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Chronic Skin Allograft Rejection in Terrestrial Slugs

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ABSTRACT—To know whether or not molluscs are capable of recognizing tissue alloantigens, dorsal skin-allografts were exchanged between adult terrestrial slug, *Incilaria fruhstorferi*. We succeeded for the first time in orthotopic transplantation of allografts and observed chronic rejection of allografts. During the first two weeks after transplantation (WAT), in all grafts, both foreign (allo-) and self (auto-), many macrophages infiltrated from the host toward the grafts. This phenomenon is seemed to heal wounds. In the case of autografts, many macrophages observed in the grafted site until 8 weeks, whereas at 4 WAT, grafted tissues such as muscle fibers and mucous cells begun to regenerate slowly and the regeneration of these cells had been over at 20 WAT. However, in the case of allografts, regenerative phenomena were not observed, rather than muscle fibers had been actively attacked by macrophages. Numerous macrophages which phagocytosed cell debris were observed in host connective tissues during this experiment. These observations strongly suggest that an allorecognition system is present in molluscs, and in the case of terrestrial slugs dorsal skin transplantation is a useful assay system for analyses of immunological incompatibility.

INTRODUCTION

We have already reported that hemolymph cells (macrophages) of the terrestrial slug can recognize and phagocytose biotic and abiotic non-self materials (Furuta *et al*., 1987; Yamaguchi *et al*., 1988; Furuta *et al*., 1989; Furuta *et al*., 1990), which are agglutinated and opsonized by body surface mucus (Furuta *et al*., 1995). The mucus contains three C-type lectins named incilarin A, B and C (Yuasa *et al*., 1998). Invertebrates lack inducible immunoglobulin or components of the complement system. Instead, lectins are believed to be recognition molecules. It is important to know whether or not these lectins can recognize slight differences in tissue or cell molecules from other individuals. However, allorecognition has not yet been definitely clarified in molluscs.

When digestive glands of *Helisoma duryi normale*, the head/foot and digestive gland tissues, and the heart of *Biomphalaria glabrata* were implanted into the cephalopedal sinus of the other individuals, these heterotopic allografts had been encapsulated by hemocytes (Cheng and Galloway, 1970; Jourdane and Cheng, 1987; Sullivan *et al*., 1992). On the other hand, when hemocyte-producing organ was implanted into the hemocoel, this kind of allograft not only survived without being encapsulated but hemopoietic activity was also still retained (Sullivan, 1990). In *Lymnaea stagnalis*, when *vasa deferentia* was implanted into the cephalopedal blood sinus, hemocytes had aggregated transiently at the cut surface at 24 hr after implantation and, thereafter, small groups of hemocytes were remained in contact with the graft (Sminia *et al*., 1974). Recently, Sullivan *et al*. (1998) had found no conclusive evidence for chronic allograft rejection in *B. glabrata* regardless of tissue that was transplanted. It is conceivable that these contradictory results may be due to the site, i.e., the heterotopic position. Thus, to elucidate whether or not allograft can be recognized in molluscs, we deemed the examination of orthotopic transplantation is indispensable.

Only two reports have been described so far on orthotopic transplantation in molluscs. According to Rögener and Renwrantz (1984), all small pieces of skin of the head-foot (autografts) of *Helix pomatia* were destroyed within 6–9 days. In this case, the skin grafts may have been deficient in size for survival. However, allo- and congeneric xenografts of cerebral ganglia were tolerated in the mesocerebrum which were removed from *H. aspersa* (Gomot and Gomot, 1996). Since results from mammals seem to indicate the presence of a barrier, considering the brain as a privileged site for transplantation is still under discussion and this may apply in even to molluscs.

Because these two experiments produced inconclusive results, we performed experiments on orthotopic transplanta-

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tion of dorsal skin from the terrestrial slug, *Incilaria fruhstorferi* and observed morphological features of auto- and allografts up to 20 WAT (138 days). Epidermis of the dorsal skin is arranged as simple columnar epithelial cells that possess surface microvilli. A great amount of mucus, which covers the dorsal surface skin is secreted by two mucous cell types whose necks reach the apical portions of the dorsal surface skin; these cells were present among the epithelial cells (Furuta *et al.*, unpublication). As the mucus rendered the physical attachment of the graft to the host bed difficult, secretion was inhibited by anesthetizing slugs on ice in our experiments. On the other hand, bacterial infection of the graft appears to be inhibited after transplantation by lectins in the mucus (Furuta *et al.*, 1995; Yuasa *et al.*, 1998). Thus, it was unnecessary to use antibiotics in the present experiments.

This is the first report of successful orthotopic transplantation in molluscs that gives us important information to discuss on phylogenetic significance of allorecognition systems especially in complex invertebrates.

MATERIALS AND METHODS

Slugs

The largest terrestrial slug in Japan, the *I. fruhstorferi* is not an aquatic animal and has no shell unlike snails. These characteristics can allow for the support of the holding of donor tissue to host site. Slugs were collected from fields in Mibu (closed area, *ca.* 0.25 km^2) and Ishibashi in the Kanto district areas which were separated by about 8 km from each other. They were kept in glass petri dishes containing pieces of sweet potato on a wet filter paper and placed in a cabinet at 25–28°C for at least one week before transplantation. At the time of experiments, slugs were 75±10 mm long and weighed 8.5±1.8 g.

Transplantation

In order to prevent slugs from moving and secreting mucus, they were anesthetized on ice for 30 min before transplantation. Small pieces of dorsal skin (2.5×4.5 mm in size and 0.1–0.2 mm in thickness) were peeled off with iridectomy scissors and immersed in slug SA solution (Furuta and Shimozawa, 1983). Then, one of the grafts of slug collected from different area was placed on the host graft bed from which mucus was gently wiped off with cotton tip sticks. In this procedure, grafts were in contact with the graft bed. Slugs were maintained in a glass petri dish (ϕ = 90 mm, h = 18 mm) which was placed on wet filter paper. In order to prevent mucus from floating the graft away, the mucus immediately in front of the graft was often wiped off with cotton tip sticks during 2 hr after transplantation. Each dish was kept in a cabinet at 4°C for 2 days. Then, the dish was placed at room temperature again, the mucus was wiped off again several times for 2 hr. Slugs had been reared normally from 3 days after transplantation until using them for following experiments.

Light and electron microscopy

Slugs were observed dissecting microscopically every week and photographed. In both auto- and allotransplantation, for light and transmission electron microscopy, 2, 1, 3, 1, 3 and 2 slugs at 2, 3, 4, 5, 8 and 20 WAT were sacrificed, respectively. Areas of skin wall containing the grafted tissues were cut out and fixed in 1% paraformaldehyde-3% glutaraldehyde solution for 2 hr, postfixed with 1% OsO_4 for 1.5 hr, and dehydrated in a graded ethanol series. Specimens for light microscopy were embedded in JB-4, sectioned in 3–5 μm thickness, stained with hematoxylin-eosin (HE) and observed with a light microscope (Nikon, Japan). Specimens for electron microscopy were embedded in Epon 812 epoxy resin. Ultrathin sections (100 nm) were stained with uranyl acetate and lead citrate, and observed with a JEM-1210 transmission electron microscope (JEOL, Japan).

