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# Transmission Electron Microscopic Studies on Bacterial Gill Disease in Rainbow Trout Fingerlings*

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**Abstract** Gill epithelia of rainbow trout fingerlings with bacterial gill disease were examined by transmission electron microscopy. Morphological alterations of the diseased epithelia started at hypertrophy of the lamellar epithelium. The hypertrophy was characterized by transfiguration of the epithelial cells into a cuboidal or columnar shape and of the chloride cells into an oval or spherical shape, and by infiltration of wandering cells. This was followed by the appearance of globoid structures on the lamellae and plasma membrane vesiculation (or blebbing) suggesting cell injury. The formation of these globoid structures progressed by macrophagial clearance of the degraded cytoplasm of transfigured epithelial and/or chloride cells leaving the flat peripheral cytoplasm and nucleus. Further, the hyperplastic lesion progressed in the order of fusion of adjacent gill lamellae, profuse proliferation of epithelial cells, clubbing of gill filaments and fusion of adjacent gill filaments. The relationship between the progression in hyperplasia and morphological changes in epithelial, chloride, and mucous cells was also examined. Ruthenium red staining clearly revealed adhesion between the cell wall substance of bacterial cells and the surface of the outermost layer of cells in the gill epithelium, appearance of gap junction between epithelial cells in the process of fusion of adjacent gill lamellae, and plasma membrane vesiculation due to cell injury.

Bacterial gill disease is known to be highly fatal to salmonid fingerlings in many hatcheries and is histopathologically characterized by hyperplasia of gill epithelia. In spite of the controversy concerning the causative agents of the disease, histopathological descriptions have contributed to its understanding (see Rucker et al., 1952; Wood and Yasutake, 1957). The question of proliferation of epithelial cells is of particular interest and attraction. Recently, we have frequently observed fusion at the distal tips of adjoining gill lamellae in diseased trout fingerlings. This is also of great interest in relation to the binding or interaction between epithelial cells. No attempts to resolve these questions have been made, at least, in the bacterial gill disease. Further, there is little useful ultrastructural description to help resolve the questions in the fields of experimental morphology and physiology, except for a few reviews (Hughes, 1980, 1982; Laurent, 1982). Therefore, in addition to descriptions of gill filament and lamellar epithelia (Bettex-Galland and Hughes, 1973; Morgan and Tovell, 1973) and on secondary lamellae development (Morgan, 1974), it is of great importance to understand the ultrastructural differences between the cell organization of normal and diseased gill lamellae and filaments. To understand the underdeveloped gill epithelium, especially the gill lamellar epithelium, the use of trout fingerlings as material is significant because of the higher susceptibility of these animals to bacterial gill disease. Detailed knowledge of epithelial organization is essential to histo- and cytopathological studies on the disease (Kimura and Kudo, 1979).

From the diseased gills of *Salmo gairdneri* and *Oncorhynchus masou* (Kimura et al., 1978) we have recently isolated the bacterium transmitting the gill disease. This isolation may contribute not only to the elucidation of the sequence of pathological events of this disease but may also reveal some of the complex mechanisms for the proliferative induction of epithelial cells and fusion of gill lamellae or filaments. To dissect in detail the morphogenetic events of bacterial gill disease, therefore, we designed a series of experiments. Before these experiments could be done, however, we found it necessary to have a detailed cytopathological portrait of the steps of the disease and an ultrastructural description of the lesions. The purpose of this paper is to de-

* Ultrastructural Studies on Bacterial Gill Disease in Rainbow Trout Fingerlings—I

fine the ultrastructural differences between gill epithelia before and following bacterial infection, the developmental process of fusion of gill lamellae or filaments, and to use these findings for a better understanding of the formation of hyperplasia, the new binding between epithelial cells on adjoining gill lamellae, and the recovery of hyperplastic lesions in an experimentally induced gill disease.

