Chronic transplantation immunity in Japanese newts, urodele amphibian

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Contents

Abbreviation

CD: cluster of differentiation

CY: cyclophosphamide

HE: hematoxylin and eosin

FITC: fluorescein isothiocyanate

MHC: major histocompatibility complex

MST: mean survival time

NK: natural killer

PBS: phosphate buffered saline

SD: standard deviation

ST: survival time

Introduction

The evolution of immune systems in vertebrates accompanies the development of more fascinating, well-defined and more sophisticated body controls. Assessment for the allotransplantation immune activity has been classically and widely employed for a long time to investigate the biological significance of acquired immune responses in classes of animals. The degree of the strength of the allograft rejection is determined based on the phylogenical status of the graft recipients in animal evolution.

However, it has long been an enigma that the adoptive immune activity in urodele amphibian, which posses a high potency of tissue and organ regeneration, is unexpectedly poor when I look their position on the evolutional tree. Indeed, urodele amphibians are reported to be quite inferior in immune activities to lower classes of bony fishes, especially in cell-mediated immunity for anti-allotransplantation antigens. Thus, graft rejection in newts proceeds in a chronic manner in strong contrast to that in bony fishes, which posses cytotoxic T cells and reject allografts in an acute manner, as seen in anuras and higher classes of animals.

Because these data on urodeles are from relatively small numbers of species, quantitatively and qualitatively sufficient data from variable species of urodeles are required, I investigated the transplantation immune activities in Japanese newts. I explored first the cellular dynamism during the allograft rejection process under the different temperature conditions. The data are reported in the Part 1 entitled "Temperature susceptibility of an effector phase in allo-skin graft rejection", where I obtained interesting results showing the newt-unique temperature-shift effects, and compared these results with those from other echtothermal vertebrates showing acute immune responsiveness. Based on the discussion in terms of the differences in the

temperature susceptible phase of the immune responses, the immune cells engaged in chronic responsiveness were postulated with their biological significance.

As shown in the Part 1, the chronic responses in newts may reflect their lack of cytotoxic lymphocytes whose functions should be controlled under the MHC system. In other words, the chronic responses are probably due to no engagement of MHC-class I molecules, as usually seen in general acute responses; this suggests that urodeles do not develop an immune system associated with MHC-class I molecules. If this is the case, urodeles would also lack accelerated responses even to xenografts, to which other MHC-class I-controlled effector cells of NK cells are known to participate in destroying xenografts, because xenogeneic, but not allogeneic, MHC-class I molecules on the target cells could not provide suppressing signals for the NK functions. Therefore, I extended the investigation in the xenotransplantation immune responses and reported in the Part 2 entitled "Lack of acute responses even to xenografts", where I analyzed the dynamism in the xenograft rejection in urodeles, Japanese newts and Asiatic salamanders, comparing with frogs, and obtained very interesting results: urodeles rejected xenografts in a chronic manner, as seen in allografts. On the other hand, the frogs did reject the xenografts by accelerated acute response, as expected. Thus, newts showed no discrimination between allo and xenotransplantation responsiveness, or they may possess no functional MHC-class I-mediated immune activities. If cytotoxic T or NK cell activity, both of which are thought to be developed and work in the immune surveillance on the generation of malignant neoplasm, are not engaged in newts, how do they prevent tumor development? The immune surveillance mechanism in newts was discussed in correlation with their strong regeneration capacity.

Part 1:

Temperature susceptibility of an effector phase in allo-skin graft rejection

Abstract

Urodele amphibians are unique because they have a much lower capacity of immune responsiveness than bony fishes that possess acute immune responsiveness. Thus, in newts the mean survival time of allogeneic skin grafts in the transplantation immunity was 48.8 ± 8.3 days in a chronic manner at 25℃. The graft rejection process was categorized into three stages: a latent stage with frequent blood circulation, or the immune induction phase, a vascular stoppage stage with dominant infiltrating cells of T cells, and a rejection stage showing the change of the dominant cells to monocytes/macrophages, probably as effector cells, temporarily referred to as the immune effector phase. The immune induction phase is susceptible for the cyclophosphamide (CY) mitosis inhibitor, but not for the temperature shift from 18 to 27℃, while the immune effector phase is susceptible for the temperature shift but not for the CY-treatment, although the temperature shift failed to shorten the graft survival time to less than 25 days, which almost equals that of the secondary set of grafts where the lack of complete blood circulation is remarkable and graft rejection is resistant to CY-treatment. On the other hand, a very low temperature (5-10℃) completely prevented the effector generation in newts; in frogs, however, it is reported that such a low temperature did not prevent the generation of effectors. Taken together, the data suggests that chronic responses in newts are due to effector cells other than cytotoxic T cells, and thus possible effector cells are discussed.

