A MICROSCOPICAL STUDY OF GLOCHIDIAL IMMUNITY[1]

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ONE PLATE (NINE FIGURES)

AUTHOR'S ABSTRACT

Glochidial cysts on the gills of immune fishes form in the same manner as normal ones, but they tend to grow larger and become more irregular. The increased thickness is due to additional cellular connective tissue in the wall. The gill tissue indicates the existing biological incompatibility only by the presence of eosinophiles, extruded chromatin spherules, and eosinophilic plastids.

In natural, or racial immunity many glochidia are promptly destroyed by cytolysis, accompanied by an invasion of host cells. These disintegrating glochidia may occur in close proximity to unaffected glochidia and apparently are merely less resistant individuals that succumb to a critically adjusted reaction.

In both natural and acquired immunity the normal retention of glochidia and the accompanying metamorphosis are replaced by premature shedding. After the first day, the cyst thins by the removal of stroma cells back into the filament until the wall is reduced to a thin envelope. Both intact and destroyed glochidia, and apparently their cyst coverings, are sloughed at about the second day. Repair of the resulting notched filament is prompt.

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INTRODUCTION

Within recent years it has been learned that a definite immunity is associated with the development of fresh-water mussels. This immunity is operative at a time when the larval glochidium normally becomes a temporary parasite on its fish host, and is expressed through the refusal of the fish to serve as host. The immunity may be either natural or

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acquired. In both instances glochidia attach and encyst in the superficial tissues of gill or fin, but are not retained longer than one to three days.[2] Underlying natural, or racial immunity is the fact that the various mussel species are usually restricted to one or more particular fish hosts. Fishes that are not hosts will receive and encyst the glochidia of any mussel that can attach, but will not retain them. On the contrary, acquired immunity is an individual reaction whereby natural hosts that have experienced two, three, or more infections[3] become incapable of carrying subsequent ones through the metamorphic period.

As a part of a program which has included experimental and microscopic studies on free and encysted glochidia (Arey, '21, '23 a, '24, '32 a, '32 b, '32 c) there was included an investigation of immunity, both natural and acquired. The objectives sought were to determine the histological conditions in host and parasite during their brief association, together with the changes anteceding rejection. A preliminary communication of the conditions found has already been published (Arey, '23 b).

A clear instance of natural immunity is afforded by the yellow sand shell, Lampsilis anodontoides, whose parasitism is restricted to the gar pikes. Infections upon such a non-host as the large-mouth black bass, Micropterus salmoides, are not retained beyond the second day. A closely graded series was collected and studied both totally and in serial section. The seventy-eight gill cysts are distributed by age as follows:

Age of cysts in hours	*Number of cysts examined*	*Age of cysts in hours*	*Number of cysts examined*
9	7	36	2
15	16	39	12
21	9	40	4
24	13	42	6
31	9		

[2] This and all subsequent statements refer to conditions as found in infections at midsummer temperature at Fairport, Iowa.

[3] In some instances one or two infections produce acquired immunity, but such animals are large and doubtless have harbored natural infections before capture (Arey, '23).

For acquired immunity the fat mucket, Lampsilis luteola, on the gills of its natural host, the large-mouth black bass, was used. These bass had become immune at the third infection and had promptly sloughed the fourth attempt at encystment. The series collected came from the fifth infection, when it was certain the immunity was well established. Some supplementary material was taken from the fifth infection of another lot. Fresh and preserved gill filaments, total mounts, and sections were utilized. The serial sections were of forty-four complete cysts, timed as follows:[4]

Age of cysts in hours	Number of cysts examined	Age of cysts in hours	Number of cysts examined
4	2	33	4
9	7	47	9
21	6	50	6
27	2	67	6
30	2		

All gill filaments received Zenker fixation within a few seconds after removal from the fish. Sections 6 μ thick were stained in hematoxylin and eosin.

OBSERVATIONS

Gross formation of immune cysts

The manner of attachment and the process of encystment conform to the normal procedure described in detail elsewhere in these reports (Arey, '21, '24, '32 a, '32 b). Yet an important size difference soon becomes apparent. In the acquired immunity of the Lampsilis luteola series the glochidia are about half-covered over at thirty minutes, and at that time the growing cyst still appears normal. At two and one-quarter hours the glochidium is two-thirds enclosed, but sits in a crater with thick lips and broad, rolling edges. Shortly after four hours it becomes completely overgrown. These immune cysts tend to grow larger than normal ones and often are strikingly bulky (fig. 1). They also show less restraint and commonly become irregular, malformed tumors

[4] Stages at fifty and sixty-seven hours were from the fifth infection material last mentioned.

with prominent irregularities and horizontally spreading wings. Toward the end of the first day they reach a maximum size and thereafter reduce in a manner to be described presently.