## Materials and methods

Thirty rainbow trout fingerlings, *Salmo gairdneri* Richardson, suffering from bacterial gill disease and twenty healthy ones were used for the present investigation. Their average body length and body mass were approximately 39 mm and 1 g, respectively. Gill tissues were excised from fresh fingerlings and fixed for 2 to 3 hrs in 2.5% glutaraldehyde buffered with an ice-cold, 0.1 M cacodylate buffer (pH 7.3) followed by thorough washing with the same buffer containing 5% sucrose and osmication with the buffered 1% osmium tetroxide. The gill tissues were dehydrated by a graded series of cold ethanol and embedded in Epon 812. Ultrathin sections, doubly stained with uranium and lead, were examined by a JEM 7A or JEM 100C type transmission electron microscope.

Ruthenium red (RR) staining (Luft, 1971) was applied to gill tissues to clarify the adhesion relationship between the bacterial cells and gill epithelial cells, degeneration of the plasma membrane and fusion of adjoining gill lamellae or filaments.

## Results

**Normal gill epithelium.** Only those findings necessary to understand the morphology of bacterial gill disease will be described because the authors have recently reported on the ultrastructure of the gill epithelium of healthy trout fingerlings (Kimura and Kudo, 1979). The epithelium of gill lamella examined consisted of a single or double layer of cells (Fig. 1). Cell organization was usually characterized by a mosaic arrangement among the three cell types of epithelial, chloride and mucous cells, such as a double layer of the epithelial cells (proximal and distal or outermost), proximal epithelial cells and somewhat flat chloride or mucous cells, or a single layer of epithelial or chloride cells. The mosaic pattern varied in each gill lamella. In ultrathin sections the greater part of the lamellar epithelium, except the tip, was sometimes covered with flat chloride cells. Cytoplasmic extensions of cells facing the free surface were usually connected to one another by junctional complexes, and by desmosomes with underlying adjacent cells. Between a double layer of epithelial cells, there was sometimes overlapping or interdigitation of cytoplasmic projections in a rather complex and irregular manner. The free surface of the outermost epithelial cells had varying numbers of irregularly arranged microvillus-like projections. In sections tangential to their free surface, branching and anastomosing structures were observed. These were thought to be identical with microridges seen in scanning electron microscopic observations (Kimura and Kudo, 1979). The epithelium of the gill filaments was comprised of a double or triple layer of the same types of cells as those in the gill lamellae.

In addition to flat epithelial cells, the cytoplasmic matrix of which looked moderately electron dense, there frequently appeared epithelial cells with a relatively less dense and thick cytoplasm, especially in the lamellar epithelium (Fig. 2). These did not appear in adult gill lamellae. These cells often had vacuoles corresponding to autolysomes in which were contained a flocculent material and meandering or vesicular membranous elements. They could also be observed in enlarged intercellular spaces, suggesting a partial degradation or diminution of the cytoplasm. The degradation never progressed towards the free surface of the cells. Decrease in number of the vacuoles (or autolysomes) in the epithelial cells was correlated with both flattening of the cells and the decrease of vesicular membranous elements in the intercellular spaces.

**Diseased gill epithelium.** Etiological bacterial cells in bacterial gill disease were slightly separated from the surface or the tips of microvillus-like projections of epithelial, chloride and mucous cells (Fig. 3). RR staining revealed that the interstices of both cells were caused by positive substance of the bacterial cell wall (Fig. 4). Many of the bacterial cells frequently fell away from the surface of the gill epithelium during the histological preparation. This may have been caused by