Cell count and statistics

Macrophage numbers were calculated as the number of cell debris-laden macrophages in more than 3 sections randomly selected for each sample. The data in auto- and allografts and their beds under a light microscope were shown as the mean±SEM of 10 visual fields. Statistical comparisons were performed using the Student's *t*-test.

RESULTS

Fate of auto- and allografts

In auto-implantation, slugs (20 of 23) had survived for more than 5 weeks and the rate of successful transplantation experiments amounted to 87.0% except for 7 slugs which were sacrificed for 2–5 WAT. And in allo-implantation, 32 of 44 grafts had survived and the rate reached to 72.7%. There was not a significant difference between the rate of auto-implantation and its allo-implatation. In both cases, although only 2 slugs died for 5–7 weeks, 8 slugs had been able to survive for more than 25 WAT without moving grafts from implanted sites.

Dissecting microscopical observation

Grafts were observed with a dissecting microscope every week and photographed. All grafts, both auto- and allografts, were initially healed within 2 weeks and then became firmly attached to the host (Fig. 1).

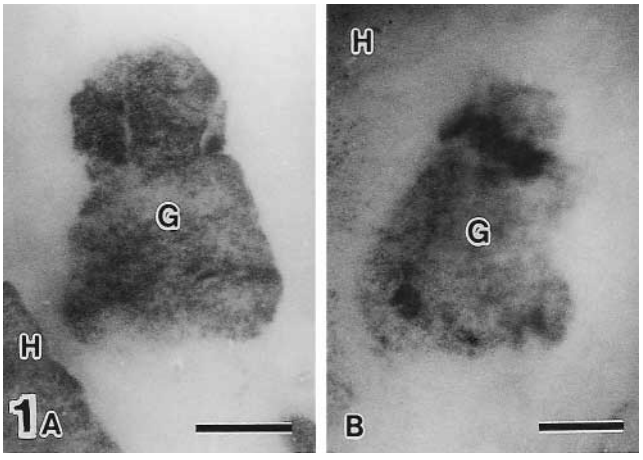

Fig. 1. Dissecting micrograph of auto-(A) or allotransplanted site (B) at 2 WAT. In the cases of auto- and allotransplantation, grafts had firmly attached to graft beds within 2 WAT and they had not moved away during experimental periods. G, graft; H, host epidermis. Scale bar = 1 mm.

Histology of normal slug skin

External surface of the dorsal skin epidermis is composed of a single microvillous columnar layer which hold in place the covering of mucus and underneath epidermal cell layer, numerous pigment cells with many dendritic processes exist.

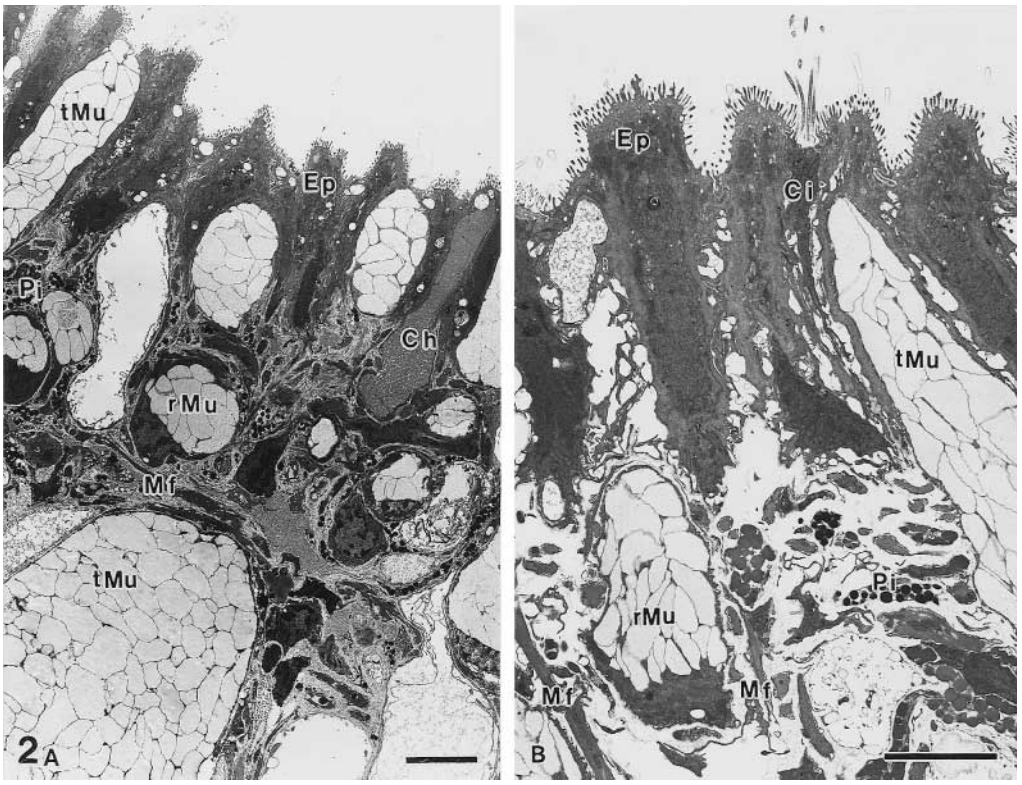

Fig. 2. Transmission electron microscopic (TEM) photographs of normal dorsal skin of slug. Epidermis of normal dorsal skin of slug, *Incilaria fruhstorferi*, is composed by 5 types of cells; surface epithelial cell (Ep), ciliated cell (Ci), round mucous cell (rMu), tubular mucous cell (tMu) and channel cell (Ch). Pigment cells (Pi) existed underneath epidermal cell layer. The epidermis is supported by a mat of connective tissue through which run muscle fibers (Mf). Scale bar = 5 μm.