The same general history holds for the naturally immune Lampsilis anodontoides series. In both instances, the greater growth exuberance in immune stages over normal ones is manifest. As a whole, the cysts are notably larger and more irregular. It seems as though some regulatory principle effecting terminal growth ceases to operate. A comparable circumstance is found in the formation of cysts on the excised gill filaments of normal hosts (Arey, '21).

Histological changes in the host tissue

Structurally the early immune gill cyst is like the normal, but with thicker walls. This massiveness is due wholly to additional cellular connective tissue. In another publication (Arey, '32 b) it is shown that encystment results from an active migration of cells which cover the glochidium, and not from mitotic proliferation. All the immune material, procured and studied subsequently, has confirmed this earlier conclusion. Elsewhere (Arey, '32 a) the point has also been stressed that the gill cyst is in no sense an exclusively epithelial cyst, if indeed it can properly be called a cyst at all. The prevalent error of interpretation has resulted from confusing the epithelium with the cellular stroma beneath it. The two are usually poorly demarcated, and often appear confluent. Some of the present series show these boundaries with greater distinctness than has been observed before (compare fig. 1; right margin of cyst). The 'cyst,' then, at its height is a tumor-like mound of connective tissue in which the glochidium lies buried; externally is a mantle of epithelium continuous with the general gill covering (figs. 1 and 6).

As with the method of formation and general structure, so also in histological composition the immune cyst adheres closely to the normal. An interesting feature is the occurrence of eosinophiles. In the naturally immune Lampsilis

anodontoides series, scattering eosinophiles are found in the vicinity of many glochidia. These have been seen in the earliest stages examined (four hours), but do not occur in the normal bass gill.[5] In the Lampsilis luteola sections eosinophiles are much more abundant, and are most plentiful in the younger stages. They tend to accumulate, at least as early as the four-hour stage, in the immediate vicinity of the glochidium, and frequently occupy a pocket or even a channel bordering the larva (fig. 1). In the later hours of encystment they are not as numerous, but occasionally may be seen scattered throughout the gill substance.

Both natural and acquired immunity lead to the production of eosinophilic plastids in the stroma. These are infrequent in the filaments infected with Lampsilis anodontoides, but can be found. About the Lampsilis luteola parasites they are commoner and occur at least as early as twenty-one hours.[6] The origin of such elements is problematical.

Another characteristic component of the Lampsilis anodontoides material is a small, spherical, deeply basophilic mass. Many such may be seen in a single section. They appear to be extruded chromatin, for identical bodies occur in the nucleus, in the cytoplasm adherent to the nucleus, free in the cytoplasm and interspersed between stroma cells. Where such bodies are frequent the nuclei tend to be pale and poorly chromophilic; often this is strikingly true.

It would seem that the basophilic spherules, and probably the eosinophilic plastids and marshaled eosinophiles, are a visible expression of tissue incompatibilities associated with immunization. They represent the only morphological index of the subtle tissue reactions undoubtedly taking place in

[5] A graded series of Lampsilis anodontoides on its natural host, the gar pike, did show some eosinophiles in the stroma of filaments. But this occurred only in certain filaments, all of which perhaps came from one or more of the six individuals used. It possibly represents a partial acquired immunity, for all the fish not only had received one previous infection, but their earlier experience as hosts while wild is unknown. One speculates on a possible correlation with the presence of fish lice which infest the gills of these animals.

[6] No stages were available in my series between nine and twenty-one hours.

the host. We shall see presently that a deleterious effect is plainly discernible in the parasite.

Sometimes cyst-like vesicles, which may contain a cellular mass, occur. These are usually too small to have held a glochidium, and the absence of cuticular or other diagnostic remains (vide infra) furnishes definite disproof of such origin. In certain instances they communicate with true glochidial cysts or are attached like blebs to their peripheries. Of somewhat the same nature are tumorous masses which externally appear like true cysts, but prove to be made of solid gill tissue with at most a spongy cellular interior. Both presumably represent proliferations expressive of the reactive exuberance of the stimulated immune filament. This tendency to overgrowth is further illustrated by the size and irregularity of the true glochidial cysts. Not only are irregular outgrowths very common, but the cyst tissue as a whole often spreads outward along the filament for some distance.