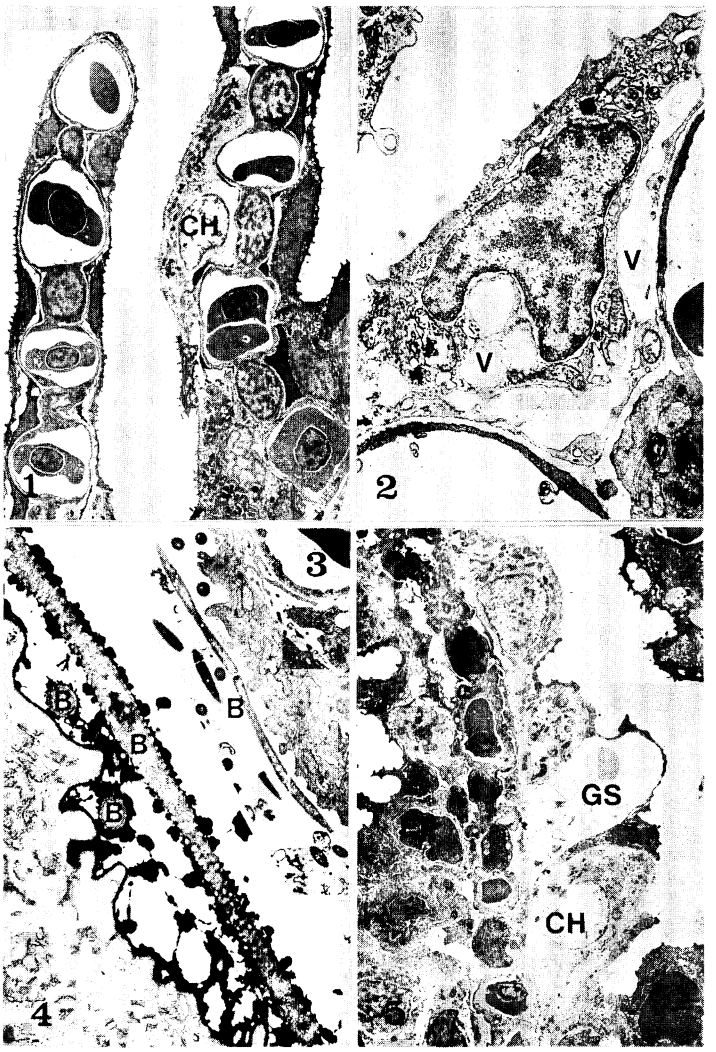


Fig. 1. Gill lamellae in a healthy trout fingerling. The epithelium consists of a single or double layer of cells. CH, chloride cell. ×1,820.

Fig. 2. Lamellar epithelial cell with a relatively less dense and thick cytoplasm. A few vacuoles (V) containing membranous vesicular elements are seen in the cytoplasm and intercellular space. ×5,740.

Fig. 3. Contact of bacterial cells with a lamellar epithelial cell. Two chained bacterial cells (B) in a longitudinal section are in contact with the cytoplasmic projections of the lamellar epithelial cell with narrow interstices. ×4,060.

Fig. 4. Relationship between a bacterial cell wall substance and the surface of a lamellar epithelial cell. RR staining shows that the positive granular material of bacterial cell wall adheres to the surface of the epithelial cell. B, bacterial cell. ×10,430.

Fig. 5. Hypertrophy of a gill lamella. The morphology of epithelial and chloride cells alters remarkably as compared with that of the cells in normal trout fingerlings. Cell debris is seen in a globoid structure (GS). CH, chloride cell; EP, epithelial cell. ×1,260.

the discharge of mucous substance from mucous cells. In the comparatively wide region between a morphologically normal portion and an advanced pathological portion, a remarkable hypertrophy of gill lamellae occurred usually starting at the extreme distal tip of the lamellae.

