THE ELIMINATION OF FOREIGN PARTICLES INJECTED INTO THE COELOM OF THE HOLOTHURIAN— *CUCUMARIA STEPHENSONI* D. JOHN

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INTRODUCTION

The fate of foreign particulate matter injected into holothurians has received more attention than have similar phenomena in most other groups of invertebrates. It has been studied by Schultz (1895), Bartels (1895), Clark (1899), Cuénot (1902), Bertolini (1937) and Millot (1950), while other authors, including Bordas (1899), Kindred (1924), Kawamoto (1927) and Ohuye (1934, 1936), have made observations which are relevant to the topic. The materials most commonly injected by these and other workers have been India ink and suspensions of carbon or carmine, though Millot (1950) injected iron saccharate into the body cavity of *Thyone*. These substances are not altogether adequate in that they display a wide range of particle size, even when most carefully prepared, the largest particles and aggregations often being too large to be phagocytosed. More serious is the difficulty of determining the routes taken by laden phagocytes leaving the body, for the investigation relies of necessity on direct visual observation of dissected animals and of sections. Under such circumstances quite important migratory pathways may be overlooked and it is very difficult to assess the relative importance of different routes. The fact that some authors studying this phenomenon have failed even to cut sections of their experimental animals increases one's lack of confidence in their results. Almost no information is available on the rate and degree of elimination of foreign particles from the bodies of holothurians.

The injection of "Thorotrast" (a stabilized suspension of thorium dioxide) provides a more elegant and reliable technique, which has already proved its value in similar studies on marine Gastropoda (Brown 1964, 1967b; Brown & Brown 1965) and on the land pulmonate, *Helix* (Brown 1967a). In sea-water or invertebrate body fluids, the particles of thorium dioxide form aggregates which are very uniform and of a size conveniently phagocytosed. As thorium is extremely opaque to X-rays, the routes taken by laden phagocytes can be determined from radiographs of either living or dead animals. Animals may also be killed and sectioned at suitable stages after the injection of Thorotrast, the sections being viewed by combined dark-ground, phase-contrast illumination in order to show up individual particles of thorium dioxide in the tissues and body fluids (*vide* Baxter 1960).

MATERIAL AND METHODS

Individuals of *C. stephensoni*, varying between 10·0 and 13·5 cm in length when fully extended and between 9·5 and 14·7 gm in weight, were obtained from rock pools and gullies along the False Bay coast of the Cape Peninsula. They were kept in sea-water tanks in the laboratory at a temperature of 15C ($\pm$1·0). This temperature is consistent with temperatures they experience in their natural habitat. Sand, small stones and other debris from intertidal pools were

included in the tanks. After 4 days, between 0·5 and 0·6 ml of Thorotrast was injected into the coelom of each test animal, while controls were subjected to dummy injections of the same amount of filtered sea-water. In a subsequent series of experiments, in which the test animals were again treated as specified above, the controls were injected with a 25% solution of tapioca-dextrin, the suspension medium of Thorotrast.

From time to time fluid was withdrawn from the body cavities of both control and test animals, for examination of the coelomocytes. Cell counts were made, using the haemocytometer techniques usually employed for vertebrate animals but with the modifications introduced by Yeager & Tauber (1935) for marine invertebrates. At first these counts were found to be extremely inconsistent but reproducibility was later greatly improved by shaking the animal vigorously for one minute before withdrawal of the sample.

Two, and sometimes three, animals were killed each day for 9 days and preserved in 75% ethyl alcohol. Three more were killed on the fourteenth day after injection of Thorotrast and the last two on the twenty-first day. Radiographs of the preserved animals, and of some of the controls, were taken by means of a Siemens diagnostic X-ray machine with enlarging facilities, the exposure being 2·0 seconds at 65 kV and 16 mA at a shutter-to-film distance of 120 cm. The animal to be radiographed was placed approximately mid-way between shutter and film, giving a direct enlargement of twice to three times natural size.

Sections were subsequently cut of some of the preserved animals for more detailed study of the distribution of the thorium particles. Many of these were relatively thick frozen sections but in some cases thin, permanent mounts were made after wax-embedding.