Cytolytic destruction of glochidia

The attached glochidium may meet a prompt death, accompanied by the dissolution of its cellular components (figs. 2 to 4). This has been observed only in the Lampsilis anodontoides infection, which was utilized as a type to illustrate the natural immunity peculiar to non-hosts. In the Lampsilis luteola series, attached to its natural host which had acquired immunity by repeated infection, glochidial disintegration was not found. As the examination included representatives of two different L. luteola infections, it would appear not to be characteristic of acquired immunity in this mussel.[7] Whether

[7] In contrast should be noted Reuling's ('19) statement for the same host and parasite. He subjected Lampsilis luteola glochidia to the blood of normal black bass and to blood from animals that had acquired immunity. The normal control remained for forty-eight hours unchanged. After twelve hours the glochidia in the immune serum desquamated their mantle epithelium, which then broke up into cellular débris; in some cases the valves opened. Finally, the entire internal structure was destroyed. Also he reports that glochidia examined after twenty-four hours' attachment to the gills of immune bass "showed a certain amount of disintegration." It should be recorded that most of the Lampsilis luteola material used by me in this study came from Reuling's later infections. It is puzzling to account satisfactorily for the discrepancy in our results.

such a conclusion will apply as a generalization to all cases of acquired immunity cannot be predicted.

Not all of the Lampsilis anodontoides glochidia are destroyed by cytolysis. Many[8] remain intact until lost (figs. 2, at right, 5 and 6). Those that do succumb may show early disturbances—an advanced state of degeneration has been observed at fifteen hours (fig. 2, at left). During this decline the glochidial tissues become necrotic, fragment into groups, and are more or less dislocated from their natural positions. The end result is often a state of disarrangement, disorganization, and disintegration (figs. 2 to 4). The adductor fragments, and its fibers swell and stain feebly as they disappear. Representatives of all the glochidial structures are recognizable for a relatively long time, but eventually the only remnants that can be surely identified may be the cuticular components of the valves (fig. 4, *h*).

With the early death of a glochidium, and the consequent relaxation and disruption of the adductor (fig. 3), the elasticity of the hinge spreads the valves apart in many instances (figs. 2 to 4). This distends the cyst and changes its shape, so that in such a disorganized condition it usually simulates two communicating cysts. The bitten host tissue may then appear like a partial partition.

Coincident with glochidial necrosis goes an invasion of host cells in greater or smaller numbers (figs. 2 to 4). Some early stages show an attack from one side only. These invasive elements are apparently cells of the stroma, to which eosinophilic elements, and perhaps other leucocytes, may be added. It is difficult to identify special cell types in most instances, and hence origins are obscure. Although it is tempting to infer a sequential relationship between this invasion and the accompanying retrograde changes, such is not amenable to proof. The two may be parallel phenomena, or the invasion may be secondary to the incipient degeneration and induced by it.

[8] Fifty-one cysts, twenty-one hours or more in age, were examined. Of these, nineteen were necrotic.

An astonishing feature is that highly necrotic glochidia occur on the same filament and often in apposition with glochidial cysts that show no apparent regressive changes (fig. 2). Advantages of position or nidus are not responsible. The phenomenon must depend either on local differences in the intensity of the immune principle, or to a variability in glochidial vitality and resistance. The first alternative is difficult to reconcile with prevailing ideas as to the nature of immunity.

Unaffected glochidia of the later hours, before shedding occurs, sometimes show mitoses in the organ-anlage mass, thus indicating that, like the normal larva, they are capable of attempting differentiation. This start toward metamorphosis was illustrated by the larva sectioned obliquely in figure 5.

The sloughing of glochidia

The manner in which glochidia are finally shed demands consideration next. Those glochidia which are not destroyed internally, according to the method just described, are lost intact. Such loss occurs frequently in natural immunity and was the only method observed in the material illustrating acquired immunity.

In neither instance do the glochidia show distinctive signs of injury. The bitten host tissue undergoes in situ (extracellular) digestion in the usual manner (figs. 1 and 6). This is accompanied by the coincident loss[9] of mantle zymogen granules and the ingestion of bits of host cells by the mantle.[10] After the normal latent period, mitoses appear in the mass of cells from which the future organs develop; such dividing cells are found even to the time when the glochidium is about to be cast off.

Structurally, the course of events leading to sloughing of the glochidium is not spectacular. The period of the bulky, thick-walled cyst is followed by a phase of thinning, during

[9] In Lampsilis anodontoides this was a diminutional rather than total loss.
[10] These events are described fully in another contribution (Arey, '32 c).

which the glochidium approaches an uncovered condition. This reduction often progresses unevenly, so that one portion of the cyst is much thinner than the rest (figs. 7, *a*, and 8, *a*). In total examination such cysts appear to be entirely uncovered on all or part of their exposed surfaces (figs. 7, *a*, and 8, *b*). When sectioned, however, this proves to be illusory and none of the glochidia actually shows bare areas. The cyst wall is merely very thin (fig. 6). Now, this is exactly what happens in normal encystment (Arey, '32 a), except that in immunity the thinning is more likely to be carried to an extreme.

