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The Initial Phase of Encapsulation of Silicone Oil Injected in *Samia cynthia ricini* (Lepidoptera, Saturniidae): The Innermost Structure of the Developing Capsule

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ABSTRACT—When 10 μl of silicone oil was injected into the hemocoel of a 5-day-old 5th-instar larva of *Samia cynthia ricini*, the globular oil was encapsulated by hemocytes. The initial process of encapsulation was examined with scanning electron microscopy (SEM). Within 2 min after injection, granulocytes attached to the oil surface, and a thin membrane of amorphous substance occurred around the granulocytes. The cells immediately discharged numerous granules. A reticular network appeared around the degranulated cells, resulting in the formation of the innermost layer of the developing capsule. Compaction of capsule-forming hemocytes occurred after completion of the innermost layer of the developing capsule. These results suggest that a localized coagulation of hemolymph on the oil surface, which resembles ordinary hemolymph coagulation, constitutes the initial phase of encapsulation.

INTRODUCTION

Cellular encapsulation is a common defense reaction in insects. In general, the reaction leads to an aggregation of hemocytes around parasites or biotic implants, and to disintegration and melanization of the innermost cells of the cellular envelope [7, 11, 13–16, 18, 19]. Abiotic implants are also encapsulated by hemocytes, sometimes, without melanization [7, 12, 18, 21, 23]. Brehélin *et al.* [5] reported the immediate occurrence of hemolymph coagulation around cellophane implants, involving coagulocytes in *Locusta migratoria* and *Melolontha melolontha*. Ennesser and Nappi [7] examined encapsulation of implants of altered allogenic nerve cords in *Periplaneta americana*, and observed that the initial-cellular response was the lysis of granulocytes on the surface of the implant, and the discharged cellular components, together with an amorphous substance, formed a layer of debris which coated the foreign surface. Their studies suggest that the initiation of cellular encapsulation is a cooperative reaction between cellular and humoral immune system, but it remains uncertain how this system is truely activated against a foreign material to encapsulate it.

In the present study, we injected a small volume of silicone oil as a foreign material into 5-day-old 5th-instar larvae of *Samia cynthia ricini*. The injected oil was globular in the hemolymph and encapsulated by hemocytes. After removing the oil, the innermost region of developing capsules was examined by SEM. Morphological events which successively occur at the initial step of the encapsulation are described.

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MATERIALS AND METHODS

Host insects

Larvae of the eri-silk worm, *Samia cynthia ricini*, which has been maintained for over 10 years in our laboratory were reared with leaves of *Ricinus communis* at 25°C under a photoperiod of 14-hr light and 10-hr dark. Five-day-old 5th-instar female larvae, each weighing about 7 g, were used as host specimens.

Silicone oil as a foreign material

Silicone oil, polydimethylsiloxane,(KF95®, Shinetsu Chemical-Co. Tokyo) was used as a foreign material.This is a chemically inert material commonly used as lubricant. It is also used as an antifrothing agent for medical use.

When a small amount of the oil (less than 30 μl) was injected into the hemocoel of larvae, it became globular in the hemolymph, and was encapsulated by hemocytes. In this study, 10 μl of silicone oil was chosen as a foreign material.The oil was kept in a vacuum chamber to remove air bubbles until use.

Injection of oil

Each of 50 specimens was injected with silicone oil using a glass micro-syringe with a 27-gauge needle. Groups of 5 hosts were held for 2, 5, 10 ,20 or 30 min, or 1, 10, 20, or 72 hr at 25°C. Specimens which were held for 2 or 5 min were killed by injecting 0.5 ml of cold 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). After 1 hr of incubation on ice, the killed hosts were dissected in the Grace's medium (Gibco, Gland Island, New York). The capsules were excised together with surrounding tissues. Each capsule was placed on a thin plate of cellulose acetate and then was all sunk carefully into 2.5% glutaraldehyde fixative for 4 hr. While other specimens which were held for the later times, were killed by injecting 0.5 ml of cold 2.5% glutaraldehyde fixative. One hour later, they were dissected in the Grace medium and fixed again in 2.5% fixative for 4 hr. The capsules were excised together with surrounding tissues, and transferred to the fixative for 2 hr. All the capsules were processed by use of standard electron microscope techniques.

