Arthropod Immune System. II. encapsulation of Implanted Nerve Cord and “Plain Gut” Surgical Suture by Granulocytes of Blattella germanica (L.) (Dictyoptera: Blattellidae)

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ABSTRACT—In the German cockroach, Blattella germanica only the granulocyte (GR) encapsulates the nerve cord implants from the American cockroach and “plain gut” surgical suture implants in the abdominal hemocoel. Encapsulation commences about 40 min after implantation and is completed in 10 days. The completed capsule is often 6.5 \(\mu\)m thick, consists of inner and outer regions, which together are composed of about 20 layers, and a 64 \(\mu\)m thick outer sheath; the latter when completed, does not show any further attachment of GRs to it, and in fact marks the end of encapsulation. Early on in capsule formation, the innermost GR layer of the inner region seems to secrete melanin, and later other layers produce random melanin deposits in the entire capsule. It seems that in this cockroach, the GR provides both the phenols and the phenol-oxidizing enzyme to produce melanization. Desmosome-like-, intermediate-, B-type gap, and septate cell junctions are present, the latter reported for the first time.

INTRODUCTION

Encapsulation of foreign or nonself tissue is a cellular reaction that is accomplished by plasmatocytes (PLs) and/or granulocytes (GRs), previously referred to as immunocytes [1, 2], because of their ability to distinguish between self and nonself tissues. Several aspects of this cellular immune reaction vary in different insects and are controversial [2, 3]. For example, the structural changes in the immunocytes during capsule formation, including the degree of flattening, necrosis, and the formation of membrane-bounded granules vary in different insects. Similarly, there are differences in the immunocytes of the middle and the outer regions in terms of the occurrence of gap junctions, desmosomes, microtubules, and mitochondria. Furthermore, the degree of melanization and the hemocytic origin of the phenols and the phenoloxidase that produce melanization are controversial, because, in addition to the GRs, coagulocytes and oenocytoids have been reported to provide both these compounds [4, 5].

Although encapsulation in the German cockroach, Blattella germanica has not been previously reported, it has been described in the American cockroach, Periplaneta americana [6-11]. According to Ennesser and Nappi [10], both the immunocytes (PLs and GRs) participate in capsule formation in P. americana, the GRs playing the major role. During an investigation of the immune system of B. germanica, we noticed several specific differences in the encapsulation process in this species from those in the American cockroach. For example, only the GRs form the capsule in B. germanica. Furthermore, early on in capsule formation, one layer of GRs secretes melanin and later other layers produce scattered melanin deposits in the entire capsule; the completed capsule has an outer sheath that shows no further attachment of GRs to it. In addition, the capsule has desmosome-like-, intermediate-, gap- and septate

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junctons. In this paper, we present these details of encapsulation and point out the significant differences from those in the American cockroach.

MATERIALS AND METHODS

Adult males of *B. germanica* from colonies, maintained at 28 ± 2°C on Purina laboratory chow and water, were used. After implantation, the insects were kept separately.

**Implantation and fixation of implants**

We implanted 1mm-long pieces of fixed (surface-altered) and unfixed nerve cord from *Periplaneta americana* and commercially available pieces of "plain gut" (sterile, absorbable surgical suture) (Ethicon, Inc., Somerville, NJ). Surface alteration was carried out by fixing in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1.5 hr and in 1% buffered osmium tetroxide for 1.5 hr. After rinsing the fixed and unfixed nerve cords and unfixed gut pieces in sterilized buffer for 1 hr, we implanted them in the abdominal hemocoel through an incision in the intersegmental membrane between the first and second abdominal tergites. The insects were temporarily immobilized with CO₂ prior to the implantation procedure. All instruments involved in surgery were sterilized and the abdomens of the host insects cleaned with 70% ethanol. Some of the implants from the host insects at 10, 20, 40 min, 1, 2, 4, 8, 16 hr and 1, 3, 10, and 20 day intervals (ages) were placed in 2.5% glutaraldehyde at 4°C, and others in a mixture of 4% tannic acid and 2.5% glutaraldehyde (1:1) in 0.1 M cacodylate buffer (pH 7.4), containing 0.13 M sucrose, at room temperature. They were then washed in buffer for 1 hr, placed in 1% buffered osmium tetroxide for 1.5 hr, washed again in buffer, dehydrated through a graded ethanol series, and embedded in Epon-Araldite mixture. The sections, stained in uranyl acetate and lead citrate, were observed in a Philips 300 electron microscope.

