number was remarkably reduced when rinsed for more than 2 h (P<0.01), and drifted to a low level 24 h after the fish were rinsed with water, although this was not significantly different from the level recorded at 2 h (P>0.05).

![Fig. 1. Relationship between the number of microspheres present in the integument and rinse time. Rainbow trout were immersed in a microsphere suspension for 5 min, then rinsed with water for 1 min, 2 h or 24 h. Mean+SD, n = six fish per group.](image)

Distribution of microspheres in the body surface

The microspheres present in the glycerol-gelatin mounted specimen appeared to be internalized in the skin, fins and gills at the rinse period 24 h after the fish had been immersed in the suspension for 24 h. The internalized microspheres formed clusters in the skin and fins. In the skin, the clusters measured approximately 0.2–1 mm in size (Fig. 2A). These clusters were scattered and diversified with patches. In fins, microsphere clusters accumulated together along the peripheral edges, forming a band approximately 0.1 mm in width (Fig. 2B). In contrast the internalized microspheres in the gills were randomly scattered (Fig. 2C).
Figs. 2–5. Micrographs of the body surface of rainbow trout immersed in microsphere suspensions. 2. Fluorescence micrograph of a glycerol-gelatin mounted specimen in vertical view of the body surface. In this case, 24 h-immersion was followed by a 24 h-rinse with water. A: skin; B: caudal fin; C: gill (primary lamellae with secondary lamellae). MP: melanophore. Note the internalized microspheres forming a cluster in Fig. 2A & B. Eight microspheres are visible in Fig. 2C. Bar = 200 μm. 3. Light micrograph showing artificial wounds to the skin after 5 min-immersion. Hematoxylin and eosin (H-E) stain. Arrow: swollen cells; B: bleeding; M: muscle fibers. Bar = 100 μm. 4. Artificial wounds to the skin after 8 h-immersion. H-E stain. A: light micrograph; B: fluorescence micrograph. Arrow: exposed tissue showing partial loss of the sheet of the migrating epithelial cells; arrow head: edematous induration or fibrous elements. Note re-epithelialization (RE) of the concave surface and microspheres in Fig. 4B. Bar in A = 100 μm, Bar in B = 25 μm. 5. Artificial wounds to the skin after 24 hour-immersion. H-E stain. A: low power magnified micrograph; B: high power magnified micrograph of re-epithelialized epidermis (RE) in Fig. 5A. Note Fig. 5B showing eosinophilic (degenerative) and basophilic (microsphere) granules in RE. Bar in A = 100 μm, Bar in B = 5 μm.
Migrating epithelial cells and microspheres in artificial wounds

In cross sections of skin incisions, the wound was observed to be lacerated and concave, and the bottom of the wound cavity reached the muscles. The wound showed tissue reactions after each period of immersion (5 min, 8 h and 24 h) elapsed following the incision (Figs. 3–5).

After a 5 min-immersion (Fig. 3), the edge of wounds had swollen epithelial cells which stained faintly with hematoxylin or toluidine blue. The concave surface of the incision which extended to the dermis and the muscle layer, was covered with swollen cells and edematous fiber elements, accompanied by necrotic cells, cellular debris and bleeding. Under fluorescence microscope, microspheres were found to adhere to those superficial layers including the swollen epithelial cells, in the wound.

After an 8 h-immersion (Fig. 4A), the swollen epithelial cells of the wound edge had sloughed off and disappeared. Instead, superficial tissues of the concave surface in the wound consisted of approximately 2 to 5 tiers of epithelial cells. Although the center of the bottom of the concavity was still exposed, individual epithelial cells in the sheet appeared to migrate from the wound edge to the bottom. The concave surface was characterized by re-epithelialization or epithelial hyperplasia in the healing wound. Such migrating epithelial cells, each of which was more elongated than normal and frequently connected with large intercellular spaces, took up microspheres (Fig. 4A & B), and occasionally contained eosinophilic granules in the cytoplasm (in such as Fig. 5B). In addition, necrotic epithelial cells were scattered in the sheet consisting of the migrating epithelial cells. The edematous induration or fibrous elements in the dermis were strongly eosinophilic, and microspheres remained in those tissues with edematous fiber elements and necrotic cells. The epithelial sheet appeared to seal such tissues containing microspheres (Fig. 4A & B).

After a 24 h-immersion (Fig. 5A & B), the concave surface consisting of more than 10 tiers of the epithelial cells exhibited further advanced hyperplasia, and these epithelial cells frequently contained more eosinophilic granules (Fig. 5B), than in the case of the 8 h-immersion. Although round cells such as lymphocytes were scattered around as an inflammatory response to the injuries, the relationship between the tissue reaction and microsphere location in the wound was essentially the same as that in the 8 h-immersion.