RESULTS

(*a*) RADIOGRAPHS

Radiographs of animals killed 24 hours after injection of Thorotrast showed well-developed, highly contrasted shadows involving the entire length of the respiratory trees and the anterior part of the water-vascular system, including the ring canal and the radial canals. On some radiographs of animals killed between 1 and 3 days after injection, the tentacular branches of the water-vascular system could also be made out, while in one the polian vesicle appeared to be involved.

In one of the animals killed on the second day, the shadows cast by the respiratory trees were darker than any of those observed on the first day but in the other two animals the shadows were somewhat fainter. The shadows of the trees were markedly fainter in two animals killed on the third day and by the fourth day they could barely be distinguished; indeed in one of three animals killed on this day they could not be made out at all. By the fifth day the respiratory tree shadows had completely disappeared.

The shadows associated with the anterior part of the water-vascular system faded less rapidly and, while certainly most intense between the first and third days, could still be seen clearly on the fifth day and, with one exception, on the sixth. A single radiograph of one of the animals killed 7 days after injection still showed a faint shadow. After 8 days no shadows were apparent and the opacity of different parts of the body was no greater than that of control animals killed at the same time.

(*b*) SECTIONS:

Sections cut through various parts of the bodies of two holothurians killed the day after injection revealed the presence of large numbers of thorium-laden cells in the walls and lumina of the respiratory trees, in the wall of the mid-gut and in the anterior canals of the water-vascular system. A few could also be seen in the body wall, particularly of the tentacles and around the mouth, in the posterior water-vascular canals and in the blood system.

Sections through an animal killed after 3 days showed far fewer laden cells and none of those observed appeared to be free. They were seen, however, in the epithelia of the respiratory trees, in the wall of the gut and particularly adhering to the sides of the ring canal and radial canals of the water-vascular system and migrating from these canals and their tentacular branches to the exterior of the body. No thorium-laden cells were seen in the coelom or in the blood system.

Very few thorium particles could be identified in sections of an animal killed on the eighth day and an animal killed after 14 days revealed no thorium particles in any part of the body.

(*c*) THE COELOMOCYTES

Hyman (1955) lists nine different types of free cell to be found in holothurian coelomic fluid. I was able to distinguish five distinct types in the coelomic fluid of *C. stephensoni*. Using Hyman's terminology, these were haemocytes, amoebocytes with colourless spherules, homogeneous amoebocytes, phagocytes and minute corpuscles. Total cell-counts varied from 365,000 to 984,000 cells per ml of fluid in animals kept for four days under the controlled conditions. The reproducibility of counts from individual animals was found to lie within 14,000, so that the differences recorded between individuals are highly significant and represent a real variation in coelomocyte populations. Haemocytes were invariably the most abundant cells seen and accounted for between 72% and 90% of the total, the percentage being consistently higher the higher the total cell count.

Amoebocytes with spherules were present to an extent which varied between 15,000 and 47,000 cells per ml. These cells, similar to those described as "morula-shaped cells" by Endean (1958), displayed a wide range of size in some samples of coelomic fluid, though not in all. The smallest cells possessed only relatively few, ill-defined spherules, much of the available space being taken up by a large nucleus; this nucleus did not appear to increase in size with the size of the cell and in the largest cells (10—13μ in diameter) it was completely obscured by the large number of spherules lying around and above it.

Minute corpuscles were not observed in all the samples studied. They never gave counts of more than 4,000 cells per ml. Homogeneous amoebocytes were always present, however, varying between 32,000 and 59,000 cells per ml of fluid. Phagocytes were present in all but one of the samples examined from untreated animals; they were identified as such by the presence of solid-looking objects of various sizes in the cytoplasm and the complete absence of "spherules". Their concentration varied from less than 1,000 to 23,000 cells per ml.

Examination of samples of coelomic fluid withdrawn from test animals, after injection of Thorotrast, led to several relevant findings. Firstly, in no sample withdrawn after the fifth hour could a single phagocyte be found; this state of affairs persisted until the fourth day, when phagocytes again appeared in the samples. their numbers then and subsequently ranging between 13,000 and 28,000 per ml. More unexpected was the appearance on the second and

third days of large numbers of homogeneous amoebocytes, reaching counts of up to 123,000 cells per ml. These high numbers did not persist, however, and by the seventh day had fallen to between 11,000 and 43,000. Variation in the counts of the other types of cell—haemocytes, amoebocytes with spherules and minute corpuscles—was neither consistent nor statistically significant. On no occasion could particles of thorium dioxide be detected in any of the cells withdrawn, though free particles could be seen in the coelomic fluid for the first 5 to 7 hours after injection.