**Freeze fracture and etching**

One to 7 days after implantation, the implants were fixed in situ with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 hr, rinsed in 30% glycerol buffer and kept at 4°C. The implants were placed on standard gold disc and quickly frozen in melting Freon 22 at −160°C for 10 sec and then quickly transferred to liquid nitrogen. Platinum-carbon replicas of etched specimens were produced in a Blazer High Vacuum Freeze-Etch Unit BAF 301 at a temperature of −100°C and a vacuum of 2 × 10⁻⁶ Torr [12]. Clean replicas were observed in a Philips 300 electron microscope.

The total (THC) and differential (DHC) hemocyte counts were determined according to the technique by Chiang et al. [13].

RESULTS

We have reported elsewhere [13, 14] that, in addition to the 2 major immunocytes (plasmatocytes (PLs) and granulocytes (GRs)), the German cockroach possesses prohemocytes, oenocytoids, adipohemocytes, and spherulocytes. We identified these hemocytes according to Gupta’s [15–17] classification.

**Normal and activated granulocytes**

Normal or resting (unactivated) GR (Figs. 1 and 2) is a flattened, discoid cell, and contains, among other organelles, microtubules that are arranged in the form of bundles in its peripheral region in the plane of flattening. The activated (postimplantation) GRs (Figs. 3 and 4) show different ultrastructural details; they are characterized by irregular outline, presence of numerous cytoplasmic extensions, and pronounced development of peripheral microtubules (Figs. 3, 4 and 17).

**Encapsulation**

In *B. germanica*, only GRs participate in capsule formation, which commences about 40 min after implantation, and is completed in 10 days. Because the reactions of the GRs to both the nerve cord and the artificial gut implants were very similar, we have presented the encapsulation of only the nerve cord implants.

Encapsulation begins with the lysis of some GRs. On the surface of 40 min to 8 hr old implants, we noticed flattening and lysis of some GRs (Fig. 3); lysis is evidenced by the presence of
lysosomes, free nuclei, mitochondria, smooth endoplasmic reticulum (Fig. 6), microvesicles, granules, and other cell debris.

The completed capsule (Fig. 18) consists of an inner and an outer region in this roach, the latter being externally limited by a sheath. No further attachment of GRs to the sheath occurs. The structural equivalent of the outer region of other insect capsules is not formed in the capsule of B. germanica.

The inner region

The inner region is completed any time between 8 hours to 3 days in various specimens. It is about 4 μm thick and generally shows 6–9 layers (Fig. 7), the first or innermost of which is consistently covered with a coating of melanin that appears as 2 sublayers, on its inner and outer surfaces, each sublayer being 50 nm thick in cross section. The GRs forming the innermost layer are considerably

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**Fig. 1.** Cross section of a normal or resting (unactivated) granulocyte (GR), showing membrane-bounded structureless granules (MG), microtubule bundles (arrow head, MB) in apical region, Golgi apparatus (GA), Golgi vesicles (GV) and nucleus (N).
Fig. 5. Section of an 8 hr old capsule showing, loosely aggregated GRs. Note some degenerated GRs, membrane-bounded structureless granules (MG), nucleolus (NL), lipid droplet (LD), and desmosome-like junctions (DJ).

Fig. 2. Section of a normal GR along its plane of flattening, showing microtubule bundle (arrow head) close to the plasma membrane in the plane of flattening.

Fig. 3. Section of an activated GRs 40 min after implantation. Note the irregular outline of GR and features of lysis in them. CD = cell debris; LGR = lysed GR.

Fig. 4. Section of an activated GR 8 hr after implantation, showing cytoplasmic extensions and pronounced development of microtubules (MT).

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flattened and show several structural changes. For example, the Golgi apparatus is well developed, and the number and the electron density of the membrane-bounded granules are reduced. In addition, microvesicles exocytosed from intact cells and mitochondria are also visible (Fig. 8). The plasma membranes of cells are not obscured.