The features of the artificial wounds in the upper caudal fin were similar to those in the skin mentioned above. The basement membrane in the fins, however, was thicker than that in the skin and more prominent with the edematous changes.

Microspheres in spontaneous injuries of the skin and fins, and in the gills

In most cases where the skin and fins of the fish were immersed in the suspension for 4 or 24 h, microspheres were found in the epidermis and/or dermis of microscopic injuries which were regarded as spontaneous (Fig. 6). In spite of the variety of changes in such injured tissues, these findings were basically equal to those of the artificial wounds in the case of the 5 min- or 8 h-immersions mentioned above. The concavity of the injuries was irregular, and so tiny that the injuries affected only the epidermis or both the epidermis and the superficial layer of the dermis.

In some cases of the 24 h-immersions, microspheres were present in the completely re-epithelialized tissue of the injuries, and histological features hardly differed from those of the 24 h-immersion in the artificial wounds, except for the fact that no concavity was found in the re-epithelialized injuries. There were no microspheres in the normal control region that had no injuries in the skin and fins.

Regardless of whether fish were immersed in the microsphere suspension for 4 or 24 h, on the surface of the gill tissues, a cluster of microspheres was connected with a clump of mucus and debris lodged between the secondary lamellae. Furthermore, in the gill tissues,
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Microspheres were taken up more in cells of the primary lamellae than those of the secondary lamellae, but the tissue reaction was latent in both.

**Electron microscopic (EM) observation of the skin, fins and gills**

In the skin and fins of fish immersed in the suspension for 4 or 24 h, we reconfirmed by EM observation that microspheres were present in the microscopic injuries and in the completely re-epithelialized injuries, both of which occurred spontaneously. On the surface of the epidermis with microscopic injuries, microspheres adhered to the exposed tonofilaments of swollen superficial filament-containing epithelial cells (Fig. 7A). Some of the microspheres were associated with the exposed tonofilaments in the bottom of the injury cavity, which was surrounded by the migrating epithelial cells (Fig. 7B). By EM, the migrating epithelial cells were characterized by their elongated shape, the presence of the tonofilaments in the cytoplasm, and the large intercellular space surrounded by cell membranes and desmosome conjunctions (Figs. 7 & 8). We regard these features as those of migrating filament-containing epithelial cells. Such migrating epithelial cells took up microspheres (Fig. 8A & B). We also regard the eosinophilic granules, which were found in the epithelial cells under light microscopy, as the phagosome in the migrating filament-containing epithelial cells.

In the skin, microspheres were also taken up by macrophages which were present in both the primary lamellae and the secondary lamellae. Macrophages which were characterized by the presence of a phagosome in the cytoplasm and the lack of junctional complexes in the cell membrane, were sometimes located in intercellular spaces.

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In the case of epidermis with re-epithelialized injuries, microspheres were taken up by the filament-containing epithelial cells. No microspheres were found in intercellular spaces.

In the dermis, microspheres were embedded in either the edematous collagen matrices or the necrotic cells with electron-lucent cytoplasm (Fig. 9A). Macrophages and lymphocytes occasionally intruded into both the epidermis and dermis of the re-epithelialized injuries, and microspheres were endocytosed by these macrophages that were located in the scale pocket with edematous collagen matrices (Fig. 9B).

In gill tissue, microspheres were also taken up by macrophages which were present in both the primary lamellae and the secondary lamellae. Macrophages which were characterized by the presence of a phagosome in the cytoplasm and the lack of junctional complexes in the cell membrane, were sometimes located in intercellular spaces.

**Fig. 7.** Electron micrograph showing microspheres (arrows) in the swollen superficial filament-containing epithelial cells of the caudal fin. A: microsphere adhering to the exposed tonofilaments (TF). B: microsphere involved in the exposed tonofilaments of the filament-containing epithelial cells in the bottom of the microscopic injury cavity (MIC). Asterisks: migrating filament-containing epithelial cells; IS: intercellular spaces; DS: desmosome conjunctions. Bar in A = 1 μm, Bar in B = 7 μm
the most superficial layer, and the cell membrane of the macrophage was partially exposed to the environment (Fig. 10).

Discussion

Kiryu and Wakabayashi (1999) determined that suspended microspheres adhered to the surface of microscopic injuries in trout integument. In the present study, we successfully characterized the fate of this adherence according to the time elapsed since injury. Firstly, the quantitative analysis (Ex. I) showed that the majority of such microspheres were taken off in a 2 h-rinse with water, as illustrated by Fig. 1. Secondarily, morphological findings (Ex. II–V) showed that a few of the microspheres were internalized both in the epidermis and dermis while migrating epithelial cells covered the exposed injury surface for re-epithelialization. In the former, such a period of the time within 2 h to eliminate most of the adhering particles implies a particulate clearance, which is thought to be a self-defence mechanism against pathogen entrance or microorganism colonization in fish. In the latter, the findings of internalized microspheres elucidates the important role of injury sites as a

