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MINIREVIEW

Transplant rejection in terrestrial molluscs

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Abstract

To know whether or not molluscs are capable of recognizing tissue allo-antigens, 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. Cellular changes in the rejection process continued over for 40 days. Two functional types of "effector" cells were recognized at the rejection site, but they were observed to be macrophages possessing perforin granules and phagocytosing damaged cells of the allograft. Three days after transplantation, the perforin-positive cells were recognized only in the recipient tissue surrounding the allograft. Five days after transplantation, these cells started to appear in the graft, while they disappeared from the host tissue. However, TUNEL-positive cells (apoptotic cells) were not observed throughout the graft-rejection process. Electron microscopic examination of the graft tissue revealed autophagic degeneration of epithelial cells, mucous cells, pigment cells, fibroblasts, and muscle cells. These observations suggest that the slugs have the capability to recognize differences in cell-surface molecules between the allogeneic and recipient tissue, and that an allograft is chronically rejected due to a type of immunocyte (macrophage) that can induce perforin-dependent cell death.

Key Words: allogeneic rejection; molluscs; orthotopic transplantation; autophagic cell death; perforin

Indroduction

All metazoan animals need an internal defense system to protect themselves against foreign materials that succeed in getting past the external defense. It is well known that the internal defense mechanism in vertebrate consists of non-specific humoral and cellular factors, and specific cellular and humoral ones. Vertebrates are able to respond against invading microorganisms first by non-specific element, such as lysozyme, complements, interferon, lysine, transferin and then by typical immune reactions of a clonal nature. Moreover, macrophages are involved in part or all of those sequential defense processes both as phagocytes and antigen-presenting cells in some way and T- and B-lymphocytes are engaged in only the later stage of specific defense reactions. However, invertebrates including the molluscs do not possess classical immune recognition molecules of vertebrates such as immunoglobulins, T-cells, MHC or antigen receptors.

But they still manage to normally keep their internal body fluids sterile. They possess the capacity to distinguish not only between self and non-self, but also among non-self materials which differ in chemical properties (Bayne, 1990; Cooper *et al.*, 1992). Phagocytosis is considered to be the primary clearance mechanism in molluscs.

Incilaria fruhstorferi, the largest slug native of Japan, possesses only macrophage, a kind of hemocyte, being able to recognize and phagocytose biotic and abiotic non-self materials (Furuta *et al.*, 1987; Yamaguchi *et al.*, 1988; Furuta *et al.*, 1990). The macrophage performs intracellular digestion of non-self materials.

The destruction of tissue allografts in mammals is thought to be mediated primarily by cellular immune mechanisms, an interpretation supported by histological observations of the dense infiltration of recipient cells into the organs to be rejected. Detailed and precise studies of the nature and the function of such cells are essential in order to clarify the mechanisms of transplant rejection. In mammals, the principal target of the immune response to allografts is the major histocompatibility complex (MHC, H-2 in mice). However, MHC molecules have not previously