External to the innermost layer are 4 other layers (2nd–5th), whose ultrastructural changes resemble those of the innermost layer, except that they lack the melanin sublayers. These 5 layers are surrounded by 2 (18–23 nm thick) laminae (Fig. 7), which appear to be composed of amorphous coagulated hemolymph. Between the 2 laminae is a GR layer represented by fragments of electron-dense material. In the next 3–4 layers (6th–9th), the GRs are less compactly arranged, but ultrastructurally resemble the preceding 5 layers. The 9th, or both the 8th and 9th, layers show necrotic GRs (Fig. 8). Desmosome-like-, B-type (=E-type) gap-, separte-, and intermediate cell junctions are found in this region (Figs. 5, 9, 10, 13-15); the gap junctions occur less frequently in the inner region than in the outer region, and the septate junctions are very few in this region and absent in the outer region.

The outer region

The outer region is completed in a 3-5 day old capsule. It is about 2.5 μm thick and separated from the inner region by a narrow band (in which distinct layering is obscure) of loosely aggregated

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**Fig. 6.** Section of an 8 hr old capsule, showing attachment of GRs to the implant at the beginning of encapsulation. Note a very thin peripheral cytoplasm (arrow head) against the implant (IM). SER= smooth endoplasmic reticulum.

**Fig. 7.** Section of a 3 day old capsule, showing the inner region. Note that the innermost layer of this region is covered with a melanin sublayer (†) on its inner and outer surfaces. External to the innermost layer are 4 other layers and 2 (18–23 nm thick) laminae (L) surrounding the 5 layers. Between the 2 laminae is a GR layer with some electron-dense materials (EDM). Note necrotic GRs in the following 2 layers. GA=Golgi apparatus; IM = implant.

**Fig. 8.** Micrograph showing GRs in a 3 day old capsule. In the region of the capsule facing the hemocoel (HC), the GRs are less compactly arranged. Note necrotic GRs (NG), several vacuoles, and lysosomes (LS), and well-developed Golgi apparatus, mitochondria (M), laminae (L), RER, and microvesicles (MV) exocytosed from intact cells.

**Fig. 9.** Section of a 3 day old capsule, showing gap junction (arrow head) in the inner region. Whenever GRs first contact each other, they generally form gap junctions.

**Fig. 10.** Freeze fracture and etching of the inner region of a 3 day old capsule, showing gap junctions (GJ). Of the 2 B-type (=E-type) junctions, one shows greater dispersion of the particles. A=A (=P) fracture face; B=B (=E) fracture face.

**Fig. 11.** Freeze fracture and etching of the outer region of a 3 day old capsule, showing gap junction. The intramembranous particles on the B (=E) fracture face are 20 nm in diameter, and their number is approximately 640/μm².

**Fig. 12.** Section of a 3 day old capsule, showing gap junction in the outer region. Plasma membrane of the GR is 7.5 nm thick and is distinguishable as 2 electron-dense layers and 1 electron-lucent region between the 2 layers, the thicknesses of the 2 layers and the electron-lucent region being 2.5 nm; that of the intercellular space between the 2 GRs forming the gap junction is also 2.5 nm.

**Fig. 13.** Section of a 3 day old capsule's inner region, showing septate junction (arrow heads). The separation of the membranes within the junctional region is about 3.5 nm wide and the septa are often about 15 nm wide but may vary in width. NG=necrotic GR.

**Fig. 14.** Section of the inner region of a 3 day old capsule, showing intermediate junction (IJ). The 2 adjacent plasma membranes run parallel to each other, the electron-lucent space between them always being 9 nm wide.

**Fig. 15.** Freeze fracture and etching of the inner region of a 3 day old capsule, showing septate junction. The septate junction reveals rows of particles (8–14 nm in dia.) on the A (=P) face and corresponding furrows (6 nm wide, 16 nm center to center) on the B (=E) face.

**Fig. 16.** Section of a 5 day old capsule, showing the outer region, which is formed by continuous layering of GRs, and is separated from the inner region by a band of loosely aggregated necrotic GRs (NG).