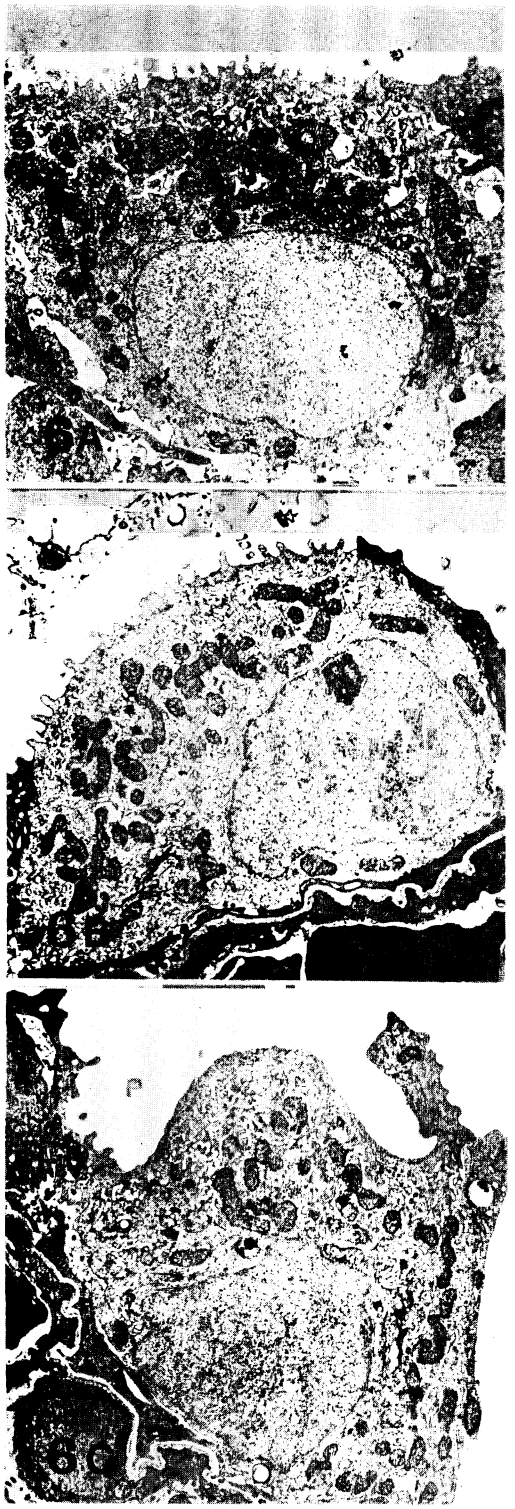

Fig. 6. Relationship between dome formation and density of cytoplasmic projections in the chloride cell. A, cytoplasmic projections are thin and high in number in the cell lacking the dome formation. B, cytoplasmic projections are thicker and lower in number than in A. C, a remarkable dome lacks cytoplasmic projections. ×4,000.

This hypertrophy was characterized by morphological changes of epithelial and chloride cells, and the infiltration of wandering cells such as macrophages or neutrophil leukocytes. The lamellar epithelial cells, especially the outermost (or distal) ones, were characterized by a columnar or cuboidal shape which often jutted out to the outside, and the thickening and simultaneous obvious decrease of microvillus-like projections (Fig. 5; also see Kudo and Kimura, 1983). In such regions, there was no cell proliferation in the lamellae. Chloride cells were, in general, spherical or oval in shape, with a surface which domed from the lamellar epithelium. On their free surface, different quantities of microvillus-like projections were found which varied from cell to cell. In general, the degree of cell surface dome formation appeared related to projection density, with some exceptions: the closest and thinnest showed lack or poorness of the dome (Fig. 6A) and vice versa (Fig. 6B, C). The plasma membrane vesiculation at projection tips was most remarkable in the former and poorest in the latter. The decrease in microvillus-like projections was nearly parallel to their thickening, shortening and irregularity such as branching, and also to the decrease of plasma membrane vesiculation. Therefore, the relationship between the decrease and the thickening might be caused by incorporation of some of the projections. The plasma membrane vesiculation was characterized by the formation of vesicles suggestive of a partial exfoliation of the tip plasma membrane. The vesicles frequently contained one to three smaller membranous vesicles of various sizes. In such, or more advanced lesions plasma membrane vesiculation also occurred in the epithelial cells, although not always at the tips of microvillus-like projections. RR staining clearly revealed the vesiculation to be a partial exfoliation of the plasma membrane from the cytoplasm (Fig. 7), but the vesicles did not contain smaller vesicles as in chloride cells. Such epithelial cells had a relatively small number of thick microvillus-like projections on the free

surface. Further, large ring-like or globoid structures (14~27 $\mu$m in diameter) frequently appeared on the gill lamellae. Their wall was constituted by thin cytoplasmic extensions of two or three epithelial cells, with occasional participation by chloride cells. While they were sometimes empty, they generally contained material relating to cell death and degeneration such as cell debris, degenerating cell organelles, flocculent material, or sometimes macrophages showing structural variations. The globoid structures are probably identified with "plaques". They frequently appeared at the tip and flat lateral sides of the gill lamellae, suggesting their possible formation through morphological alteration (Fig. 8A, B, C). In such regions hyperplasia of the interlamellar epithelium and fusion of the gill lamellae were in progress. Plasma membrane vesiculation also was very striking in the epithelial and chloride cells.