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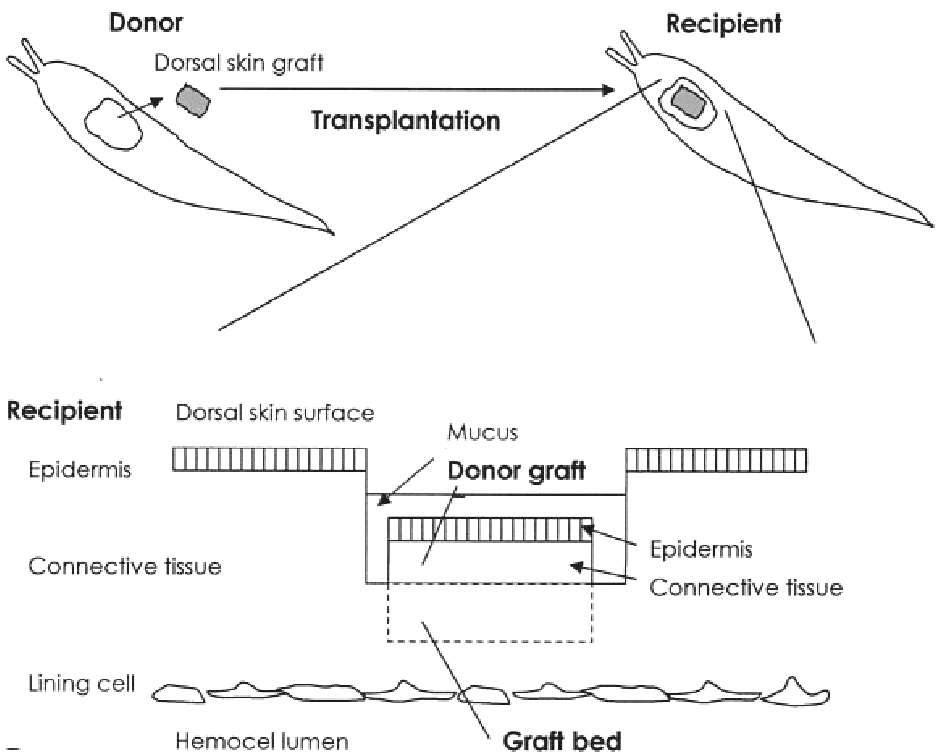

Fig. 1 Procedure of allotransplantation of dorsal skin graft in the slug, *Incilaria fruhstorferi*. A small piece of dorsal skin (2.5x4.5 mm in size and 0.1-0.2 mm in depth) was peeled off and placed on the recipient graft bed of other individual.

been reported in invertebrates, and invertebrates possess neither antigen-specific lymphocytes nor any form of secreted immunoglobulins. Yet, despite the lack of a highly developed specific immune response, those animals apparently have thrived for hundreds million years in a world full of pathogens.

Invertebrates possess several types of hemocytes and at least one of these functions as a phagocyte. Phagocytes are known to play a crucial role in the internal defense system in invertebrates. In addition, a body of evidences suggests that invertebrate animals, especially molluscs, may possess the capability to recognize and reject allogeneic transplants. This is controversial. When digestive glands of *Helisoma duryinormale*, the head/foot and digestive gland tissues, and the heart of *Biomphalaria glabrata* were implanted into 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 hemocel, 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 h after implantation, and thereafter small groups of hemocytes were contact with the graft (Sminia *et al.,* 1974). Sullivan *et al.* (1998) had found no conclusive evidence for chronic allograft rejection in *B. glabrata* regardless of tissue that was transplanted.

The above results show that some grafts can often survive in a recipient for many months, whereas, others undergo varying degrees of hemocytic encapsulation and degenerative changes. These contradictory results may be due to the site, *i.e.*, the heterotopic position. Only two reports have been described so far on orthotopic transplantation in molluscs. According to Röegener 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 xeno-grafts of cerebral ganglia were tolerated in the mesocerebrum which were removed from *Helix aspersa* (Gomot and Gomot, 1996). Since results from mammals seem to indicate the presence of a barrier, considering the brain as privileged site for transplantation is still under discussion and this may apply in even to molluscs. In order to unravel these confused results mentioned above, orthotopic transplantation would be required. Obstacles in performing orthotopic transplantation seem to lie in the technical difficulty of holding the donor tissue at the appropriate site in the recipient. We overcome this difficulty by a method recorded elsewhere (Yamaguchi *et al.*, 1999). We attempted to elucidate in our examination cytological change in both grafted and recipient tissue in response to orthotopic transplants of allogeneic skin using the terrestrial slug, *I. fruhstorferi.*