Electron microscopy

The capsules, after washed in the buffer solution, were post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 20 min and washed again in the buffer. Capsules were then dehydrated through a graded series of acetone. During dehydration, they were cut into several pieces to remove silicone oil. For SEM, the pieces were placed in isoamyl acetate and dried in liquid carbon dioxide by the critical point method. The preparations were coated with gold and examined with a Hitachi S430 SEM. For TEM, pieces of the post-fixed capsules were embedded in Spurr resin. Ultra-thin sections were poststained with uranyl acetate and lead citrate, and observed in a Hitachi HU12A TEM.

Hemocyte count and cell type determination

Larvae were bled by amputation of dorsal spines. Fresh hemocytes were taken over a Toma's cell count slide and immediately photographed under a Nomarski interference microscope. The total hemocyte count (THC: cell count/mm^3 hemolymph) and the differential hemocyte count (DHC) were determined from the micrographs. Cell types were classified and identified according to their cytoplasmic inclusions from TEM studies [10, 17, 19], or estimated by the external shape of the cells observed by SEM [10, 17].

RESULTS

Effects of oil injection on the number of circulating hemocytes

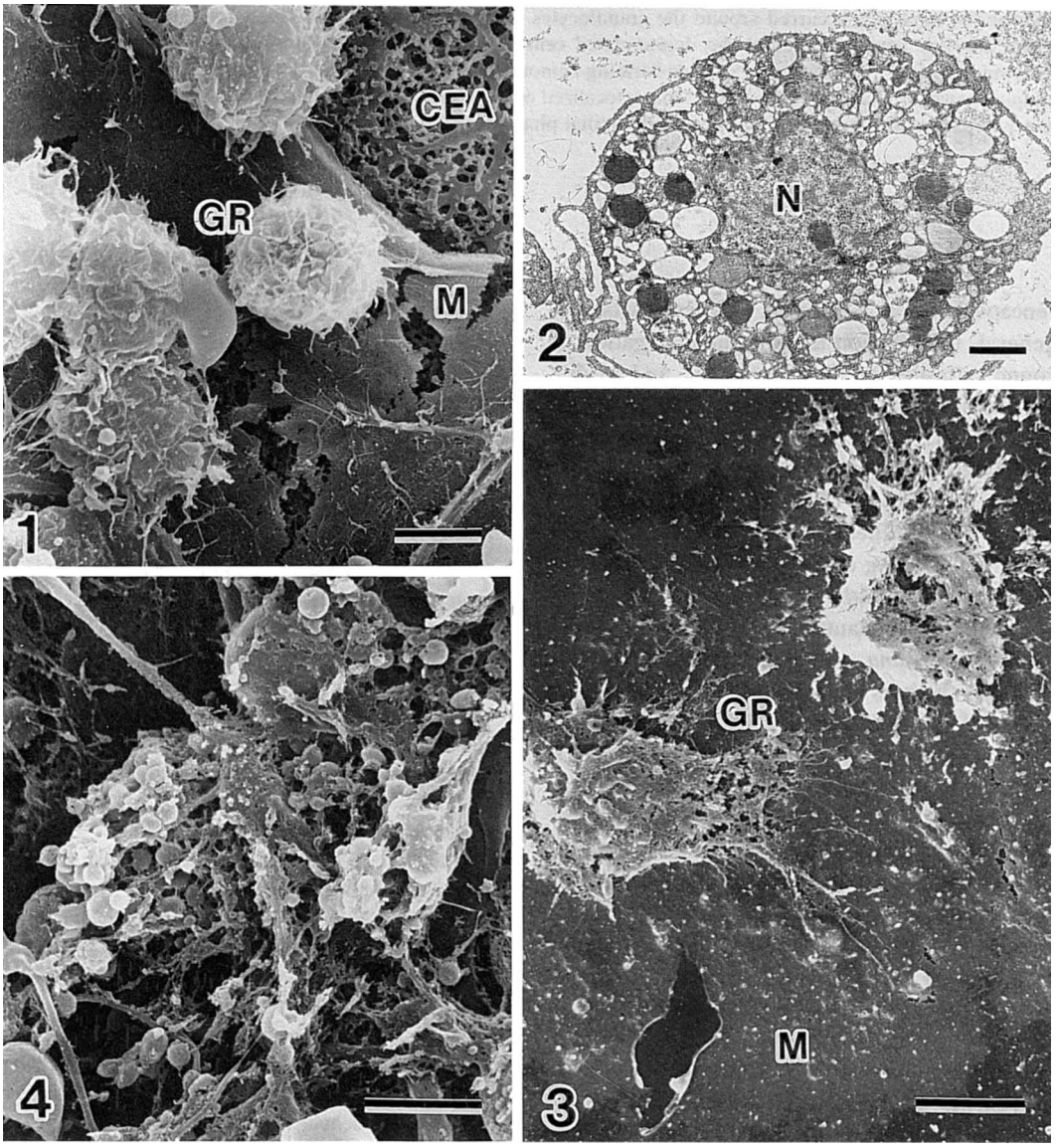

FIG. 1. Granulocytes retrieved together with silicone oil 2 min after injection. Note that these cells are attached to an amorphous membrane. GR: granulocytes. M: amorphous membrane. CEA: plate of cellulose acetate. Bar=5 μm

FIG. 2. TEM of granulocyte. Electron dense, electron lucent and structural granules, and numerous vesicles are seen. N: nucleus. Bar=1 μm.

FIG. 3. The surface view of a 5-min-old capsule. Lysing granulocytes extend filopodial processes on amorphous membrane. GR: granulocyte, M: amorphous membrane. Bar=5 μm.