Introducution

Amphibians stand on a unique position of the evolutional branch toward the high classes of vertebrates on the phylogenic tree based on a shift from an aquatic to a terrestrial lifestyle. Among them, urodela is unique in terms of their extremely large genome size and the fascinating potencies of organ-regeneration, especially in adult newts (Oviedo and Beane, 2009). These small amphibians may provide a big problem against or an exception in the paradigm that claims that the higher the position on the phylogenic tree of animals, the more sophisticated and the higher potential of their body-control systems. Consider their inferiority in immunological potentiality to much lower vertebrates, for example, bony fishes, which show acute responsiveness in allotransplantation immunity with good immunological memory formation and strong antibody responses, as seen in anura, which is comparable to mammals (Cohen and Borysenko, 1970; reviewed in Murakawa, 1971; Hildemann et al., 1981). In contrast to these animals, the immune response of newts and salamanders is in a chronic manner without the detectable cytotoxic cell activity that is evident in fishes and anura (Horton, 2001; Nakanishi et al., 2002).

Because such unusual chronic responsiveness in the transplantation immunity is also observed in lungfish (Richard, 1982; Ruben et al., 1988), which also bear a very large genome size, there might be same biological relationship among these unique characteristics. Therefore, since the mechanisms and the real dynamism of chronic immune responsiveness seem crucial in evolution studies, I explored anti-allogeneic skin graft responsiveness in Japanese newts.

As mentioned above, since newts have unique and good properties of large cells and chronic immune responsiveness (Murakawa et al., 1973), the slow dynamics of cellular events can be externally observed in a real time scale stereomicroscopically,

as first reported by Murakawa (1965). Furthermore, temperature shift experiments on the graft-rejection process of the induction and effector phases, could be suitably dealt with (Cohen, 1966c). My primary purpose in this study is to determine the cellular events that were greatly affected by temperature variation within a physiological range for newts (18 to 27℃) and was extended to the effect of such very low temperature shift (5-10℃) as in hibernation, in order to compare my results with the effects reported in frogs (Macela and Romanovsky, 1969).

I obtained interesting results showing the newt-unique temperature-shift effects, and I compared these results with those from other echtothermal vertebrates showing acute immune responsiveness (Simnett, 1965; Marchalonis, 1977). Based on the discussion in terms of the differences in the temperature susceptible phase of the immune responses, the immune cells engaged in chronic responsiveness were postulated with their biological significance.

Materials and methods

Animals

Adult newts were collected from the north area of Niigata prefecture and kept in a water tank whose temperature was controlled at 23 ± 1℃. They were given pig liver chops every five days.

Reagents and antibodies

One-third saturated ethyl *p*-aminobenzoate (Wako, Osaka) was used for anesthetization. Cyclophosphamide (CY) (Endoxan, Shionogiseiyaku Co., Osaka), an inhibitor of mitogenesis, was first dissolved in a 0.6% physiologically balanced salt (PBS) solution at a concentration of 10 mg/ml as a stock solution and intraperitoneally injected at a dose of 100 μg/g of body weight, according to the manufacture's prescription for therapeutic treatment; the drug treatment did not cause any external change in appetite, body weight, or behavior for the three-month experimental period. For the skin-infiltrating cell observation, a Maygruenwald and Giemsa solution (Wako, Osaka) was used, and for the histological staining, a Hematoxylin and Eosin solution (Sakura-finetic Japan, Tokyo) was used. Rabbit anti-human CD3ε antibody (DAKO, Glastrup, Denmark), FITC-labeled goat anti-rabbit IgG antibody (Bethyl Laboratories, Montgomery, Tx), and goat IgG (Rockland, Gilbertsville, PA) were used for the T cell-staining (Keresztes et al., 1996; Park et al., 2005), as described previously (Fujii et al., 2007).

Skin transplantation

Skin transplantation was performed basically according to the report of Murakawa (1965, 1968). After complete anasthetization of the recipient newt, around 6-mm square of dorsal skin was carefully peeled off to prepare the graft bed. Meanwhile, as a graft, red ventral skin was also carefully peeled off without muscle

adhesion and trimmed to around 5-mm square with small scissors. The obtained graft was put on and expanded over on the graft bed. These procedures are shown in Fig. 1. The graft-bearing newt was laid on a plastic tray with a wet sheet, not in water, overnight, resulting in natural acceptance of the skin graft.

