In vitro antigen trapping by gill cells of the rainbow trout: an immunohistochemical study

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Summary. An *in vitro* assay was used to study the involvement of gill cells in the trapping and processing of particulate antigens. Gills were routinely processed for light microscopy after being placed in medium containing either *Yersinia ruckeri* O-antigen-labelled fluorescent beads, unlabelled fluorescent beads, *Y. ruckeri* O-antigen or formalin-killed *Y. ruckeri*, for 0, 30 s, 1, 5 and 30 min. *Y. ruckeri* formalin-killed cells, *Y. ruckeri* O-antigen and fluorescent beads labelled with *Y. ruckeri* O-antigen were taken in by gill epithelial cells as soon as 30 s after administration. In contrast, unlabelled fluorescent beads adhered to the epithelial cell membranes, but did not occur inside the gill cells. These results are discussed principally in relationship with the specificity of antigen trapping.

Key words: Antigen-trapping, Gills, O-antigen, Rainbow trout, *Yersinia ruckeri*

Introduction

The route of antigen entry is of great importance in the expression of the vertebrate immune response. In fish, topical immunization by bath, spray, flush exposure or shower, has been demonstrated to be an effective method for inducing protection against various fish pathogens (Fender and Amend, 1978; Anderson et al., 1979). However, both the route of entry and the mechanism of uptake of the antigens, are largely unknown.

In bath administration, skin, gills and gut epithelium seem to be involved in antigen uptake (Fender and Amend, 1978; Tatner et al., 1984), but the gills have been claimed as the main site of *in vivo* antigen trapping and processing by most authors (Smith, 1982; Nelson et al., 1985). Moreover, Alexander et al. (1981) demonstrated *in vitro* the entry of bacteria through the

gills. The gills, especially the secondary lamella in which blood and water are separated only by two layers of epithelial cells and thin projections of pillar cells, are structurally suitable organs for antigen trapping. Nevertheless, the mechanism of antigen uptake after bath immunization, as well as the gill cells involved in it, have not been explained completely.

In previous studies we demonstrated the trapping of both formalin-killed *Y. ruckeri* and O-antigen by salmonid gill cells ultrastructurally (Zapata et al., 1987). In the present work, we have challenged gills *in vitro* with antigenic and non-antigenic materials to clarify the mechanisms involved in antigen uptake, especially the specificity of the process.

Materials and methods

Tissue samples

Gill pieces were aseptically removed from 200 g rainbow trout *(Oncorhynchus mykiss)*, anaesthetized with a solution of 0.4% MS-222 (Tricaine-Methanesulfonate, Sandoz) in water, obtained from stock fish held at the National Fish Health Research Laboratory facilities (Kearneysville, West Virginia, USA). After their *in vitro* exposure to antigens, the gill pieces were fixed in 10% phosphate-buffered formalin, embedded in paraffin and sectioned. The 7 μm sections from the samples exposed to fluorescent beads, were counterstained with Harris's haematoxylin and observed under a Nikon Labophot fluorescence microscope. On the other hand, sections of the samples exposed to *Y. ruckeri* O-antigen or to *Y. ruckeri* formalin-killed cells were immunohistochemically studied using a peroxidase-antiperoxidase (PAP) method. After blocking endogenous peroxidase activity with freshly-prepared 0.5% H_2O_2 in methanol and the non-specific binding sites with normal goat serum (1:40, 30 min), the sections were incubated with either rabbit anti-O-antigen serum or rabbit anti-*Y. ruckeri* (1:200, overnight at 4 °C), then treated with goat anti-rabbit IgG (1:40, 30 min), as

secondary antibody and, finally with rabbit-PAP serum (1:100, 20 min). All the antisera came from Dako (Denmark), except for the specific ones which were from Fish Biologics, NFHRL (Kearneysville, USA). For demonstrating the horseradish peroxidase activity, the sections were treated with 0.5% 3,3'-diaminobenzidine-tetrachloride buffered in 0.05 M Tris containing 0.15% hydrogen peroxide. Methylene blue was used for counterstaining.

Preparation of beads

Labelled fluorescbrite carboxylate microspheres (Polysciences Inc., Warrington, Pennsylvania, USA), having a diameter of 1.16 μm, were coated with *Y. ruckeri* O-antigen by heat incubation at 37 °C for 1 hour.