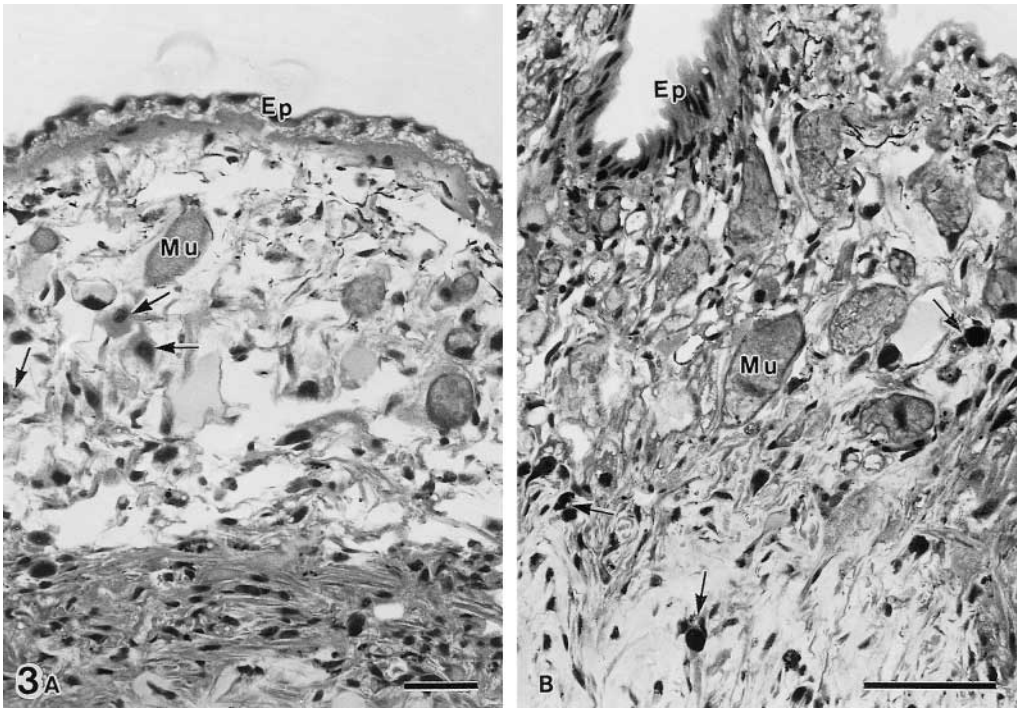

Fig. 3. Light microscopic (LM) photographs of longitudinal sections of auto- (A) and allograft (B) at 2 WAT. Morphological differences between auto- and allografts were not conspicuous. Mucous cells (Mu) whose necks did not reach apical portions of the surface of the transplanted dorsal skin and macrophages (arrows) phagocytosed cell debris were observed in graft tissue. Ep, surface epithelial cells of the graft. HE stain. Scale bar = 50 μm

The epidermis is supported by a mat of connective tissue through which run muscle fibers. Five main types of epidermal cells are distinguished (Fig. 2): (1) microvillous cells, (2) round mucous cells, (3) tubular mucous cells (4) channel cells (Fig. 2A) and (5) ciliated cells (Fig. 2B). The epidermal cells closely cling to one another by zonula adherens in the apical region of cells. The presence of unicellular mucous glands is well established in the slug, and the cells typically possess cell bodies located in the subepidermal connective tissue and secretory processes extending to the surface of the epidermis (Fig. 2). Macrophages are hardly observed in subepidermal connective tissue of normal slug skin.

Light and electron microscopical observation

Mucous cells, pigment cells and muscle fibers were provided as convenient criteria of graft viability in this experiment.

2 weeks—In auto- and allografts, grafts had connected to host beds (Fig. 3) and various macrophages congregated around, particularly, underneath the graft sites and infiltrated into the graft matrix. As macrophages were hardly contained in graft matrix before transplantation, infiltration of macrophages might be considered as migration from host site after transplantation. Numerous macrophages had already phagocytosed cells damaged by mechanical trauma (Fig. 4). As a result of it, pigment cells, mucous cells and muscle fibers decreased in number in grafts and their beds. Apparently this occurred as a non-specific response to injury, since there was no histological difference between auto- and allotransplantation.

3 weeks—In both auto- and allografts, graft matrix was taken up or surrounded by macrophages (Fig. 5). There was no morphological difference between auto- and allograft until 3WAT.

4 weeks—Mucous cells and muscle fibers of both auto- and allografts decreased in number, and various macrophages which infiltrated from host beds to grafts and phagocytosed cell debris were observed (Fig. 6). While in allografts, mucous granules were destructed in mucous cell bodies and muscle fibers were attacked or phagocytosed by macrophages (Fig. 7). At the same time, in only autografts, beginning of regeneration was observed partially; muscle fibers were slowly regenerated and mucous granules were reproducted in regenerated mucous cells (Fig. 6A). Significant differences in light or electron microscopic structures of tissues existed between auto- and allografts; mucous cells and muscle fibers slowly regenerated only in autografts.

5 weeks—In autografts, regenerations of mucous cells, muscle fibers and channel cells were often recognized (Fig.

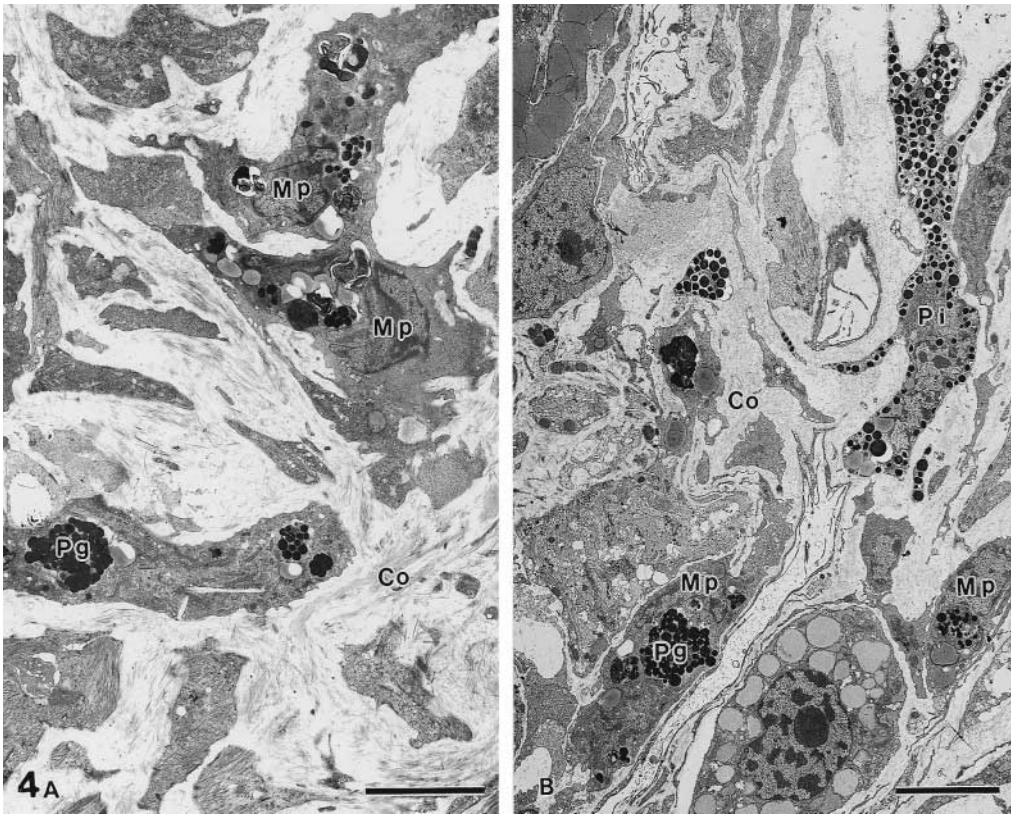

Fig. 4. TEM photographs of the beds of auto- (A) and allograft (B) at 2 WAT. Various macrophages (Mp) phagocytosed cell debris such as pigment cells were observed in connective tissues of both auto- and allograft beds. Pi, normal pigment cell; Pg, pigment granules; Co, collagen fibers. Scale bar = 5 μm.