**Fig. 17.** Section of a 10 day old, completed capsule, showing the cytoplasm of GR with pronounced development of microtubules.
Fig. 18. Section of a 10 day old, completed capsule. It is about 6.5 nm thick. Numerous microtubules, but few mitochondria (M) and RER, and flattened nuclei (N) are seen. The outer sheath (OS) surrounding the capsule is 64 nm thick. HC=hemocoel; multivesicular body in white circle.
Fig. 19. Section of a 10 day old, completed capsule, showing random deposits of melanin (MD), RER, vacuolated mitochondria (VM), and autophagic vacuoles (AV).
necrotic GRs (Fig. 16); this band of loosely packed
GRs is marked by nuclei and other cell remnants.

The first 4-5 layers of the outer region are visible
in a 3 day old capsule and consist of GRs that
especially show the same structural details as
those of the inner region. Gap junctions are
frequently found in the capsule at this stage (Figs.
11 and 12).

The outer region is completed by deposition of
additional 4-5 layers of GRs, which are extremely
flattened and marked by pronounced development
of microtubules (Fig. 17). The granules and the
Golgi apparatus gradually disappear, and very few
mitochondria and RER are visible. At this stage of
capsule formation, the innermost layer and the 2
amorphous laminae of the inner region become
indistinguishable. Autophagic vacuoles, large lipid
vacuoles, masses of endoplasmic reticulum,
vacuolated mitochondria, fragmented nuclei, ran-
domly scattered melanin deposits, and cell rem-
nants are commonly found throughout the capsule
(Figs. 18 and 19), and the plasma membranes of
the GRs are highly electron dense. No septate
junctions were observed.

The completed (10 day old) capsule as a whole
(Figs. 18 and 19) is very compact, consists of about
20 layers in some regions of the implant, and is
about 6.5 μm thick. The most characteristic fea-
ture of the capsule is the presence of 64 nm thick
outer sheath that marks the end of encapsulation.
No further attachment of GRs to the sheath
occurs. The general features of a finished capsule
include numerous microtubules, free ribosomes,
few mitochondria and RER, and randomly distrib-
duted melanin deposits throughout the capsule.
Golgi apparatus and granules are rarely observed.
Only gap cell junctions are found in the outer
region of the completed capsule. At no stage of
the capsule formation did we notice the “cylinder
inclusions” that had been observed in the capsule
of P. americana [10, 18].

DISCUSSION

Most of the general discussion on encapsulation
pertaining to other insects has been previously
reviewed by Gupta [2, 17]. Although, there is
disagreement among insect hematologists as to the
number of hemocyte types and their identification
in various insects [17], the immunocytes (PLs and
GRs) that participate in immunorecognition and
encapsulation are generally recognizable in light,
scanning, and transmission electron microscopical
examinations. In the present study, we did not find
any plasmacocytes participating in capsule forma-
tion. Why the PLs do not participate in capsule
formation may be related to the absence of
peripheral microtubules in these cells [20]. Both
altered (fixed) and unaltered (unfixed) xenogeneic
implants produced similar reaction.

Initial immunocyte response to the implant

Apparently, capsule formation both commences
and is completed much earlier in P. americana
(Blattoidea: Blattidae) than in B. germanica (Blat-
toidea: Blattellidae). Ennesser and Nappi [10]
reported that capsule formation in P. americana
commences 10 min postimplantation, and is com-
pleted in about 40 min. In B. germanica, we
noticed first signs of aggregation of GRs on the
implant 40 min postimplantation, and it took
about 10 days for the capsule to be completed.
Whether this differential cellular reaction between
the two species of cockroaches is due to taxonomic
differences (the two species belonging to two differ-
ent families), or that the immunocytes in the
German cockroach are less reactive to the foreign
tissue than those of the American cockroach, is
difficult to explain. This difference in response
does not seem to be due to lower total (THC) and
differential (DHC) counts of hemocytes, because
we did not find any significant differences in the
THCs and DHCs of GRs of the two species [13 and
unpublished observation]. At any rate, it is hard to
imagine that the slow and delayed hemocytic reac-
tion to the implants is an efficient or beneficial
immune reaction from the standpoint of survival of
the cockroach; this prompted us to investigate
whether a much faster rate of phagocytic reaction,
being often the first line of defense, compensates
for the much slower process of encapsulation. Our
preliminary results of the rate of phagocytosis of
injected microspheres suggest that this indeed is
the case (unpublished observation).
Number of cell layers

Cellular capsules vary in terms of the structural changes in the immunocytes that take part in capsule formation and the number of cell layers (regions) in the completed capsule. Baerwald [18] reported a range of 20-75 layers in various insects. In many insects, the layers of the inner region show flattened and necrotic PLs and/or GRs. In B. germanica, the most characteristic features of the inner region are the presence of the 2 sublayers of melanin on the inner and outer surfaces of the first or innermost layer of GRs and the 2 laminae, which disappear later in the completed capsule.