The query immediately presents itself as to what becomes of the cells that formerly composed the thick cyst. It is natural to assume that the reduction depends on a direct cellular loss by sloughing, yet actual microscopic observation does not confirm this view. It must be remembered that gill cysts are made up of cellular connective tissue overlaid with epithelium (fig. 1). As the size reduction goes on there is no evidence of epithelial necrosis, loosening, or desquamation. Hence, so long as the continuity of the epithelium is maintained there can be no sloughing of the underlying stroma cells (fig. 6). What, then, does occur? As the cyst becomes smaller its contour also grows smoother. Both of these occurrences appear of necessity to depend on the movement of cells from the cyst wall backward into the body of the filament. Such a process is in a sense not surprising, for it is merely the reverse of the outward migration at the time of encystment. But now the response is harder to comprehend, for there lacks the visible stimulus that originally called the cellular elements forth.

Finally, comes the problem of actual shedding, whereby intact glochidia, as well as the valves and cellular débris of destroyed ones, separate from the filament. Frequently the cyst reshapes itself until it is distinctly stalked (fig. 5), and, together with its contents, is lost as a whole. Yet this cannot be the commonest manner of removal. The majority of glochidia become thinly covered while retaining the original

broad attachment. They are then cast off, but as to what process directly precedes the loosening I am not able to state, for illustrative stages have not been observed. In some instances there is evidence of undercutting, yet this cannot be the regular method, since the broad attachment still continues as a rule in filaments from which glochidia are already being lost. Microscopically there were no noticeable changes about the bases of such cysts as have been sectioned or examined totally (fig. 6). Against these statements must be placed the fact that glochidia undeniably are got rid of in some manner. The details await further inquiry which must combine observations on living filaments with subsequent microscopic examination. It is my belief that the intact or disintegrated glochidium is cast off within the highly thinned cyst as a unit. Empty cysts have not been observed, whereas deeply scalloped filaments are common (figs. 7 and 9).

Two somewhat incidental statements are on record which are of interest inasmuch as they refer to living conditions. Reuling ('19), also utilizing black bass made immune to Lampsilis luteola, records: "On examination after 24 hours the gills of the fish which had previously been infected twice showed marked necroses and sloughing of the epithelial [sic] cyst around each glochidium." Howard ('14), working with the Quadrula group, refers to the reaction when glochidia find attachment to a non-host and says: "If not [retained], the cyst is shed by a process of desquamation of the external epithelium. I have observed this in gill infections only. A stream of water of not great force will remove the outer layers of epithelium of an infected gill about to shed the glochidia. Such a catarrhal reaction. . . ."

For a short time after glochidia are shed the filaments appear notched, each scallop indicating clearly the former location of a glochidium (figs. 7 and 9). When glochidia are closely spaced a solid stretch of filament tissue is lost with them and as a result the filament for a time is greatly narrowed locally (fig. 7, at tip). In a surprisingly few hours the filament is restored to its normal size and shape.

SUMMARY

Glochidial cysts on the gills of immune fishes form in the same manner as normal ones, but they tend to grow larger and become more irregular. The increased thickness is due to additional cellular connective tissue in the wall.

The gill tissue indicates the existing biological incompatibility only by the presence of eosinophiles, extruded chromatin spherules, and eosinophilic plastids.

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PLATE 1

EXPLANATION OF FIGURES

1 Section through a typical immune cyst of Lampsilis luteola, nine hours after attachment. The glochidium is surrounded by a zone of eosinophiles. Photo. × 147.

2 Section through neighboring normal (at right) and degenerating (at left) glochidia of Lampsilis anodontoides, fifteen hours after attachment. Among the remains of the opened, degenerating larva, the hinge (*h*), cuticula, and parts of the mantle are distinguishable. Photo. × 167.

3 Two opened and partly degenerated glochidia of Lampsilis anodontoides at twenty-one hours. A piece of the disintegrating adductor muscle is seen on each lateral side of the right-hand larva. Photo. × 167.

4 Two late stages in the degeneration of Lampsilis anodontoides glochidia after forty-two hours of encystment. In the widely opened larva on the right, the hinge (*h*) and mantle cuticula are alone recognizable; in the cyst at the left the hinge appears in another section. The two cysts communicate, but are partly separated by an incomplete partition. Photo. × 167.

5 A forty-hour pedunculated cyst of Lampsilis anodontoides. The section passes obliquely through the glochidium. Photo. × 167.

6 A greatly thinned cyst of Lampsilis luteola, fifty hours old, nearly ready to slough. Photo. × 167.

7 Gill filament with fifty-hour cysts of Lampsilis luteola. The cyst designated *a* is unevenly reduced in thickness. Extensive sloughing has carried away much tissue from the distal half of the filament. Photo. × 17.

8 Gill filament with sixty-seven-hour Lampsilis luteola cysts. At *a* the cyst has thinned unequally; at *b*, so evenly and thoroughly that the glochidium appears to be naked. Photo. × 17.

9 Gill filament with a sixty-seven-hour Lampsilis luteola cyst and several notches indicative of previously sloughed glochidia. Photo. × 17.