Preparation of bacterial antigens

Formalin-killed cells and O-antigen (obtained in accordance with Edwards and Ewing, 1972) were suspended in the culture medium, at a concentration of 125 μg/ml calibrated on a spectrophotometer (Bausch and Lomb, Inc.) at 525 nm, of 40% Transmittance.

Experimental design

Gill pieces were placed in Petri dishes with 7 ml of Eagle's minimum essential medium containing 2% fetal calf serum ($EMEM_2$). Baths of this medium contained suspensions to test antigen uptake. Two different experiments were carried out. In the first, the gill pieces were exposed for 0, 30 s, 1, 5 or 30 min to either fluorescent beads labelled with *Y. ruckeri* O-antigen or unlabelled fluorescent beads which were added to the medium. In the second experiment, the incorporation of either *Y. ruckeri* O-antigen or *Y. ruckeri* formalin-killed cells in cultured gill samples was tested. The gill pieces were placed in $EMEM_2$ containing formalin-killed cells and O-antigen. After antigen exposure for 0, 30 s, 1, 5 or 30 min, the samples were fixed, embedded and antigen detected.

In both experiments, negative controls consisting of gill samples cultured without antigens were processed and observed in the same manner as the experimental sections. Furthermore, the PAP method was applied to some immunized samples not exposed to the primary antibody. Samples from 20 fish were used in the experiment, one for each time point in each of the four experiments.

Results

At time 0, fluorescent beads conjugated with O-antigen, as well as both free O-antigen and *Y. ruckeri* formalin-killed cells occurred in clusters among the primary lamellae of the gills. Sometimes, they were also stuck to the epithelial cell surfaces of the secondary lamellae (Fig. 1). This last condition was more frequent

30 s after immunization. Then, clusters of antigens appeared principally on the apical tips of secondary lamellae cells. In contrast, fluorescent beads without antigen appeared embedded among mucous material among the primary lamellae, but they did not gain access to the gill epithelial cells.

One minute after immunization and at the following times tested (5 and 30 min), fluorescent beads conjugated to O-antigen occurred inside the gill epithelial cells (Fig. 2). Unlabelled beads, however, appeared near the gill surface but were never found inside the epithelial elements (Fig. 3).

Likewise, O-antigen *Y. ruckeri* formalin-killed cells detected by PAP method appeared in the pavement epithelial cells of gills (Fig. 4) and in gill phagocytic cells (Fig. 5). In this case, *Y. ruckeri* cells were apparently trapped more rapidly by gill cells than was the O-antigen as they occurred inside the gill epithelial cells earlier. Some images also suggested their presence outside the cells in the intercellular spaces (Fig. 6). Finally, they seemed to be present inside the endothelial cells of the blood vessels of the primary lamellae (Fig. 7).

Discussion
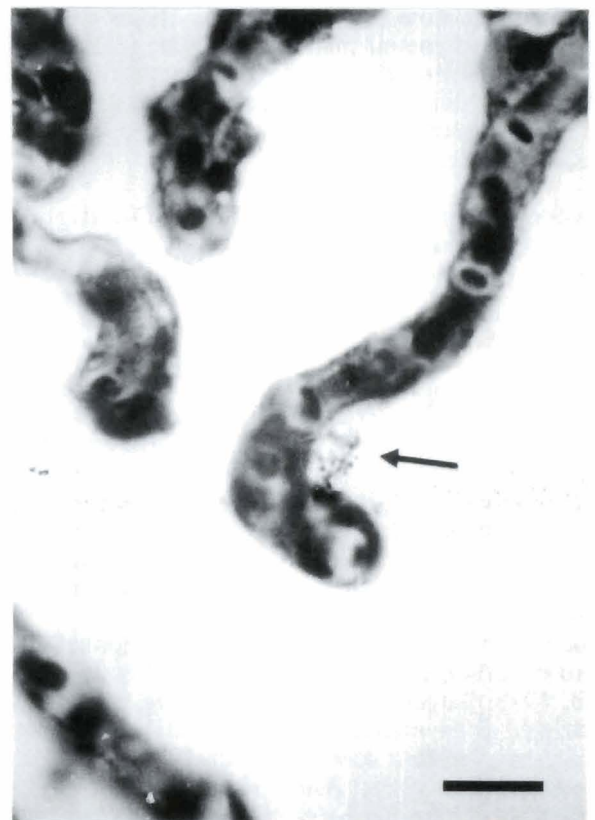
Our current *in vitro* results confirm previous data from