Melanization and sources of melanin

Although Ennesser and Nappi [10] only occasionally observed melanin layers in the P. americana capsules, we consistently found the 2 sublayers of melanin in the innermost layer of the inner region during the initial stages of encapsulation and numerous randomly distributed melanin deposits in various parts of the 10 day old completed capsules in B. germanica. The question whether melanization originates in the animate foreign tissue or in the host's immunocytes is still debated, although it has been reported in many cases that the immunocytes provide both the phenolic substrate and the phenol-oxidizing enzymes [see 2, 17 for review]. It should be stated, however, that the hemocytic origin of both the phenols and the phenoloxidases continues to be controversial. Furthermore, in addition to GRs and/or PLs, coagulocytes and oenocytoids have been reported by one author or another to provide both the substrate and the enzyme [4, 5], although Gupta [1] did not find tyrosinase in the oenocytoids of another cockroach, Gromphadorhina portentosa.

Our present studies suggest that in B. germanica both the phenols and phenoloxidase are supplied by the GRs, because we noticed the melanin sublayers even in the capsules around the artificial gut implants, which would be the most unlikely sources of these compounds. Indeed, Schmit et al. [5] have demonstrated the presence of melanin precursors in the GRs of Galleria mellonella. However, the possibility that the hemolymph could be the source of the phenols and the phenol-oxidase that produced the melanization cannot be ignored.

Lysis and other structural changes in granulocytes

That encapsulation commences with the lysis of GRs has been reported by many authors. Apart from the general flattening of the immunocytes forming the capsules, several other changes have been described in them [see 2, 3, 17 for review]. We also noticed lysing GRs on the surface of the implant and the 2 (18-23 nm thick) laminae surrounding the 5th layer of the inner region. We consistently observed these laminae, which, as far we know, have not been reported in the capsules of other insects. They seem to be transient structures, because they gradually disappear as the capsule formation progresses and are generally absent in the fully formed, 10 day old capsules. Their function remains unknown.

The pronounced development of microtubules in the GRs forming the capsule has been reported in other insects and is prominent in those of B. germanica. François [19] has shown numerous microtubules and desmosomes in 7 day old capsules of Thermobia domestica. According to Baerwald [18], the microtubules in the marginal bundles are presumably associated with desmosomes. We have reported elsewhere [20] that the pronounced development of microtubules is characteristic of activated GRs and that these organelles are absent in the PLs.

Functional significance of cell junctions in capsule formation

The presence of the intermediate-, desmosome-like-, gap-, and the septate junctions in the capsules seems to be functionally significant. On the basis of the known functions of these cell junctions in vertebrate tissue, we propose that they perform two most important functions in the formation of the capsules: 1) the gap junctions provide cell-to-cell communication during coupling of the GRs to form the various layers and 2) the desmosome-like- and septate junctions anchor the layer-forming GRs mechanically.

Gupta [2] has briefly summarized some of the literature on gap cell junctions in some arthropods. The presence of gap junctions in capsules is impor-
tant, because during capsule formation, these
junctions probably assist the enveloping GRs to
recognize one another and thus interact more
effectively in forming the capsule. In fact,
Caveney and Berdan [21] have suggested rapid de
novo formation of gap junctions in the hemocytic
capsule of P. americana. It is conceivable that
coupling through gap junctions enables the GRs in
B. germanica to set up a pathway for communica-
tion among the cells of various layers by estab-
lishing gradients of ion concentrations along the
width of the capsule, for, according to Alberts et
al. [22], “any of these gradients could serve as a
reference system for the determination of the
relative cell position,” thus instructing each GR to
attach in a certain position.