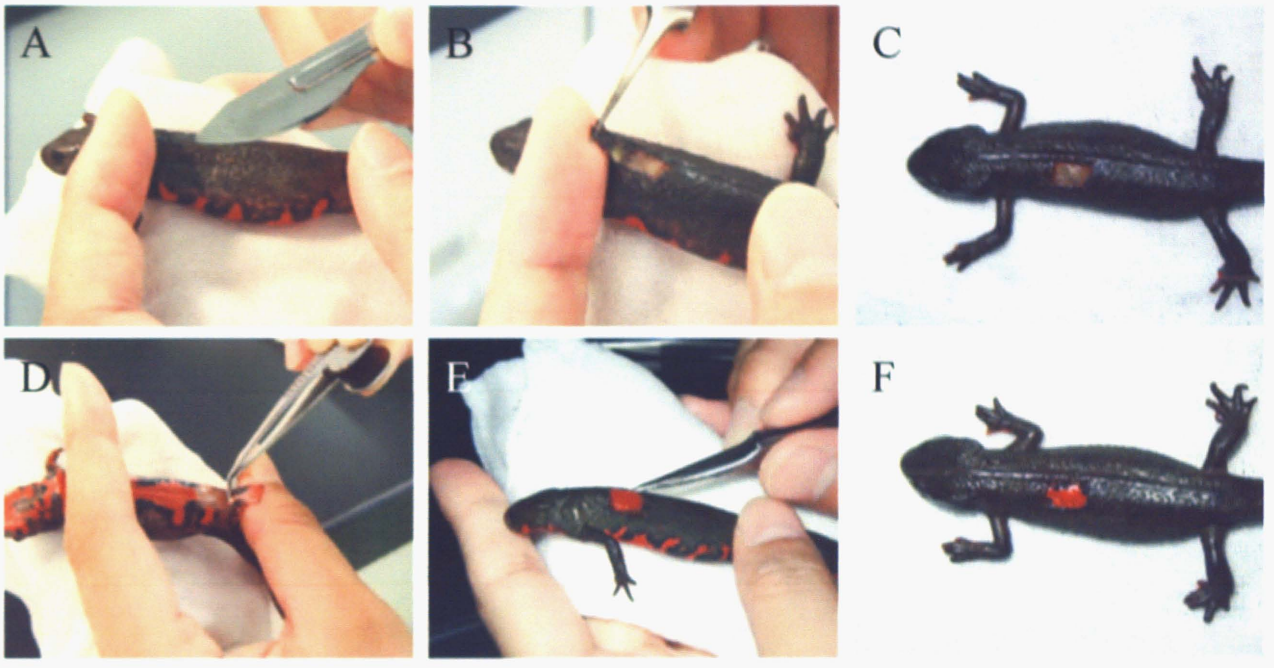


Figure 1. Process of skin transplantation. Dorsal skin of recipient was cut (A) and peeled off (B) to make a graft bed (C). On the other hand, ventral skin-graft from donor was prepared (D) and put on the graft bed, carefully (E) to complete transplantation process (F). For details, see text.

Observation of graft on rejection process

The transplanted skins were observed under a stereomicroscope every three days, unless otherwise mentioned, after the newts were anesthetized.

Cytological observation of graft-infiltrating cells

At given days after the transplantation, the grafted skin was carefully peeled off using two pairs of either sharp or dull pointed tweezers and put on a clean slide glass. The cells infiltrating the entire area of the graft were stamped on the slide glass and differentially counted for each graft after May-Giemsa staining. The infiltrating cells were calculated and expressed as cell numbers per 10 mm^2 skin graft.

Histological and immunohistological observations

The grafted skin were cut, removed, and fixed in Bouin's fluid for more than 24 hrs. These specimens were embedded in paraffin wax, and 6-μm thick sections were cut and stained with hematoxylin and eosin (HE) and examined by light microscopy.

To detect the T cells, skin-graft specimens were embedded in an Optimal Cutting Temperature compound (Miles Lab. Inc., Naperville IL. USA), and the tissue block was frozen and stored at −80℃ until the cryostat sections were cut. Six μm thick frozen sections were air-dried and fixed with cold acetone for 10 minutes. Immunohistological observations were carried out, as previously described (Touma et al., 2000). The sections were washed with PBS and incubated overnight with anti-CD3ε antibodies. After the primary antibody was washed out with PBS, sections were treated with FITC-labelled goat anti-rabbit IgG for 1 hr at room temperature and examined by fluorescence microscopy.