Fig. 1. A cluster of formalin-killed *Y. ruckeri* (arrow) occurs adherent to the secondary gill lamella sampled immediately (time 0) after *in vitro* exposure to medium containing the killed bacteria. Bar = 10 μm.

Fig. 2. Antigen-bearing beads appear in the cytoplasm of an epithelial cell (arrow) 5 minutes after bath exposure. Bar = 10 μm.
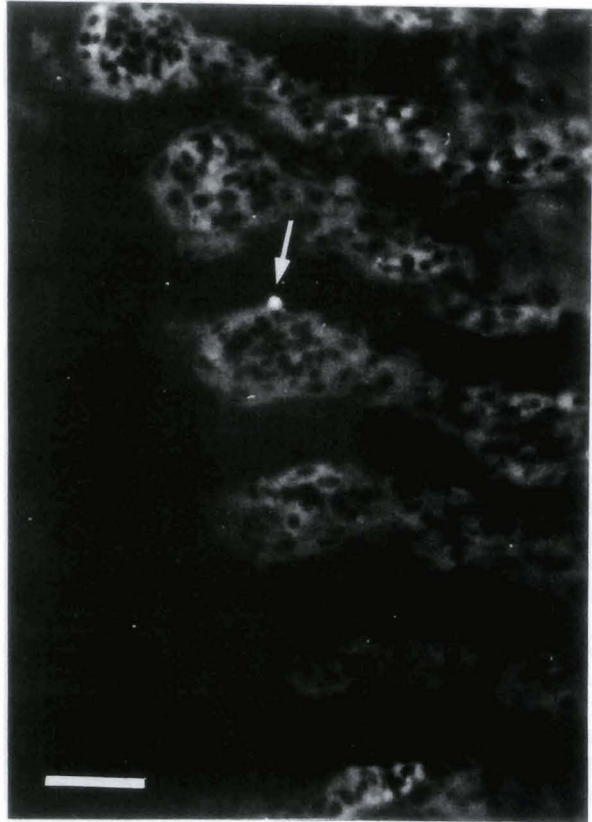
Fig. 3. Gill tissue 30 s after immunization with non-coated fluorescent beads. The beads are bound to the epithelial cells (arrow) but they never occur inside them. Bar = 10 μm.
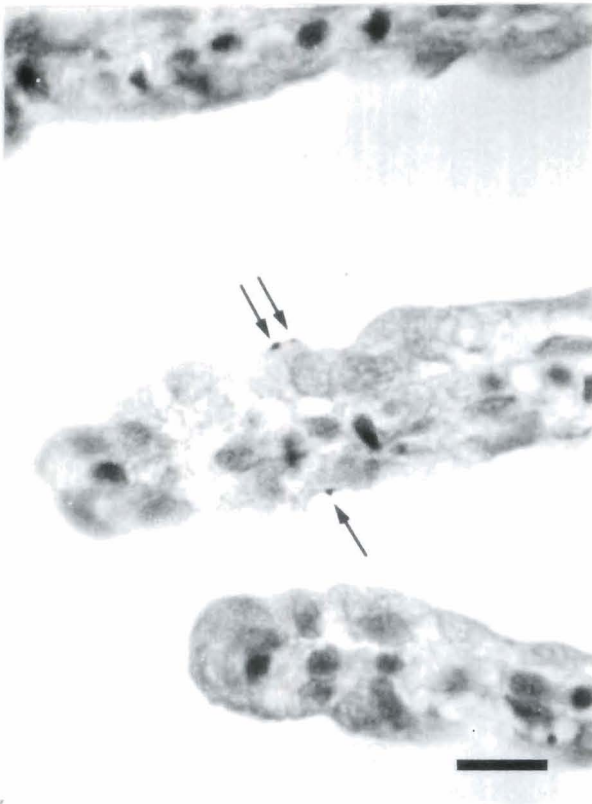
Fig. 4. Particles of *Y. ruckeri* formalin-killed cells on the surface of the epidermal pavement cell (arrows) in a sample taken 5 minutes after antigen exposure. Bar = 10 μm.
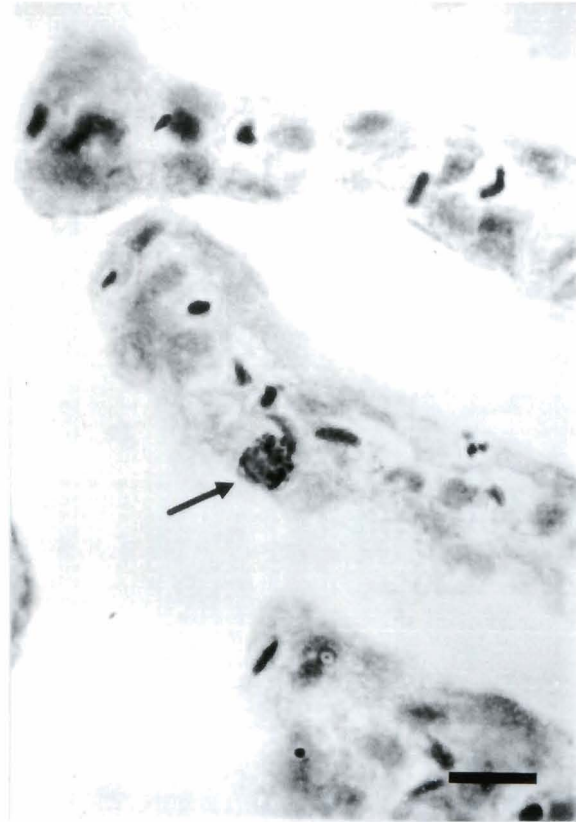
Fig. 5. Presumptive macrophage in the gill lamella containing *Y. ruckeri* O-antigen particles 5 min after exposure. Bar = 10 μm.