Although experimental demonstration of GR-
to-GR communication through gap junctions in
the capsules in B. germanica remains to be estab-
lished, Caveney and Berdan [21] have shown that
carboxy fluorescein, when microinjected into 72 hr
old hemocytic capsule of P. americana, “moved
readily from cell to cell,” via the gap junctions.
Furthermore, cell-to-cell communication has been
reported in cultured multicell spheroids of mam-
mary tumor cells of the marshall rat [23]. These
authors reported that 2 days after the formation of
the spheroids, the pores in the gap junctions
apparently closed (indicated by the fact that in-
jected Lucifer yellow dye was retained in the
injected cell and did not spread to other cells of the
spheroid). Gupta [2] suggested that if a similar
cessation of cell-to-cell communication occurred
during the capsule formation in arthropods, it
would explain the progressive retardation of the
attachment of immunocytes to the capsule as its
thickness increases. The existence of such a
mechanism remains to be confirmed, however.

In the very few arthropods in which the gap
junction particles had been studied by the early
1970s, they were generally reported to be located
on the extracellular E (= B) fracture face, and
therefore, Flower [24] designated these gap junc-
tions as “inverted type,” with reference to the
mammalian gap junctions, in which the location of
the particles is reversed (i.e. on the cytoplasmic P
(= A) fracture face). Thus, Staehelin [25] and
Baerwald [8] suggested that the extracellular E-
type gap junctions may be characteristic of arthro-
pods, although such junctions have also been
found in the coelenterate, Hydra [26]. The nem-
tode, Trichinella spiralis [27] and some mollusks
[28, 29], on the other hand, have P-type gap
junctions. In view of this, Gupta [2] suggested that
probably the E-type represents the most primitive
stage, while the P-type is a more advanced type
that originated before the evolution of the arthro-
pods, in which both P- and E-types are present.
Indeed P-type junctions are now known to be
present in several arthropods [30–32]. We counted
about 640/μm² such intramembranous particles
(connexons) on the E fracture face in the capsule of
B. germanica. According to Baerwald [18], gap
junctions are more numerous in the deeper layers
of P. americana capsules. Our observations in B.
germanica support this.

The desmosome-like- and septate junctions
probably help maintain the structural integrity of
the capsule. Desmosomes are known to anchor
cells together and are most abundant in tissues
(e.g. cardiac muscle, skin, and neck of uterus) that
are subject to severe mechanical stress [22]. Des-
mosomes have been reported in the capsules of
Carausius morosus, Locusta migratoria, Melolom-
tha melolontha, P. americana and G. mellonella,
and gap junctions only in Ephesia kuhniella and P.
americana [18].

We report for the first time the presence of
septate junctions in the capsule of B. germanica by
both thin section and freeze-fracture methods. We
should mention, however, that Grimstone et al.
[33], in their Figure 13, have mentioned “trans-
verse striations in intercellular material.” We have
reported the details of the septate junctions else-
where [34].

Continuation and cessation of layering in capsule
formation

The mechanisms, involved in the maintenance
of continuous layering in the capsule and its ces-
sation when the capsule has been completed, remain
conjectural. Grimstone et al. [33] suggested that as
each layer is formed, the surface properties of the
plasma membrane of the cells forming the capsule
changes, as a result of which they are recognized as
nonsel tissue and in turn attract new cells. Amir-
ante [35] hypothesized that as hemocytes adhere to the foreign tissue, they form “new acceptors” (some sort of lectin receptors) on their plasma membranes that attract the lectins on the plasma membranes of other hemocytes, which then form the next layer. We have suggested above the possible role of gap junctions in establishing a continuous ion gradient, which might promote continuous positioning of GRs on the developing capsule.

Whatever the mechanism(s), it must cease to operate when the capsule reaches a certain thickness or age, because no further attachment of immunocytes occurs. In fact, this cessation of attachment is not abrupt, but seems to occur gradually. How this mechanism is turned off in other insects is unknown. In B. germanica, the completed capsule has an outer sheath, which, because it is composed of the host material, seems to render the capsule as a self tissue, and no further immunoreaction among the GRs occurs. It must also act as a barrier and interrupt the cell-to-cell communication through the gap junctions. Because we found this sheath only when we allowed encapsulation to proceed at least until 10 days, it is likely that it was missed in the capsules of other insects that were not allowed to fully mature.

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