DISCUSSION AND CONCLUSIONS

Previous work such as that of Bertolini (1937) on the elimination of foreign particles from the Dendrochirota, of which *Cucumaria* is a member, and related groups, has already shown that such particles are ingested by coelomocytes which then migrate through the walls of the respiratory trees and gut. As far as migratory pathways are concerned, the main interest of the results gained in the present work thus centres on the heavy involvement of the anterior portion of the water-vascular system. This route is either peculiar to *C. stephensoni* or has been overlooked by previous workers using less sophisticated methods. The pathway must originally have been an attractive one for migrating phagocytes, for primitively the water-vascular system communicates directly with the external environment via a hydropore, a condition which is retained in some holothurians throughout life (Hyman 1955). If migration of laden phagocytes did take place via this route in primitive holothurians then the migration of such cells through the tentacular walls in *C. stephensoni* (and possibly in other species) may be considered secondary to the closure of the hydropore. Only further experimental work, particularly on those species which still possess a hydropore in the adult stage, will be able to confirm or refute this theory.

Other points of interest are the rapidity and the completeness of the elimination of thorium dioxide particles in *C. stephensoni* as compared with other invertebrates which have been subjected to the same technique. In the snail, *Helix aspersa*, kept at the same temperature and injected with a comparable amount of Thorotrast, thorium shadows reach maximum intensity only by the fourth or fifth day, while even after 12 days the animal's body is still more opaque to X-rays than are control snails (Brown 1967). In the case of *Bullia*, thorium shadows persist for up to 6 weeks, at which stage sections reveal the presence of quite large amounts of thorium remaining in the tissues (Brown & Brown 1965). In *Cucumaria stephensoni*, on the other hand, the shadows reach maximum intensity about 24 hours after injection and have virtually disappeared within 7 days, while sections of a 14-day animal show no thorium in the tissues. The only animal so far investigated which can approach this standard of efficiency is *Patella* (Brown 1967b). Hyman (1955) has said that "from what is known of the physiology of holothurians, it would seem that these animals operate on a very primitive basis, that each organ system covers more than its usual function, and that amoebocytes play a remarkable role in the economy." Indeed great efficiency in the elimination of foreign materials from the body fluids may well be considered a primitive feature and I am unaware of any report in the literature which might weigh against this hypothesis.

The disappearance of thorium dioxide particles from the coelomic fluid more or less coincides with the disappearance of phagocytes, which is understandable if fully-laden phagocytes

tend to attach themselves to surfaces. There is no evidence that any other type of cell takes up thorium particles, though it must be admitted that in section it is difficult or impossible to distinguish free phagocytes, migrating through tissues, from fixed phagocytes. However, it is clear that none of the other coelomocytes are involved in thorium uptake. This is not to say that the other types of coelomocyte are completely unaffected by the introduction of foreign material into the body, for the homogeneous amoebocytes increase greatly, though temporarily, in numbers. Endean (1958) believed that the homogeneous amoebocytes of *Holothuria* were formed in the epithelium of the lumina of the respiratory trees and were the primitive coelomocytes, giving rise to other cell types. In particular he observed intermediate forms between these cells and the amoebocytes with spherules (or morula-shaped cells); the small cells with few spherules observed in the coelomic fluid of *Cucumaria stephensoni* might also be regarded as intermediate forms. Endean does not mention coelomocytes intermediate between homogeneous amoebocytes and phagocytes and such forms were not seen in the present investigation either. Nevertheless, if homogeneous amoebocytes do give rise to the phagocytes it would explain the increase in numbers of the former followed by the reappearance of phagocytes. In this connection it may possibly be significant that no mitotic figures were observed in any of the coelomocytes.

SUMMARY

The elimination of thorium dioxide particles introduced into the coelom of *Cucumaria stephensoni* was investigated by means of radiographs, sections and the examination of coelomic fluid samples. Phagocytes laden with the particles migrate through the respiratory trees and gut, and also via the anterior water-vascular system. The elimination is remarkably efficient, being rapid and virtually complete. The numbers of homogeneous amoebocytes are affected by the introduction of Thorotrast and it is tentatively suggested that these cells give rise to the phagocytes as well as to other coelomocyte types.

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