The fusion of gill lamellae occurred in a comparatively wide region between an advanced lesion and a structurally normal portion. It generally started between the tips of lamellar epithelial cells and infrequently at the lateral sides near the tips (Figs. 9, 10). At the beginning of the fusion gap junctions were observed between the plasma membranes of adhering epithelial cells, and these were more sharply revealed by the reaction product after RR staining (Fig. 11). The fusion at the tips of adjacent gill lamellae resulted in the formation of an interlamellar cavity (Fig. 10). In such a lamellar epithelium, no proliferation of lamellar epithelial cells was found, but interlamellar (or filament) epithelial cells increased in number to narrow the interlamellar cavity. This was followed by the disappearance of the cavity and enclosing of chloride cells and sometimes of mucous cells in the interior of the hyperplastic lesion. In addition to such a pattern, proliferation of interlamellar epithelial cells also resulted in fusion of the lamellae by filling the greater part of the interlamellar space (Fig. 12). In such cases, sometimes only the tips of adjacent gill lamellae projected from the hyperplastic lesion. This hyperplastic epithelium was often made up of more than seven layers of epithelial cells, a few of these with a mitotic figure, chloride cells located only in the outermost layer and infiltration of macrophages or neutrophil leukocytes. Many of the mitotic figures

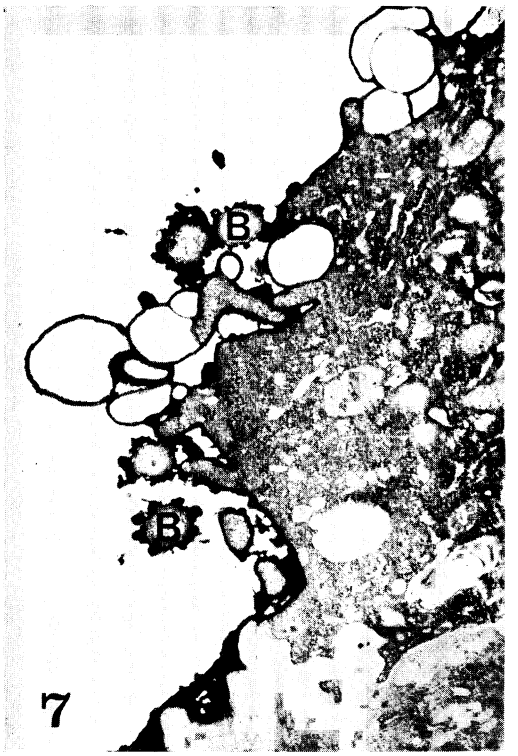


Fig. 7. Plasma membrane vesiculation. RR staining clearly shows a partial exfoliation of the plasma membrane from the cytoplasm. B: cross-sectioned bacterial cells. ×14,900.

were seen in epithelial cells of the gill filament, except for a few cases from the distal lamellar cells.

A combination of profuse proliferation of epithelial cells in gill filaments and the fusion of gill lamellae not only made it difficult to distinguish individual gill lamellae but also caused the distal end of the gill filaments to have a club-like form. Cell organization in such lesions was characterized by degenerating chloride cells, a great number of spindle-like, polygonal, or irregular epithelial cells, wandering cells such as macrophages or neutrophil leukocytes, strands of morphologically distorted pillar cells, and sometimes mucous cells and lymphocyte-like cells. The degenerating chloride cells were generally located in the interior of the lesion, along the strands of pillar cells or thin cytoplasmic extensions of flat epithelial cells which were in close conformation to the basement lamina, or were interposed among other types of the above-