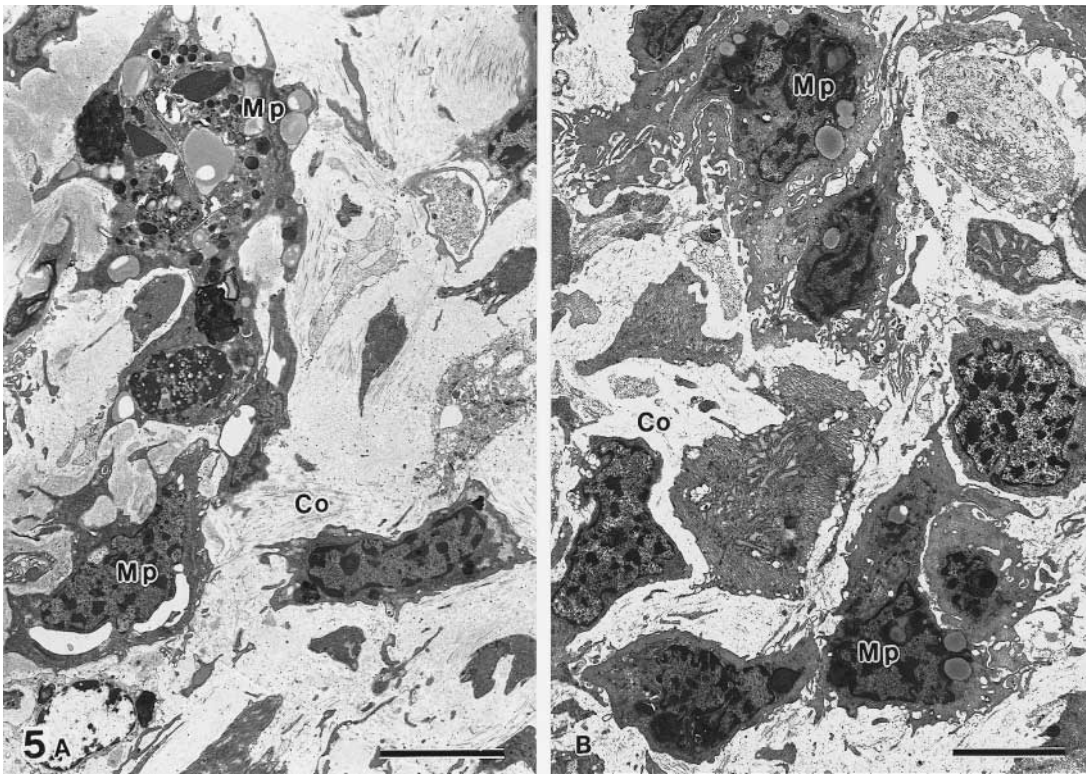

Fig. 5. TEM photographs of the beds of auto- (A) and allograft (B) at 3 WAT. Many macrophages (Mp) which surrounded and phagocytosed cell debris and graft matrix were observed in auto- (A) and allograft beds (B). Co, collagen fibers. Scale bar = 5 μm.

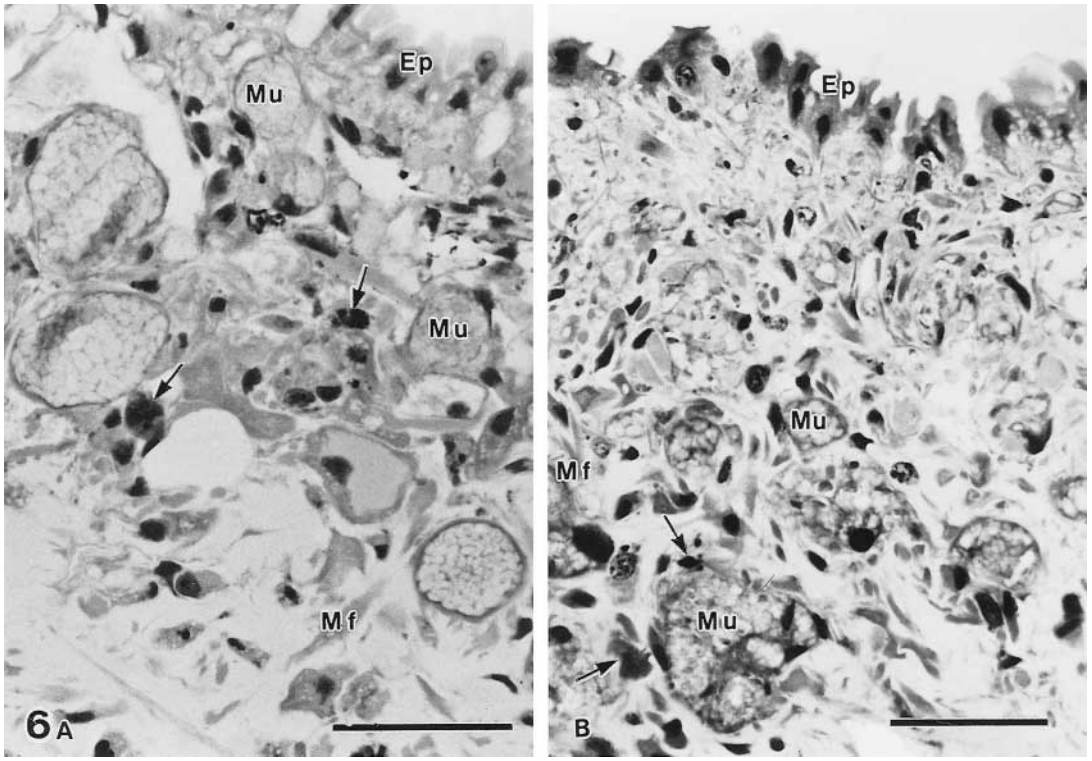

Fig. 6. LM photographs of longitudinal sections of auto- (A) and allografts (B) at 4 WAT. Mucous cells (Mu) and muscle fibers (Mf) of both auto- and allografts decreased in number. But, sign of mucous cell regeneration was observed in only autograft, although various macrophages (arrows) were present in its bed. Ep, surface epithelial cell. Scale bar = 50 μm.