FIG. 4. The surface view of a 10-min-old capsule. Granulocytes disintegrate and release numerous granules. Bar=5 μm.

Circulating hemocytes were counted in the hosts at the two stages; shortly before and 10 hr after oil injection, in order to elucidate the effect of oil injection on hemocyte population. The THCs were 9,800±860 (N=5, Mean± SD) and 10,500±1,650, respectively. This result revealed that THCs fluctuated around the level of 10,000/mm^3 during the first 10 hr after injection. Plasmatocytes (60% of circulating hemocytes) and granulocytes (38%) were predominant. The ratio of two cell populations was unchanged at the two stages. Other types of hemocytes were small prohemocytes and oenocytoids. These cells were not examined in this study.

Hemocytes in direct contact with the oil surface

Two minutes after oil injection, the surface structure of the silicone oil, which was fixed on the plate of cellulose acetate was observed by SEM. There were filopodial hemocytes and an amorphous membrane around the cells (Fig. 1). The cells contained two types of granules; electron dense and electron lucent granules, and large numbers of vesicles were also seen in the cytoplasm (Fig. 2), which enabled us to identify these cells as granulocytes [5, 10]. The amorphous membrane was so thin and fragile that it was often broken during preparation for SEM.

Formation of the innermost layer

When the oil globules were retrieved 5 min after injection, they had been almost enveloped by a thin membrane about 50–60 nm in thickness (Fig. 3).Granulocytes were extending cytoplasmic processes. The cell bodies showed lysing figures. In 10-min-old capsules, there was marked degranulation of granulocytes all around the oil surface(Fig. 4).