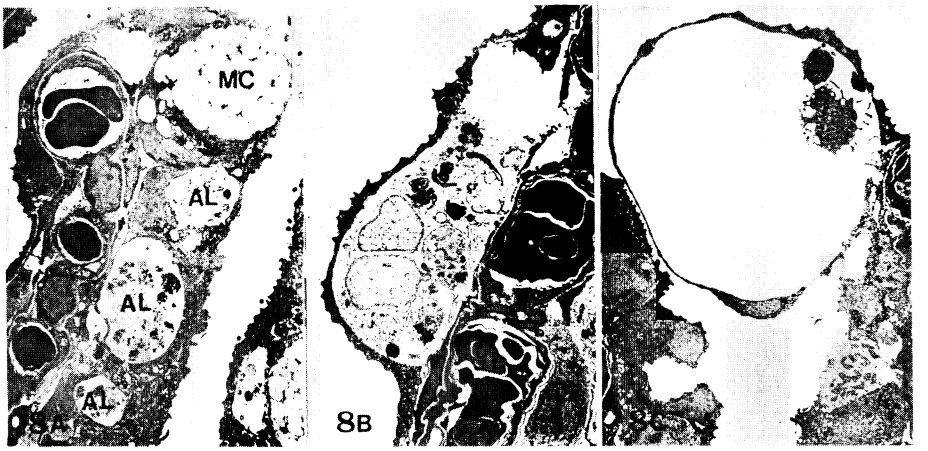


Fig. 8. Formation of globoid structure. A, autolysomes (AL) in the lamellar epithelial cells are seen, suggesting the earliest stage in the formation of a globoid structure. MC, mucous cell. ×1,260. B, macrophage containing phagocytized materials suggests clearance in a globoid structure. ×1,260. C, the interior of a globoid structure shows more progressive clearance by macrophage. ×900.

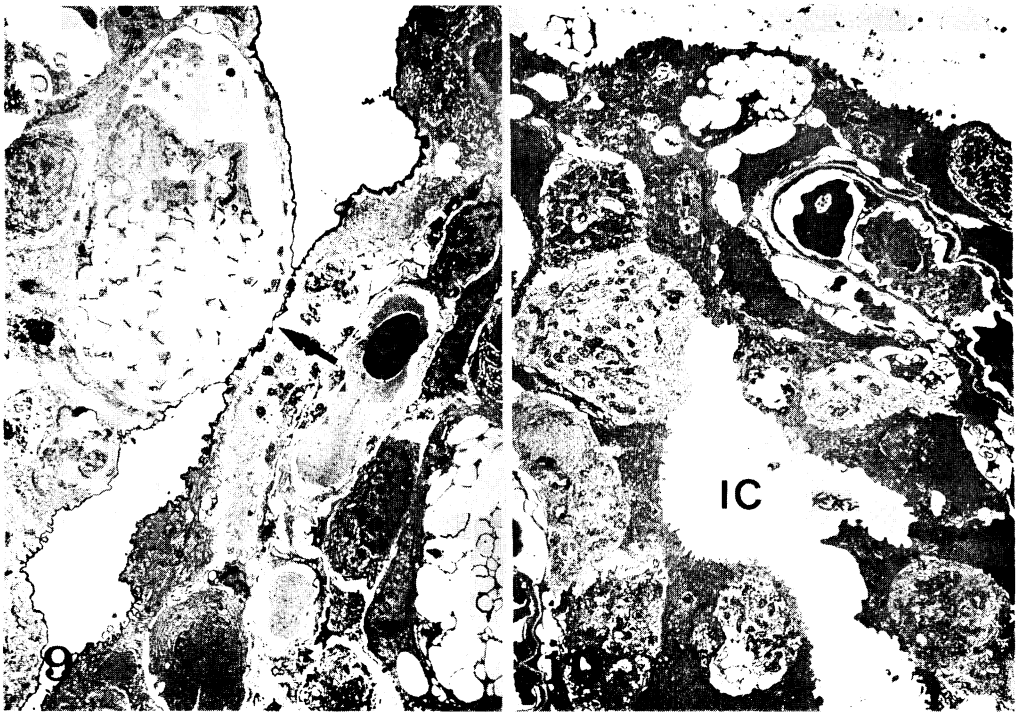


Fig. 9. Fusion of adjacent gill lamellae at lateral sides. See Fig. 11 for enlargement of area of arrow. RR staining. ×2,000.