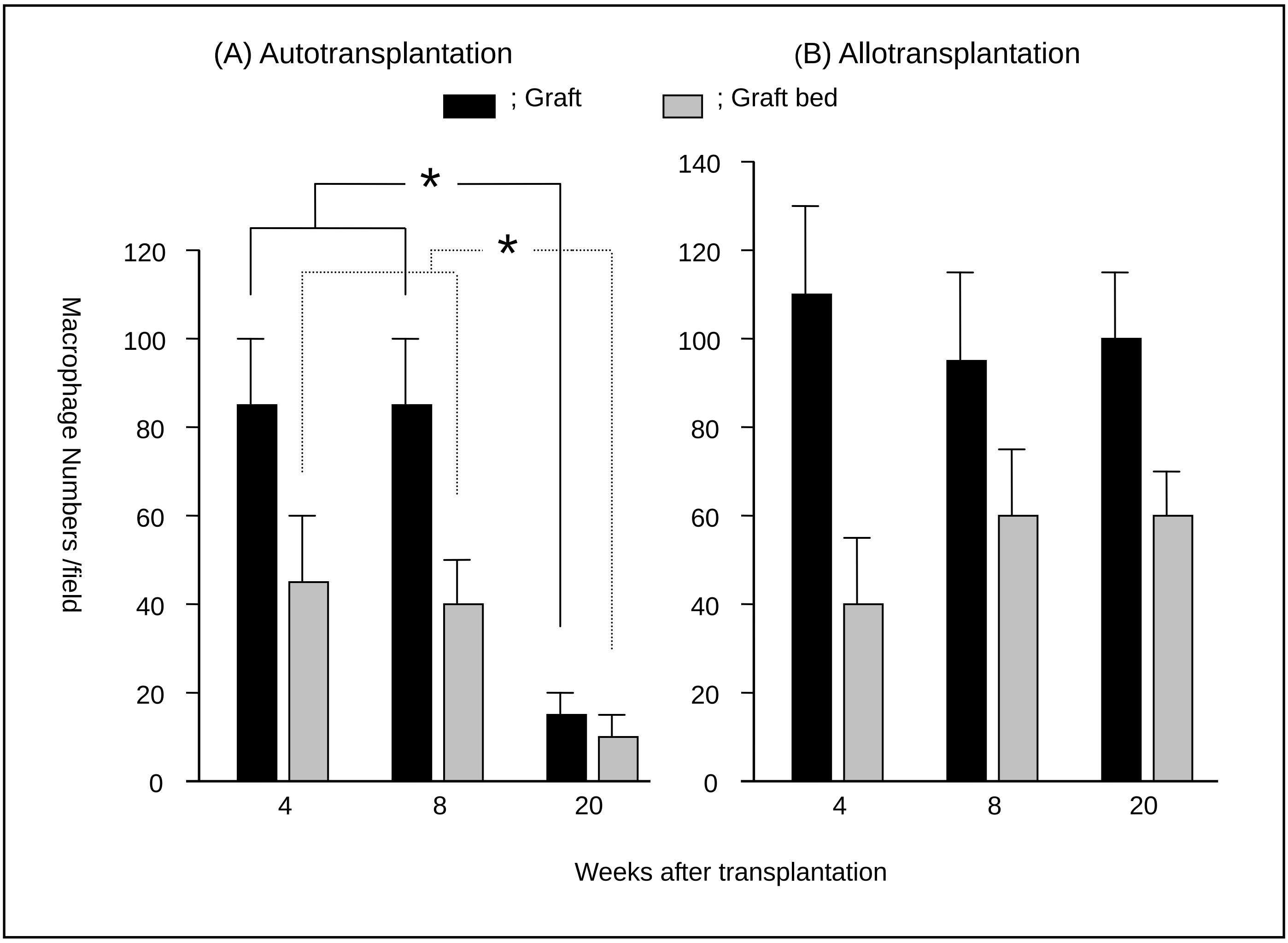

Fig. 2 Changes of macrophage numbers in auto- and allografts, and their beds after transplantation. The number of macrophages phagocytosed cell debris in grafts and their beds were counted out at 10 visual fields randomly selected for each sample. In autotransplantation, the numbers at 20 weeks after transplantation significantly decreased in grafts and their beds comparing with them at 4 or 8 weeks. Mean ± SEM (n = 10). *$p < 0.001$ (Student's t-test) (Yamaguchi *et al.*, 1999).