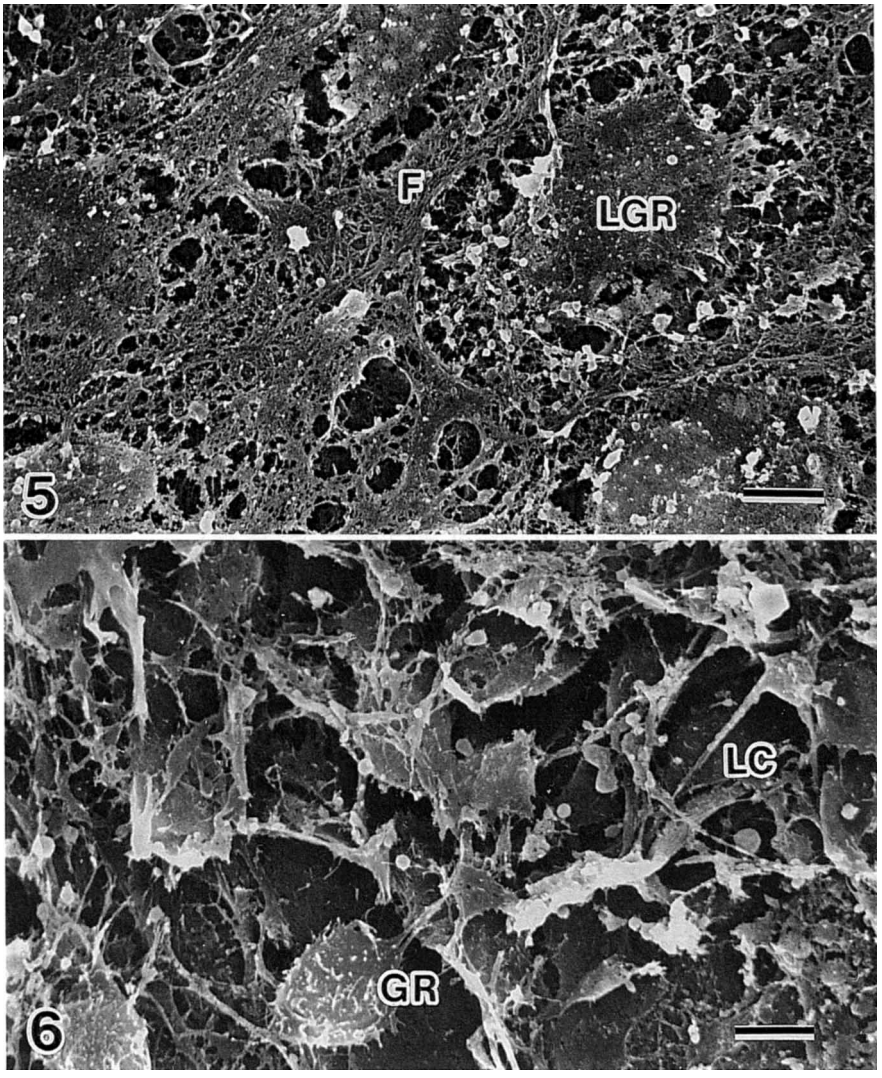

FIG. 5. The inner surface of a 20-min-old capsule. Network structure consists of lysed granulocytes and fibrillar structure. LGR: lysed granulocyte. F: fibrillar structure, Bar=5 μm.

FIG. 6. The outer surface of the 20-min-old capsule. Intact granulocytes aggregate extending cytoplasmic processes. GR: granulocyte. LC: lamellar hemocyte. Bar=5 μm

The innermost region of developing capsules

Capsules were obtained from larvae 20 min after injection, and their inner and outer surfaces were examined.The amorphous membrane was no more seen. The inner surface consisted of degranulated lysing cells which were extending cytoplasmic processes, and forming prominent network around the cells (Fig. 5). Lysing cells were arranged at intervals of about 20–40 μm from cell to cell. Numerous granules (100–300 nm in diameter) and fibrillar structure was also seen. While the outside view of this capsule was quite different from that of the inner surface (Fig. 6). Numerous intact cells were aggregating on and near the capsule. Granulocytes and lamellar hemocytes (probably modified plasmatocytes) were loosely in contact with their cytoplasmic processes.

In 1-hr-old capsules, the network structure in contact with the oil surface became more strongly reticulated by the increase of fibrillar material(Fig. 7). In 10-hr-old capsules, deposition of amorphous structure appeared on the innermost region of the network (Fig. 8).This structure developed and finally covered the inner surface of the developing capsule. The inner surface of 20-hr-old capsules had been coated withthe amorphous structure, and numerous hemocytes were aggregating outside the innermost layer(Fig. 9). These cells consisted of various types of intact cells. Spindle shaped or lamellar plasmatocytes were more frequently seen, while granulocytes and degranulating hemocytes were also observed (Fig. 10).Compaction of hemocytes was observed in the capsules of later than 20 hr after injection.