Artificial field for hibernation

A large plastic 560 mm × 680 mm × 305 mm-high, top- and bottom-opened box, was firmly set on the ground and filled with leaves. After the newts were put in the

box, a plastic cover with many punch holes was added and filled with leaves. Thermometers were set on ground and outside the box.

Statistical analyses

The significance of the differences among the experimental groups was determined by Student's *t* test. The correlations between parameters were analyzed using Pearson's correlation coefficient. A p value less than 0.05 was considered to indicate a significant difference.

Results

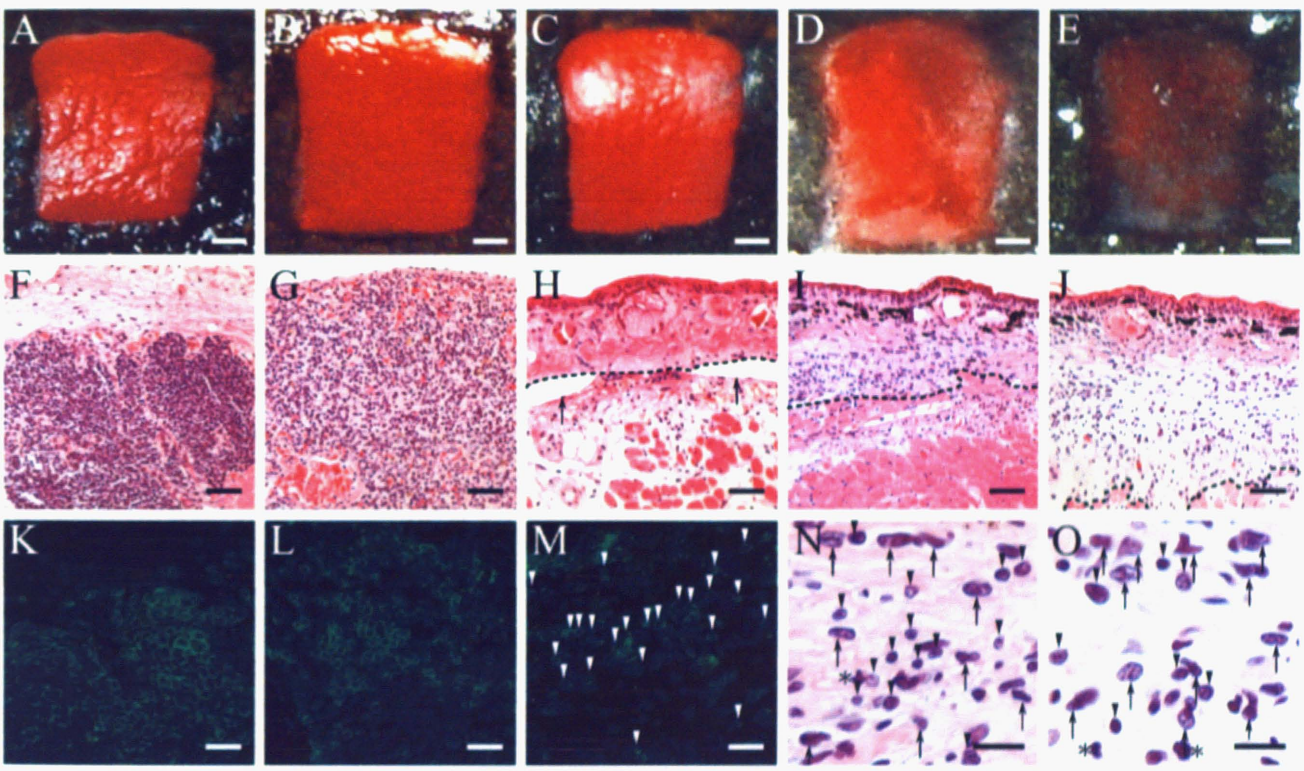
Histocytological dynamics in allografts

Ventral skin grafts have quite good properties for the observation of the graft-rejection process under a stereomicroscope, including blood circulation, damaged pigment cells of the graft, and the movement of the recipient's cells while the skin graft is being rejected. The changes of these parameters could be useful diagnosis for allotransplantation and were categorized into five stages, basically according to Murakawa (1965, 1968). Thus, the grafted skin showed the following daily or weekly changes (Fig. 2): 1) Within a few days after the grafting operation, bleeding stopped and the cut healed. The graft was completely adhered and fixed on the graft bed (healing stage). 2) This was followed by the adhesion of the blood capillaries of the donor and the recipient, vascularization started (vascular reaction stage). 3) Then frequent and smooth blood circulation on the grafted skin, as if it were on the donor newt, lasted 2 to 4 weeks (latent stage), because no external sign of the graft rejection was monitored at this stage. 4) Blood circulation became dull and partly stopped. This reaction expanded over the graft within around a week (vascular stoppage stage). 5) Finally, severe immune attack damages and lyses the color pigment cells of the graft, making transparent spots on the bottom of the graft bed. At this stage, the epithelial sheet of the recipient's melanophores is entering from the margin of the graft bed toward the center of the graft (rejection stage). The day when the graft was completely destroyed, was expressed as the last day of graft survival.