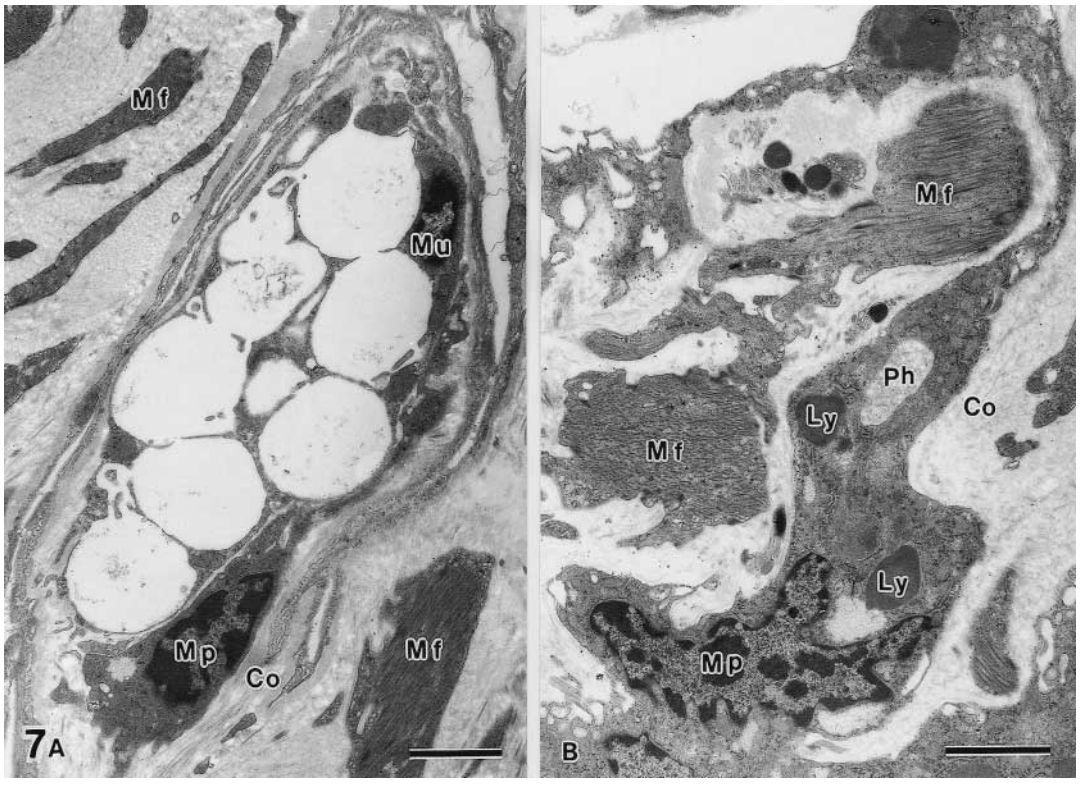

Fig. 7. TEM photographs of macrophages surrounded a mucous cell or muscle fibers in allograft beds at 4 WAT. A; A macrophage (Mp) attached to a mucous cell (Mu) of which granules lost their components. B; A macrophage (Mp) surrounded muscle fibers (Mf) and collagen fibers (Co) and it contained primary lysosomes (Ly) and a phagosome (Ph). Scale bar = 2 μm.

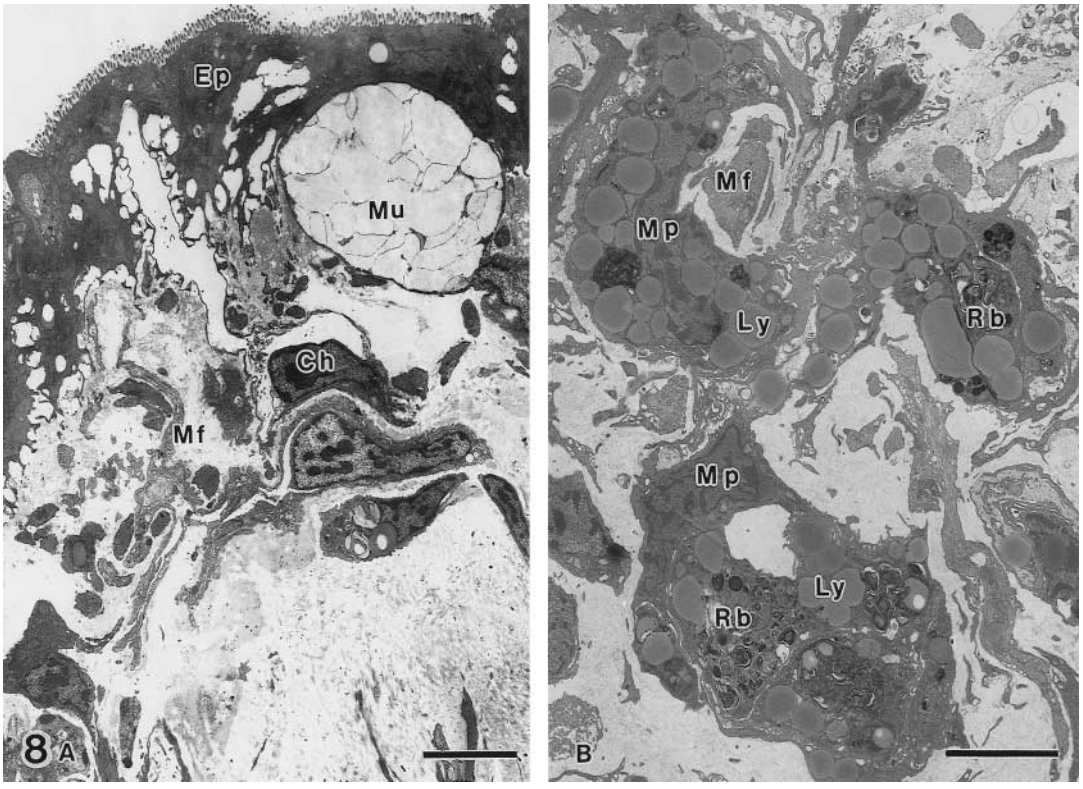

Fig. 8. TEM photograph of an autograft (A) and an allograft bed (B) at 5 WAT. Although in autograft regenerations of mucous cells (Mu), channel cells (Ch) and muscle fibers (Mf) were often observed, in allograft bed macrophages (Mp) had continue to phagocytose cell debris and they contained many lysosomes (Ly) and residual bodies (Rb) in cytoplasms. Ep, surface epithelial cell. Scale bar = 5 μm.