72-hr-old capsules of silicon oil

Milky-white capsules of silicon oil were obtained from insects 72 hr following injection. Figure 11 shows a transverse section of the capsule, which consists of about 15 cell layers, being 30–40 μm thick. The capsule can be divided into the inner and outer regions. The inner region consists of necrotic cells. Though the nuclei are present, mitochondria and other cytoplasmic structures are obscure. In SEM observation, the inner surface consisted of the amorphous structure (Fig. 12). No brown colored, melanized area was found in the inner layer of the capsule. The outer surface of the capsule was covered with numerous intact cells (Fig. 13).

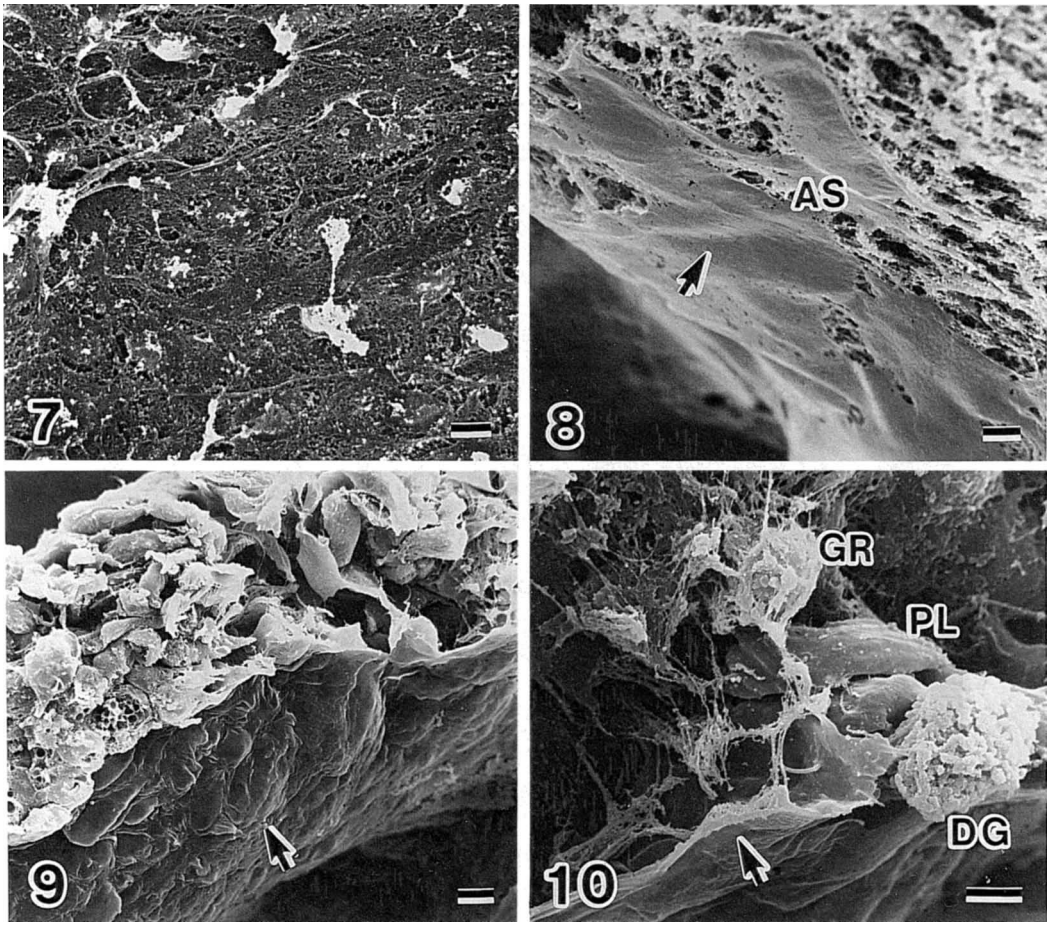

FIG. 7. The inner surface of a 1-hr-old capsule. The network structure develops to envelope the oil surface. Bar=5 μm

FIG. 8. The inner surface (arrow) of a 10-hr-old capsule. Amorphous substance seals network. AS: amorphous substance. Bar=5 μm.