These events are histocytologically concerned well with cell types involved in each stage on the skin rejection. At the healing and vascular reaction stages, infiltrating cells were mainly neutrophils that are engaged in inflammatory reactions (Fig. 3A) and are equally observed in autologous skin transplantation without skin

rejection categories (i.e., latent, vascular stoppage, and rejection stages (Fig. 3B). On the other hand, those at the vascular stoppage stages were mainly lymphocytes, which are key cells in the acquired immune responses. Consequently, they may be reduced in number at the rejection stage, while monocytes/macrophages, probably as the effector for target cell destruction, increased in number throughout the graft rejection process (Figs. 2N, O, and 3A), as will be discussed later. Note that many infiltrating cells in these stages include CD3ε-bearing cells (Fig. 2M): Such cells are seen in the thymus confluent or without medulla and in the spleen as a cluster (Fig. 2K, L).

Figure 2. External and histological aspects of allogeneic skin grafts. External aspects of allografts under immune rejection process are categorized: (A) healing stage, (B) vascular reaction stage, (C) latent stage, (D) vascular stoppage stage, and (E) rejection stage, as shown in top series of pictures. These figures are reflected by the histological dynamics, as shown in the HE-stained sections, H: latent stage, I: vascular stoppage stage, J: rejection stage. Dotted lines indicate borderline between the skin graft and dermis of host. Cells infiltrating into the grafts at vascular stoppage and rejection stages are shown in N and O: lymphocyte (*arrowhead*), macrophage (*arrow*), monocyte (*asterisk*); note that lymphocytes are dominant in the vascular stoppage stage and monocytes/macrophages are dominant at rejection stage. T cells infiltration into the graft at vascular stoppage stage was demonstrated using anti-CD3ε monoclonal antibodies (*arrowheads*) under DAPI-counter staining (M) with special references for specific staining of T cells under special localization in the thymus (F, K) and spleen (G, L) for HE and anti-CD3ε-staining, respectively. Neither typical medulla in the thymus, nor typical red and white pulp discrimination in the spleen are observed. Scale bar: A-E, 1 mm; F-J, 100 μm; K-M, 50 μm; N-O, 25 μm (in cooperation with Yoshihiro Kushida)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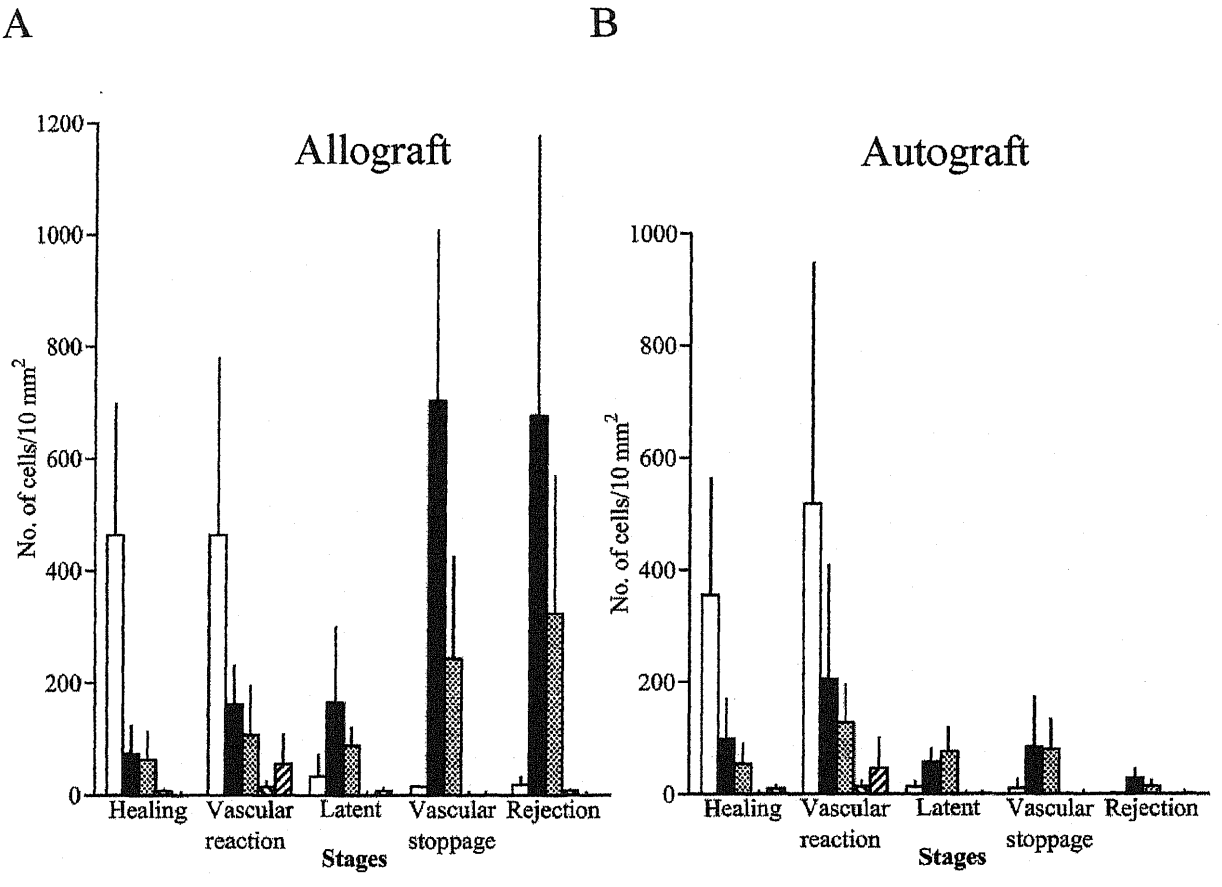