Histology of the epithelial cells of the dorsal surface skin

External surface of the dorsal skin epidermis is composed of a single layer of microvillous columnar cells which hold in place the covering of mucus and underneath epidermal cell layer, numerous pigment cells with many dendritic processes exist. The epidermis is supported by a mat of connective tissue through which run muscle fibers. Five main cell types are distinguished in the epidermis: (1) microvillous cells, (2) round mucous cells (3) tubular mucous cells (4) channel cells and (5) ciliated cells. 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 secretary processes extending to the surface of the epidermis. Macrophages are hardly observed in subepidermal connective tissue. Furuta and Shimozawa (1983), in an *in vitro* experiment, found that fibroblast of *I. fruhstorferi* differentiated into macrophage after injection of foreign materials, and the main macrophage producing site is in the cells that line the hemocel wall, which are derived from fibroblasts (Furuta *et al.*, 1994).

Tissue transplantation

The terrestrial slug was selected as the donor and recipient for implantation, because it is easy to use, being not aquatic and possessing no shell. These features are favorable because the graft is not readily peeled off from the recipient body mechanically, as by a water stream or contact with a shell. Transplants were made orthotopically: a piece of the dorsal skin (2.5x4.5 mm in size, 0.1~0.2 mm in depth) from a donor animal was placed on the cut surface at the corresponding site of the dorsal skin in the recipient (Fig. 1).

Epidermis of the dorsal skin is arranged as simple columnar epithelial cells. Although the body surface of the terrestrial slug is covered with only simple epithelium, the surface is prevented from evaporation and is protected from mechanical

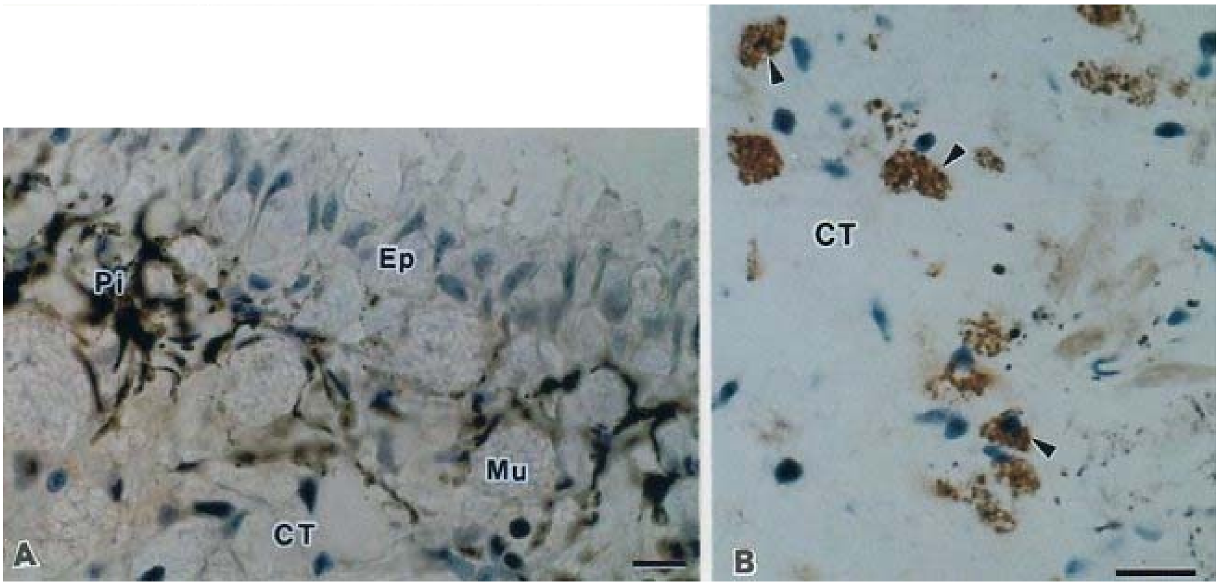

Fig. 3 Perforin immunohistochemistry of an allografted recipient site of dorsal skin, 1 day (A) and 3 days (B) after transplantation. Perforin-positive cells (arrowheads) are evident in the recipient connective tissue (CT) surrounding the graft 3 days after transplantation, but not 1day after transplantation as well as controlled immunohistochemistry. Ep, epithelial cell; Mu, mucous cell; Pi, pigment cell. Bar = 10 µm (Furuta *et al.,* 2006).

injuries. The reason is that the surface skin of the slug is covered a large amount of mucus which is secreted by two mucous cell types whose necks reach the apical portion of the dorsal surface skin; these cells were present among the epithelial cells (Yamaguchi *et al.*,1999). As the mucus rendered the physical attachment of the graft to the host graft bed difficult, secretion was inhibited by anesthetizing slugs on ice. On 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).