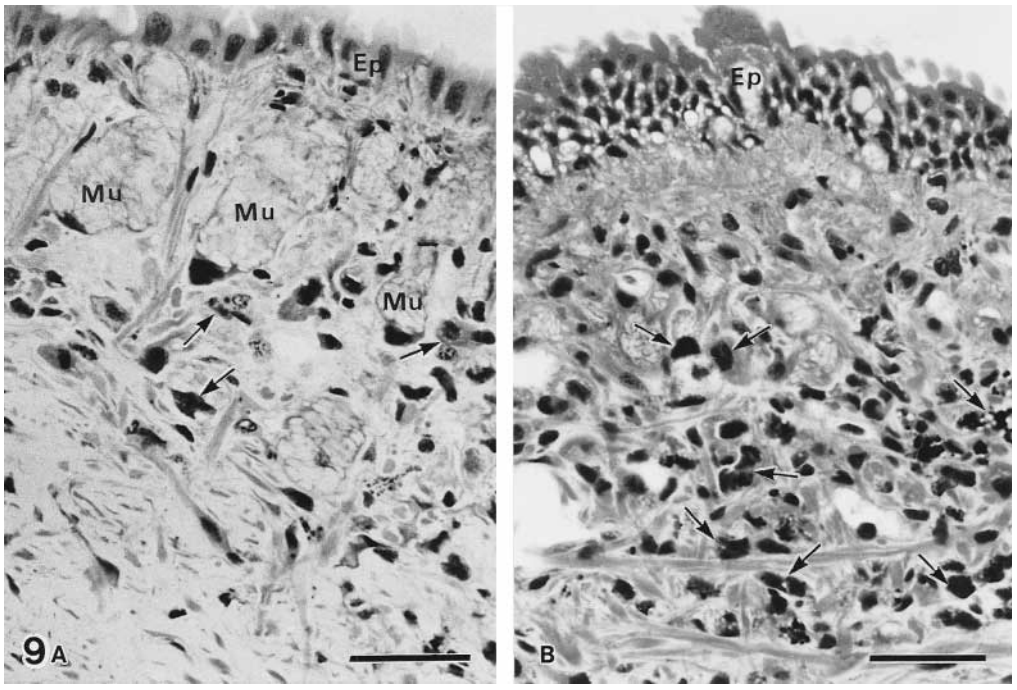

Fig. 9. LM photographs of longitudinal sections of auto- (A) and allografts (B) at 8 WAT. Elimination of cell debris and regeneration of mucous cell (Mu) were recognized in autografts, but many macrophages (arrows) phagocytosed cell debris were present in allografts. Ep, surface epithelial cells of the graft. HE stain. Scale bar = 50 μm.

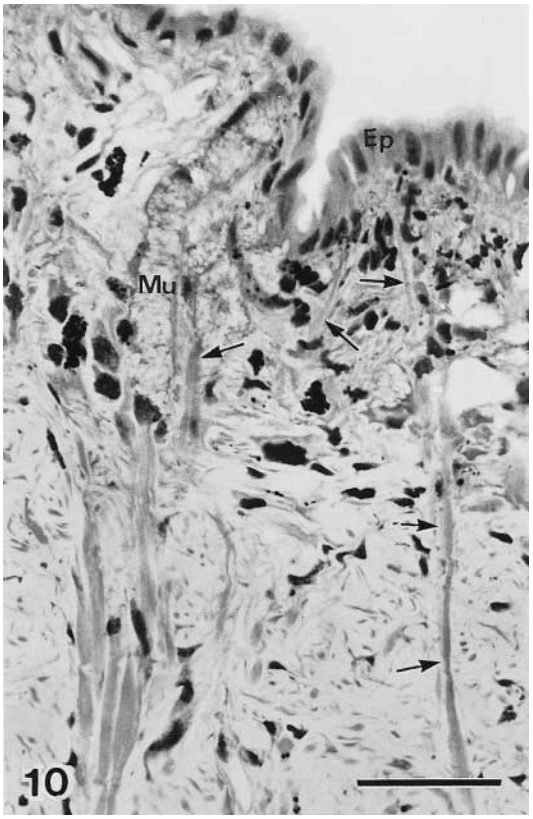

Fig. 10. LM photograph of the regenerated muscle fibers in the autograft at 8 WAT. In only autotransplantation, long and fine muscle fibers (arrows) which elongated from the host to the donor and mucous cells (Mu) were regenerated in the graft at 8 WAT. Ep, surface epithelial cells of the graft. HE stain. Scale bar = 50 μm.

8A), although many macrophages were present in subepidermal connective tissue. On the other hand, in allografts, macrophages had continued to phagocytose cell debris (Fig. 8B).

8 weeks—In autografts, although some macrophages were present in grafts and beds, regenerations of muscle fibers and mucous cells were recognized here and there through grafts (Figs. 9–11). On the other hand, many macrophages laden with cell debris were observed in allografts and their beds (Figs. 9B, 11B, 12). These observations indicate that only in autotransplantation regeneration of epidermis occurrs at this time, but in allotransplantation macrophages have continued to phagocytose tissues for excluding grafts even this time.

20 weeks—In autografts, many regenerated mucous cells and muscle fibers were observed and might recover their functions (Fig. 13A). However, in allografts, many macrophages were still present in grafts and their beds where regenerations of mucous cells and muscle fibers did not occur; phagocytosis and elimination of grafts as nonself materials had not finished even this period (Fig. 13B). Rejection period might be necessary more than 20 weeks in allografts.

Macrophages

The number of macrophages was counted in the auto- and allografts, and in their beds (Fig. 14). At any time, macrophage numbers in allografts and their beds were significantly higher than those of autografts and their beds. And the number in allografts was higher than those in beds. Furthermore, at 20 WAT, the macrophage numbers in autografts and their beds decreased significantly comparing with the numbers at