Fig. 8. Electron micrograph showing microspheres (arrows) taken up by the migrating filament-containing epithelial cells (ME) in the microscopic injury (MI) of the skin. A: low power magnified micrograph; B: high power magnification of ME in Fig. 8A. DS: desmosome conjunctions; IS: intercellular spaces; TF: tonofilaments. Note Fig. 8B showing microsphere surrounded by ribosomes with linear arrangement. Bar in A = 7 μm, Bar in B = 1 μm

Fig. 9. Electron micrograph showing a scale pocket (SP) of the skin. A: microsphere (arrow) in the intercellular matrices between a necrotic scale-forming cell (or hyposquama) and scale pocket lining (SPL). The necrotic cell shows a nucleus with high electron-density. B: microsphere in a macrophage. BP: basal plate; PS: phagosome. Bar in A = 2 μm, Bar in B = 3 μm
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It is well known that epithelial cells in wounded metatarsal pads of the guinea-pig bear the engulfment of particulate and colloidal materials (Platt, 1963). This is also known in the skin of apparently healthy rainbow trout (Peleteiro and Richards, 1990) and in cultured epithelium of Atlantic salmon (Asbakk and Dalmo, 1998). The present findings relating uptake of environmental particles by epithelial cells, were compatible with those in the skin of artificial wounds of Gasterosteus aculeatus (Phromsuthirak, 1977) and carp (Iger and Abraham, 1990) as follows: the migrating filament-containing epithelial cell appears in those wounds for re-epithelialization during the healing process, and takes up such particles. In our result, it should be emphasized that the microsphere which adheres to the surface of microscopic injuries is taken up by these migrating cells, and no free particles in water are directly taken up by these cells. The migrating epithelial cell also must play a role in internalization of suspended microspheres into the dermis, covering and sealing the exposed dermis. Once having been, as such, embedded in the dermal tissue, microspheres should be endocytosed by free or fixed macrophages in the dermis. Uptake of microspheres might be switched from the migrating epithelial cell to the macrophage though the switch mechanism is obscure. Some investigators agree that there is a relationship between the epithelial cells and the phagocytes (Peleteiro and Richards, 1990; Sanders and Wride, 1995).

The process of suspended particle uptake by the gills was well studied by Zapata et al. (1987) and Goldes et al. (1986). Zapata et al. (1987) examined the uptake by Atlantic salmon of the Yersinia ruckeri particulate antigen by electron microscopy after brief immersion in antigen suspension. Antigens were found in the gill mucus, adhering to and within pavement cells, and in mononuclear phagocytes lying directly beneath these cells. Goldes et al. (1986) studied the uptake of 5 μm clay particles by the gill of rainbow trout during immersion in suspension for up to 32 days, and reported the involvement of the same cell types. In those two reports, it was suggested that the particles are first taken up by the pavement cells, exocytosed and subsequently engulfed by underlying phagocytes. One new finding to emerge in our EM observations is that some of the microspheres which lodge between the secondary lamellae might be taken up directly by macrophages. A feature of glycerol-gelatin mounted specimens, in which internalized microspheres were scattered in the gill tissues, is presumably due to the action of macrophage endocytosing microspheres.

From our results, and in addition to the results of Moore et al. (1998), we propose that vaccine antigens, which are taken into the fish body surface tissues during immersion immunization, may do so in the same way that microspheres are taken up into the body surface tissues. To internalize more antigen into the body surface tissues, prolonged immersion immunization, such as 24 h-immersion, is known to be more effective than brief immersion (3–5 min) in concentrated suspensions (Moore et al., 1998; Ototake et al., 1998; Ototake et al., 1999). These findings are consistent with the migration of epithelial cells, as described in the present study. Additionally, once a wound cavity is sealed with migrating epithelial cells, these cells cease migrating (Bereiter-Hahn, 1986) and taking up environmental particles (Phromsuthirak, 1977). Generally, it takes 3–24 h for closure of incisions in fish skin by migrating epithelial cells (Mittal and Munshi, 1974; Anderson and Roberts, 1975; Phromsuthirak, 1977; Hickey, 1982; Rai and Mittal, 1983; Roubal and Bullock, 1988; Iger and Abraham, 1990). Thus, injuries to rainbow trout could be a valuable route for the uptake of antigen administered by immersion, 2 to 24 h after fish suffer from injuries. In order to elucidate further mechanism of the immersion immunization of fish, there is much room for a study on the relationship between the migrating epithelial cells and macrophages.

Fig. 10. Electron micrograph showing three microspheres (arrows) in a macrophage in a primary lamella of a gill. Arrow head: the cell membrane of a macrophage partially exposed to the environment; PS: phagosome; asterisk: some material (other than microspheres) in phagolysosome; N: nucleus of a macrophage. Bar = 3 μm
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