Fate of autografts

In transplant experiment of slugs, mucous cells, pigment cells and muscle fibers were provided as convenient criteria of graft viability.
Two weeks after transplantation, grafts had connected to host beds and various macrophages congregated around, particularly, underneath the graft sites and infiltrated into the graft matrix. Numerous macrophages had already phagocytosed cells damaged by mechanical trauma. As a result of it, pigment cells, mucous cells and muscle fibers decreased in number in grafts and their beds. This phenomenon is seemed to heal wounds. Until eight weeks after transplantation many macrophages observed in the graft site, whereas at four weeks after transplantation, grafted tissues such as muscle fibers and mucous cells began to regenerate slowly and the regeneration of these cells had been over twenty weeks after transplantation. At twenty weeks after transplantation, the macrophage numbers in autografts and their beds decreased significantly comparing with the numbers at four and eight weeks ($p < 0.001$, Student's *t*-test). By contrast, numerous macrophages were still present in allografts and their beds at eight and twenty weeks and revealed active phagocytosis (Fig. 2). Thus, the dorsal skin of host slug was completely repaired in autograft transplantation.

Fate of allografts

The rejection of allografts in mammals is mainly mediated by cytotoxic T-lymphocytes, whereas no comparable immune cells have been described in invertebrates. The examination was undertaken to determine whether similar cytotoxic effector cells are present when allograft rejection occurs in the terrestrial slug *I. fruhstorferi* (Yamaguchi *et al.*, 1999; Furuta *et al.*, 2006). Immunohistochemistry for perforin, detection of apoptosis by the TUNEL (TdT-mediated dUTP-biotin nick-end labeling) method and electron microscopy were performed using both donor and recipient tissues.

One day after transplantation, the grafted skin appeared normal, and both perforin-positive and TUNEL-positive cells were not recognized in the graft tissues (Fig. 3A). Three days after transplantation, perforin-positive cells (macrophages) were evident in the recipient tissue surrounding the graft (Fig. 3B), but not present in the grafted tissue. Five days after transplantation, perforin-positive cells were observed in the graft, but no these cells were seen any longer in the recipient tissue surrounding graft. Beneath the epithelial layer, the cells composing the connective tissue of the graft (fibroblasts, muscle cells, pigment cells, nerve cells, etc.) began to appear shrunken, and chromatin-condensed nuclei (CCN, pro-CCN) were scattered throughout the tissue. Autophagic