*In vitro antigen trapping by gill cells*

light (Smith, 1982; Nelson et al., 1985) and transmission electron microscope analysis (Zapata et al., 1987) supporting the gills as an important organ for antigen trapping.

The mechanism appears to be efficient since the *in vitro* experiments demonstrate antigen trapping 30 s after bath immunization. The trapping may be still faster in *in vivo* situations. Several immunization experiments support the idea that a 5 s contact time between the fish and the killed bacteria is sufficient to elicit an immune response (Gould et al., 1978; Johnson et al., 1982; Tatner and Horne, 1983; Tatner, 1987). Thirty min after immunization, the latest time tested in the current experiments, the gill cells still showed antigen trapping activity. Smith (1982) demonstrated such activity in gills 24 h after vaccination with latex-BSA. Furthermore, Tatner (1987) showed that at high antigen concentrations, lengthening the immersion time did not result in greater uptake, whereas when the antigen concentration was low, proportionally greater periods of time were needed for antigen uptake to occur. Nevertheless, in those experiments the whole body of the fish was exposed not only gills. The current finding of clusters of both *Y. ruckeri* O-antigen and *Y. ruckeri* formalin-killed cells suggests that the level of antigen trapping is very low, relative to the amount of suspended antigen.

The results also suggest some specificity in the mechanism of particle uptake by gill cells. Whereas both *Y. ruckeri* O-antigen and *Y. ruckeri* formalin-killed cells were quickly taken up by gill cells, inert non-antigenic material, such as latex (non-published results) and unlabelled fluorescent beads adhered to gill surfaces, but were not engulfed. Anderson et al. (1984) have speculated on the existence of specific receptors on gill cells involved in antigen trapping, especially in the case of natural fish pathogens such as *Y. ruckeri*. Nevertheless, further research is necessary to confirm this hypothesis.