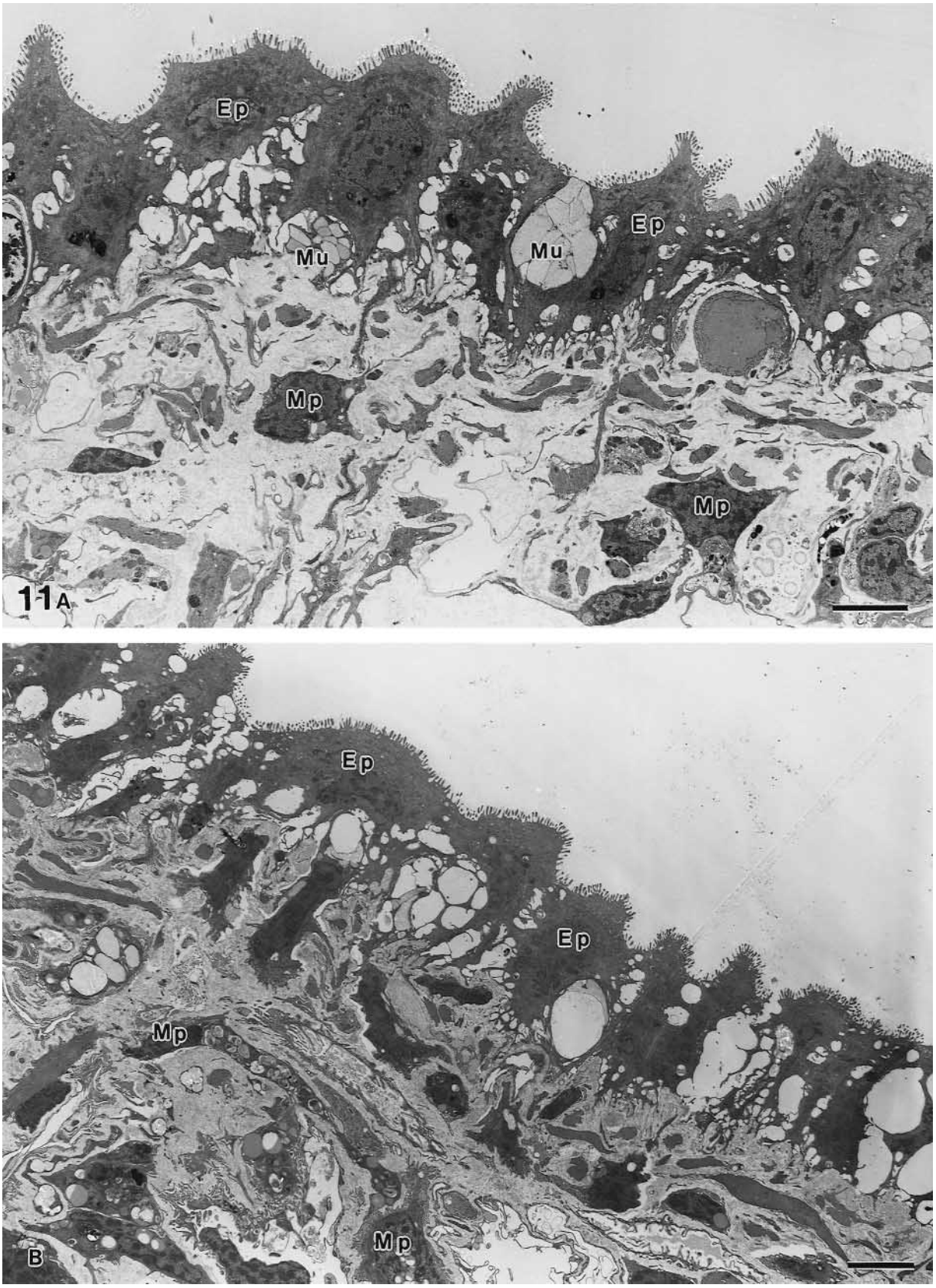

Fig. 11. TEM photographs of auto- (A) and allograft (B) at 8 WAT. Regeneration of mucous cells (Mu) were recognized only in autografts, but no regeneration occurred in allografts. Ep, surface epithelial cell; Mp, macrophage which phagocytosed cell debris. Scale bar = 5 μm.

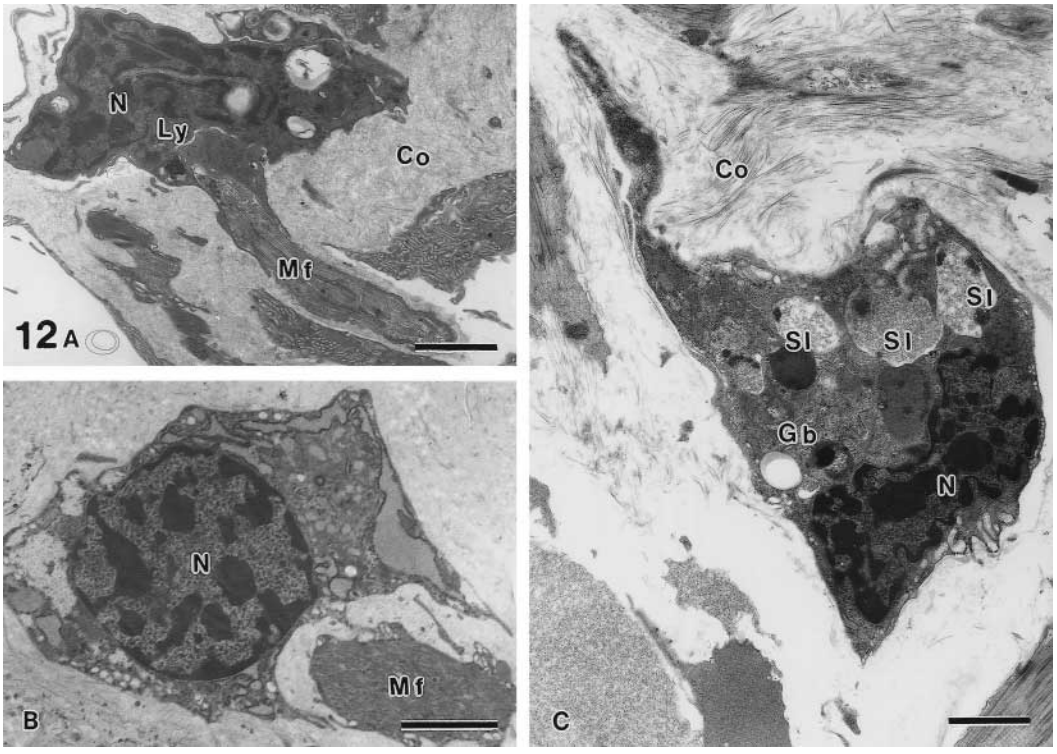

Fig. 12. TEM photographs of macrophages which phagocytosed muscle fibers and graft matrix in allograft beds at 8 WAT. Macrophages attacked to muscle fiber (Mf) (A, B) and engulfed graft matrix (C). They contained primary lysosomes (Ly), secondary lysosomes (Sl) and Golgi bodies (Gb) in cytoplasms. N, macrophage nucleus; Co, collagen fibers. Scale bar = 2 μm.