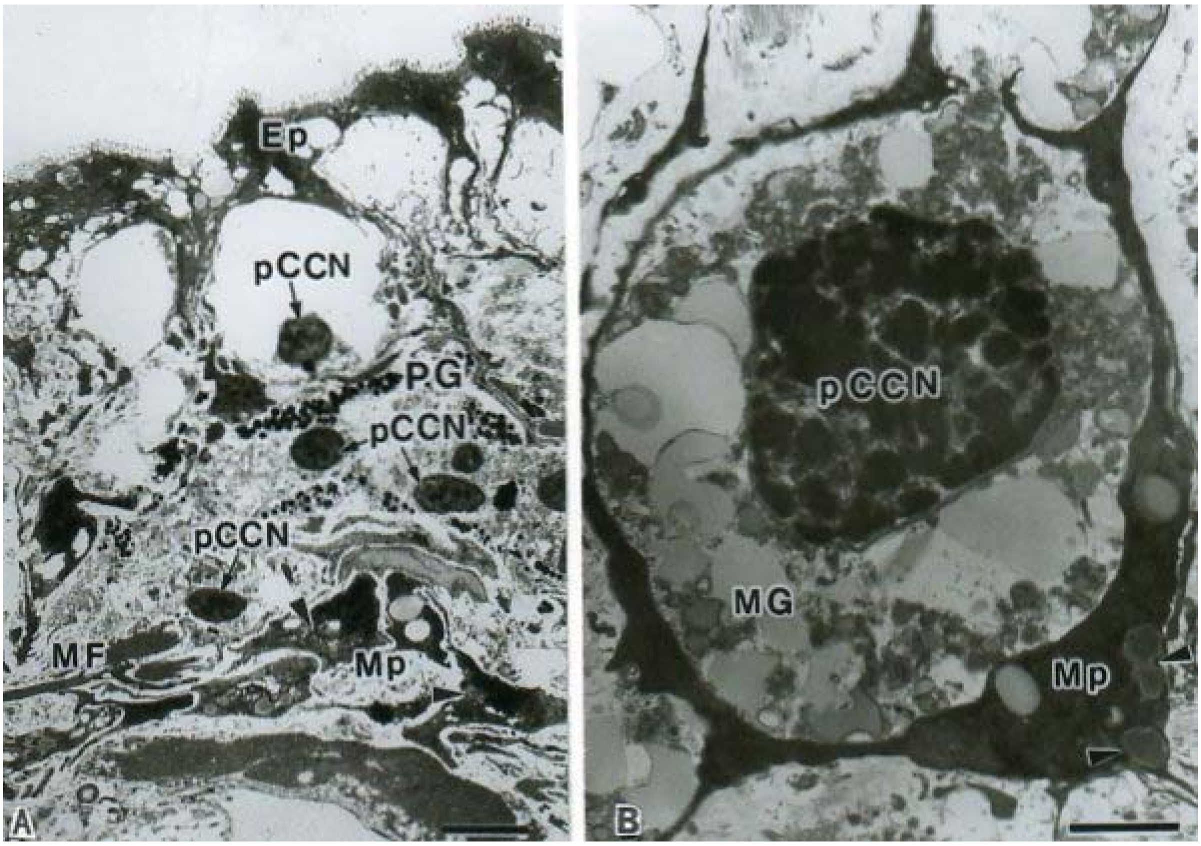

Fig. 4 Electron micrographs of the tissue of a dorsal skin allograft, 5 days after transplantation. Columnar epithelial cells (Ep) of the graft have become simple-squarmous type. Autophagic or early degenerating cells are seen in the connective tissue of the graft. A) Ep decreases in height and becomes simple-squarmous type. The cells composing the connective tissue of the graft begin to appear shrunken and contain chromatin-condensed nuclei (pro-CCN; p-CCN), and macrophages containing perforin-like granules (arrowheads) have infiltrated into the graft. Bar = 5 µm. B) A mucous cell containing granules (MG) irregular in shape becomes autophagic and is surrounded by a macrophage (Mp) containing several perforin-like granules (arrowheads). Bar = 2 µm (Furuta *et al.,* 2006).

vacuoles were observed in the cytoplasm of these cells (Fig.4A, B). Macrophages containing small perforin-like granules often appeared surrounding the cells with pCCN (Fig. 4B). The remnants of autophagic cell death were phagocytosed by macrophages that infiltrated into the grafted connective tissue. Twelve days after transplantation, the constitutive cells of the connective tissue of the graft disappeared through phagocytosis by infiltrating macrophages. In the graft tissue, cell elements were gradually lost and cell-free space was formed at the site of cell disappearance. Autophagic cell death was found to play an important role in tissue destruction. One-hundred forty days after transplantation, the grafted tissues were completely displaced by recipient tissues.

From the above data, we concluded that terrestrial slugs (molluscs) have the capability of recognizing and rejecting allogeneic tissue transplants and that they possess perforin-like molecules that lead to a cytotoxic reaction for autophagic cell death in the rejection of allografts. The presence of a putative perforin in molluscs suggests that perforin may be an immune defense mechanism conserved across phylogenetic lineages. The existence of a perforin in terrestrial slugs implies a much earlier evolutionary origin of this molecule than has been previously thought.

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