FIGS. 9 and 10. The transverse view of a 20-hr-old capsule. The inner surface (arrow) of the capsule consists of an amorphous substance by which the oil globule is completely isolated from aggregating cells. F: fibrillar strands, DG: degranulating cell, PL: plasmatocyte, GR:granulocyte. Bar=5 μm

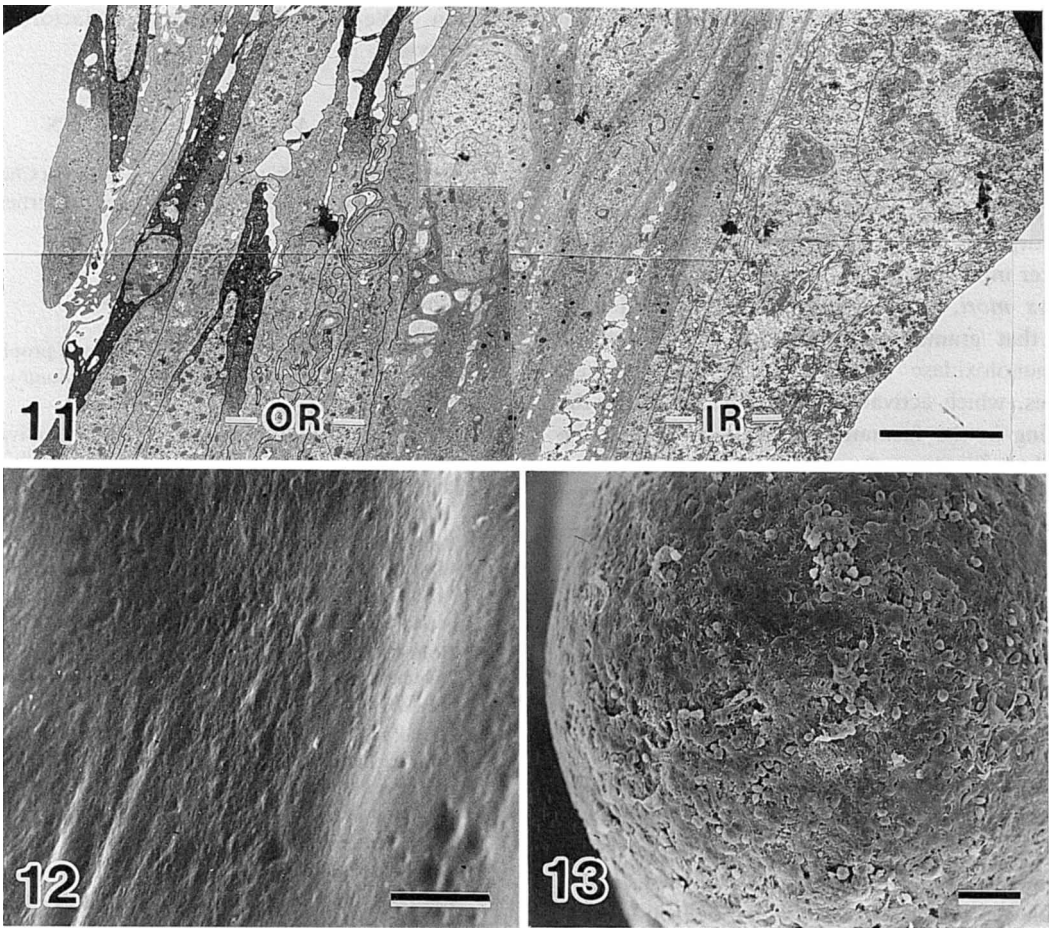

FIG. 11. TEM of a 72-hr-old capsule. The capsule consists of about 15 cells in thickness, which can be divided into two regions.The inner region (-IR-) consists of necrotic cells and the outer (-OR-) of flattened cells. Melanin is not seen. Bar=5 μm

FIG. 12. The inner surface of a 72-hr-old capsule coated with amorphous substance. Wrinkle patterns on the surface will be an artifact when the capsule is prepared. Bar=5 μm.

FIG. 13. The outer surface of a 72-hr-old capsule.It consists of numerous hemocytes. Bar=50 μm.

It seemed that encapsulation process were still being in progress [11].