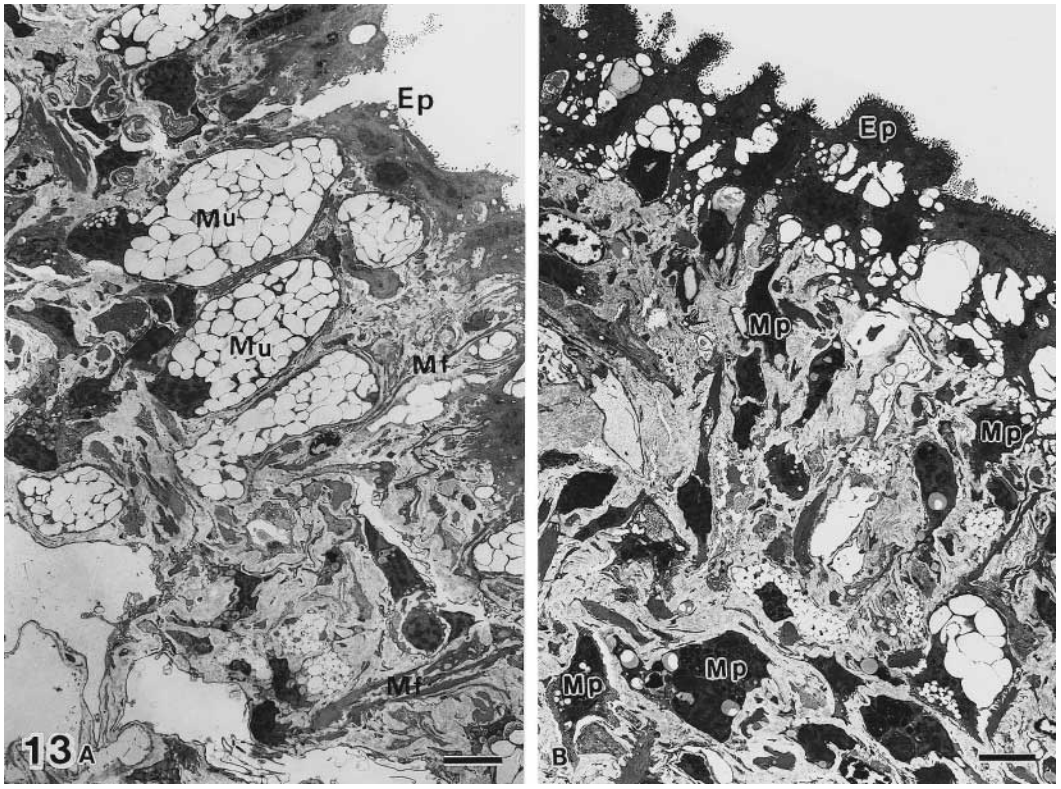

Fig. 13. TEM photographs of auto- (A) and allograft (B) at 20 WAT. In autotransplantation, mucous cells (Mu) and muscle fibers (Mf) of the graft regenerated and seemed to recover functionally, but in allografts many macrophages (Mp) still continued to phagocytose cell debris. Ep, epithelial cell. Scale bar = 5 μm.

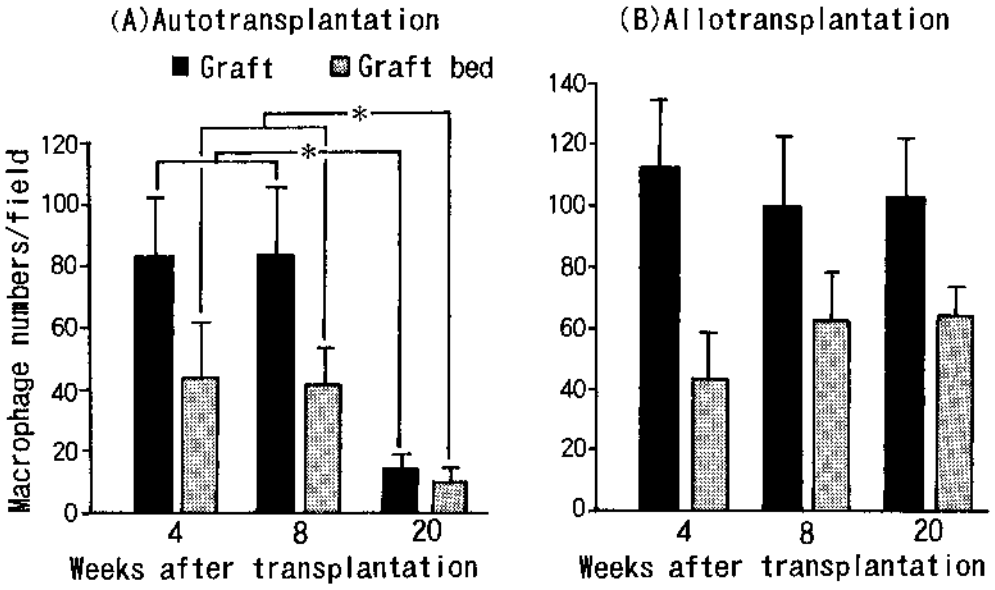

Fig. 14. Changes of macrophage numbers in auto- and allografts, and their beds after transplantation. The number of macrophages phagocytosed cell debris in grafts and their beds were counted out at 10 visual fields randomly selected for each sample. In autotransplantation, the numbers at 20 WAT significantly decreased in grafts and their beds comparing with them at 4 or 8 weeks. Mean±SEM (n=10). *, P<0.001; by Student's *t*-test.

4 and 8 weeks (p<0.001, Student's *t*-test). By contrast, numerous macrophages were still present in allograft and their beds at 8 and 20 weeks and revealed active phagocytosis.

DISCUSSION

In molluscs, because of technical difficulties, experiments of orthotopic transplantation led to inconclusive results as reviewed by Du Pasquier (1993). We were able to inhibit large amounts of mucus secretion by anesthetizing the slugs on ice until the graft connected to the host bed. Moreover, as the mucus protected grafts from bacterial infection and drying after transplantation, we required no antibiotics. This method will be profitable for studying allorecognition system and immunological memory.

The major differences between auto- and allografts by light or electron microscopical observation were as follows; 1) in allografts and their beds, many macrophages were present even at 20 weeks, but 2) in the autograft, mucous cells and muscle fibers regenerated as early as 4 weeks. The macrophage numbers in autograft and their beds at 20 weeks decreased significantly (p<0.001) comparing with the numbers 4th and 8th weeks. This result suggests that macrophages have finished eliminating cells damaged by mechanical trauma during transplantation from the site after 8 weeks in autografts. These differences enable us to consider that an allorecognition system is present in terrestrial slugs.

In other invertebrates, there are evidences of allografts rejection in Porifera *Callyspongia diffusa* (Smith and Hildemann, 1984), Cnidaria *Hymeniacidon sinapium* (van de Vyver, 1980); *Montipora verrucosa* (Hildemann *et al.*, 1977), Annelida *Eisenia foetida* (Cooper, 1969), Arthropoda *Periplaneta americana* (George *et al.*, 1987; Hartman and Karp, 1989), Echinodermata *Cucumaria tricolor* (Hildemann and Dix, 1972); *Dermasterias imbricata* (Karp and Hildemann, 1976); *Lytechinus pictus* (Coffaro and Hinegardner, 1989), and Tunicata *Botryllus primigenus* (Oka and Watanabe, 1957); *Botryllus schlosseri* (Boyd *et al.*, 1990); *Styela plicata* (Raftos *et al.*, 1987). Of these invertebrates, chronic rejection has been shown in Annelida; *Eisenia foetida* rejects the allograft in 15 to 255 days (Cooper and Rubilotta, 1969) and in Echinodermata; *D. imbricata* rejects it on the average 213 days (Karp and Hildemann, 1976). Thus, it is not improbable that molluscs reject allograft chronically.

Accordingly, we speculate that cells damaged by mechanical trauma may be phagocytosed within 8 weeks in both allografts and also their beds. Thereafter, later than 8 weeks, allogeneic cells might be recognized as nonself by a factor(s) in hemolymph such as lectins and/or cells such as a natural killer cells, although they have not yet identified in molluscs. The presence of immunological MEMORY in molluscs has not yet been revealed entirely. As considering from our results, a series of experiment for long periods is necessary to demonstrate allo-transplantation of second- and third-sets in terrestrial slugs. So, xeno-transplantation in which grafts may be rejected acutely rather than allografts is now in progress to prove the presence of it.

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