Fig. 10. Fusion of gill lamellae at the tips. The fusion forms an interlamellar cavity (IC). ×1,800.

described cells. Their morphological characteristics were the existence of varying numbers of lysosomes or a few autolysomes and networks of smooth endoplasmic reticulum whose intracisternae were enlarged, an extremely irregular outline caused by progressive degeneration in

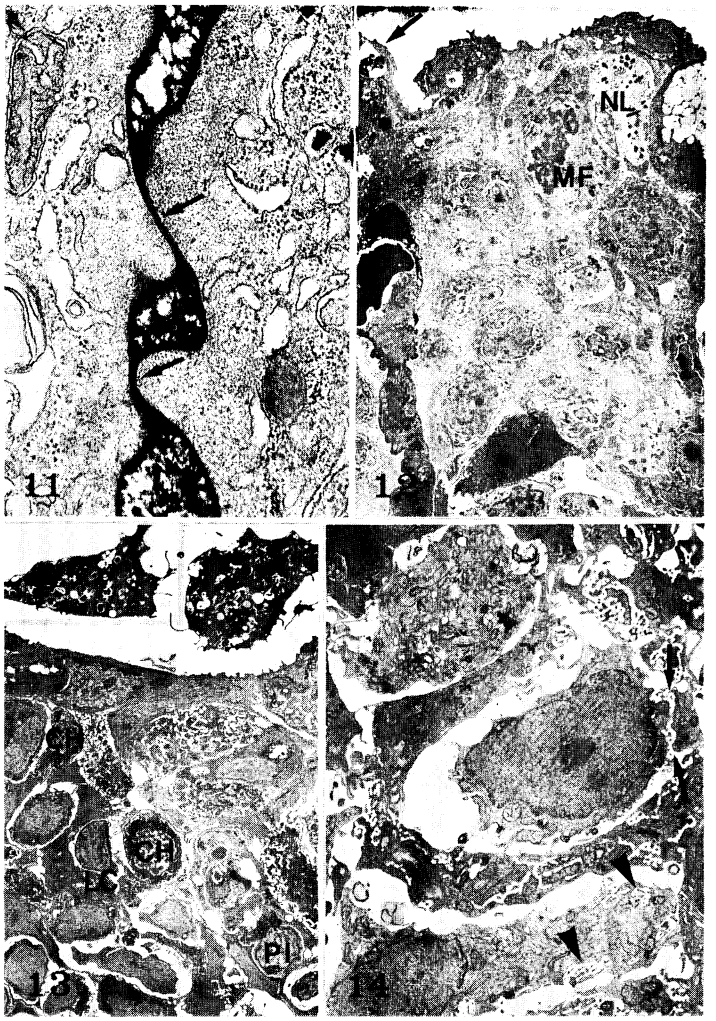


Fig. 11. Gap junctions between epithelial cells (arrow). Enlargement of portion of Fig. 9. RR staining. ×28,630.

Fig. 12. Proliferation of epithelial cells in the interlamellar epithelium. Neither chloride cell nor mucous cell is enclosed in the interior of the lesion. Arrow shows the tip of gill lamella. MF, mitotic figure of an epithelial cell; NL, neutrophil leukocyte. ×1,260.

Fig. 13. Chloride cells shut up in the lesion and lymphocyte-like cell (LC). The lesion corresponds to region B in fig. 3 (Kudo and Kimura, in press a). The chloride cells (CH) have small clusters of smooth endoplasmic reticulum in the perinuclear cytoplasm. Epithelial cells of the outermost layer are exfoliating from the surface of the hyperplastic lesion. PI, pillar cell. ×1,260.

Fig. 14. Chloride cell during the cytoplasmic "cutting-away" or "fragmentation". The cell is connected with cell debris in the range shown by arrows. Two small clusters of smooth endoplasmic reticulum are seen in an elongated cell (arrowhead). The latter morphology possibly causes a partial cytoplasmic "cutting-away" or "fragmentation" in a chloride cell. ×3,780.

the peripheral cytoplasm (Fig. 13) and by the cytoplasmic "cutting-away" or "fragmentation" of the degenerating peripheral cytoplasm, and occasionally cytoplasmic continuities with one or more pieces of cell debris around them (Fig. 14). The "cutting-away" or "fragmentation" resulted in cells with a thin perinuclear cytoplasm (Fig. 13). These were generally oval but