DISCUSSION

Morphological events of the initial process of encapsulation occurring on silicone oil were clearly shown by removal of the oil itself. Some characteristics of the encapsulation are as follows:

Silicone oil as a foreign material

Silicone oil was very useful as a foreign material for the *in vivo* experiments of encapsulation in *Samia cynthia ricini*. It was very easy to inject with 10 μl of silicone oil into the hemocoel. We could repeat the same experiments in many specimens collected from other batches, and obtained the results similar to those presented here. Silicone oil is known not only to be chemically inert and not toxic to mammals, but also to be harmless to insects. It may be considered that the encapsulation of silicone oil was initiated by the one-way response of the host immune systems to the injected oil.

Type of encapsulation

There is a correlation between high hemocyte count (more than 6,000 cells/mm^3) and the occurrence of cellular encapsulation, whereas a lower hemocyte number correlates with the initiation of humoral encapsulation [8]. In 5-day-old 5th-instar larvae, the THC was about 10,000/mm^3. The DHC of granulocytes was about 3,800/mm^3. We estimated from TEM micrographs that about 600–1,000 granulocytes/mm^2 attached to the oil surface and disintegrated within 20 min.

Initiation of capsule formation

In general,the granulocytes come in contact with the surfaceof foreign materials, disintegrate and release granules, probably containing encapsulation promoting factor(s) [16, 20]. When silicone oil was injected, granulocytes first attached to the oil surface. It is noteworthy that the thin membrane of amorphous substance had occurred on the oil

surface 2 min after injection. The amorphous substance, in appearance,looked like the hemolymph coagulum which was formed in the hemolymph taken out of the body [4, 6, 16]. If this interpretation would be correct,it will mean that within 2 min after injection, granulocytes disintegrate to release hemocyte coagulogen on and near the oil surface. However, we had no technique to demonstrate it in the short time *in vivo*. The amorphous substance changed into fibrillar structure 20 min after injection.

In *Bombyx mori*, Ashida and colleagues [1–3] have demonstrated that granulocytes discharge granules which contain prophenoloxidase (proPO) activating enzymes, serine proteases, which activate the proPO cascade in the plasma, resulting in the formation of melanin around the capsule, and the activation of proPO is necessary for the activation of immune systems of the insect. Wago [22] described that preinjection of a serine protease inhibitor, p-nitrophenyl p'-guanidino benzoate(p-NPGB) suppressed an attachment of granulocytes to a foreign surface.

On the assumption that the activation of proPO may be related to the initial step of encapsulation, we carried out a preliminary experiment [Takahashi, unpublished data]: Silicone oil suspended with fine crystals of 10 μg p-NPGB (Sigma, St.Louis) was injected into 5th-instar larvae.No encapsulation occurred against it within 20 hr. While the oil suspended with p-NPGB heated at 70°C was completely encapsulated. It is suggested that serine protease(s) and probably, the activation of proPO cascade will be necessary for the encapsulation of silicone oil, though the reaction is not accompanied with visible melanin formation. It will be an intriguing problem how the morphological events occur on the foreign surface in relation to these biochemical events.

Innermost region of developing capsules

The network structure was predominant in the innermost region of capsules later than 20 min after injection. We checked whether or not the network structure would be an artifact-product formed during preparation for SEM. Soluble components were surely removed from the innermost region as described previously [21], but this structure was commonly found in a number of SEM micrographs of capsules. It was presumably derived from humoral components and/or resulted from lysis of granulocytes.

The innermost layer has often been described as a connective tissue in some insects [12, 17–19]. This layer became gradually undetectable in the capsules elder than 72 hr old. Salt [9, 18] observed in *Ehestia* larvae that the inner layer substance moved to the intercellular spaces during cell compaction. The innermost layer has been suggested to be an actual barrier to protect the hemocoel at the initial phase of encapsulation. Plasmatocytes and other cells came toward the foreign surface during and after completion of the innermost layer. Compaction of the cells to form the capsule occurred over 20 hr later after injection. Thus, the recruitment and compaction of capsule-forming cells may be induced by factor(s) released from the innermost region. We are now interested in the factor(s) which recruit hemocytes.

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