In fish, the mode of entry of antigenic material into gill cells is a matter for discussion. Chilmonczyk (1979) was unable to demonstrate antigen entry through gills and other authors have proposed that antigens enter the internal milieu through gill epithelium disrupted or altered, due to diseases or mechanical damage (Bowers and Alexander, 1981, 1982; Alexander et al., 1982). Although the current study by light and fluorescence microscopy does not permit us to understand the mechanism of antigen entry, previous ultrastructural results have demonstrated that gill epithelial cells engulf *Y. ruckeri* O-antigen by endocytosis (Zapata et al., 1987). In agreement, particulate and soluble antigens are taken
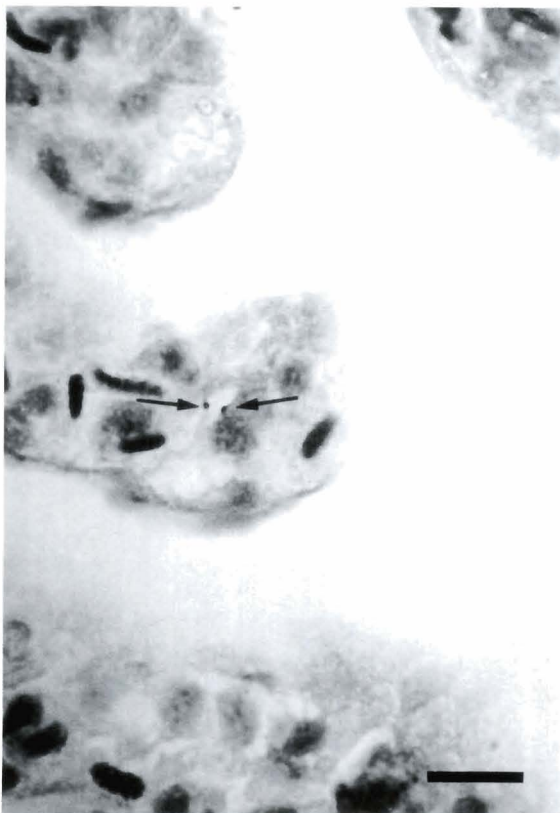
Fig. 6. *Y. ruckeri* O-antigen particles (arrows) in the intercellular space of gill cells five minutes after exposure. Bar = 10 µm.
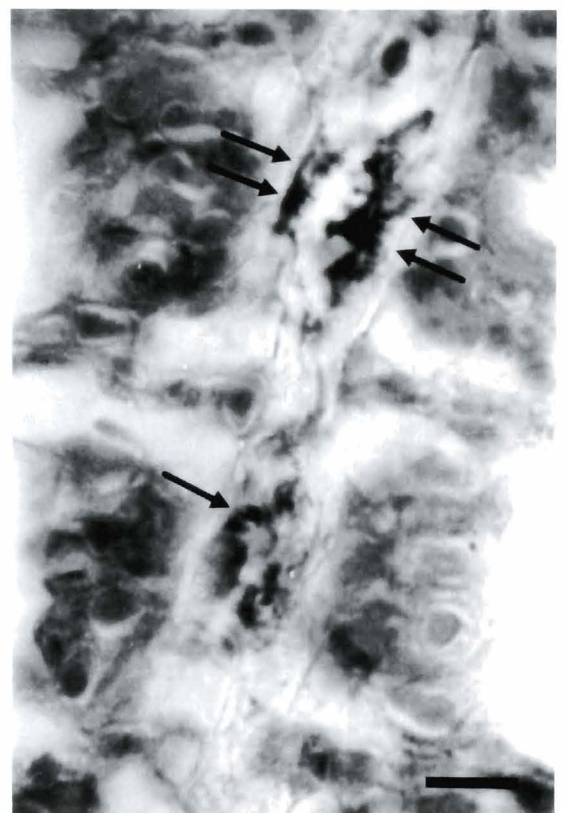
Fig. 7. Formalin-killed *Y. ruckeri* cells in the endothelial cells (arrows) of gill blood vessels 5 min after exposure. Bar = 10 µm.

up by the same mechanism by gut enterocytes (Rombout et al., 1985; Rombout and Van den Berg, 1989).

In conclusion, the present *in vitro* study confirms the involvement of gills in antigen trapping by fish subjected to bath immunization although their importance relative to other organs needs further confirmation. Moreover, the specificity of the process, as well as the mechanisms of antigen processing and transport from the gills to the lymphoid organs also need to be conclusively defined.

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