Figure 3. Lymph-myeloid cell accumulation in allogeneic skin grafts at late stages of rejection process in comparison with those in autografts. Dynamic changes in numbers of cells infiltrating allogeneic (A) and autologous (B) skin grafts were shown. For details of investigating procedure, see Materials and Methods. Mean numbers of cells per 10 mm^2 graft were obtained from eight different grafts on different recipients and expressed with SD as a vertical bar. □; neutrophils, ■; lymphocytes, ⬚; monocytes/macrophages, ▨; eosinophils, ▨; basophils

Lack of a sound vascularization in secondary grafts

Acquired immune response, which is characterized by immunological memory formation, causes faster rejection of the secondary set of a graft from identical donors, than its primary set. Indeed, the mean survival time (MST) of the graft in the primary response, 54.6 ± 9.7 days, was markedly shortened to 30.1 ± 6.6 days (n = 21) as 3-month-intervals between the first and secondary graftings (Fig. 4A). This rejection time is still slow when compared with other species of ectothermic vertebrates (Hildemann, 1958; Borysenko and Hildemann, 1969; Cohen and Borysenko, 1970; reviewed in Murakawa, 1971; Hildemann et al., 1981).

The most profound difference in the rejection process between the first and secondary grafts is that there was no typical latent stage in the latter; normal blood circulation was not observed in the secondary graft. This result shortened the MST of the secondary graft from four to two weeks when compared with the primary response.

How long the immunological memory lasted in the slow responses is interesting, and this question is important when dealing with secondary graft experiments to confirm whether grafting is under immunological memory. So I made secondary set experiments under 3-, 9-, 12-, and 18-month intervals between the first and secondary graftings. The later the secondary setting was performed, the longer the MST was, and the proportion of newts showing typical secondary responses without latent stages or immunological memory decreased (Fig. 4A). The proportions were plotted against the intervals between the first and secondary grafts (Fig. 4B), indicating that the immunological memory lasts nine months, and half of the recipients (50%) with immunological memory are around 16 months. Thus, once induced, immunological memory lasts slightly longer than MST in the primary

response. Therefore, experiments on immunological memory are usually carried out three months after the primary responses.

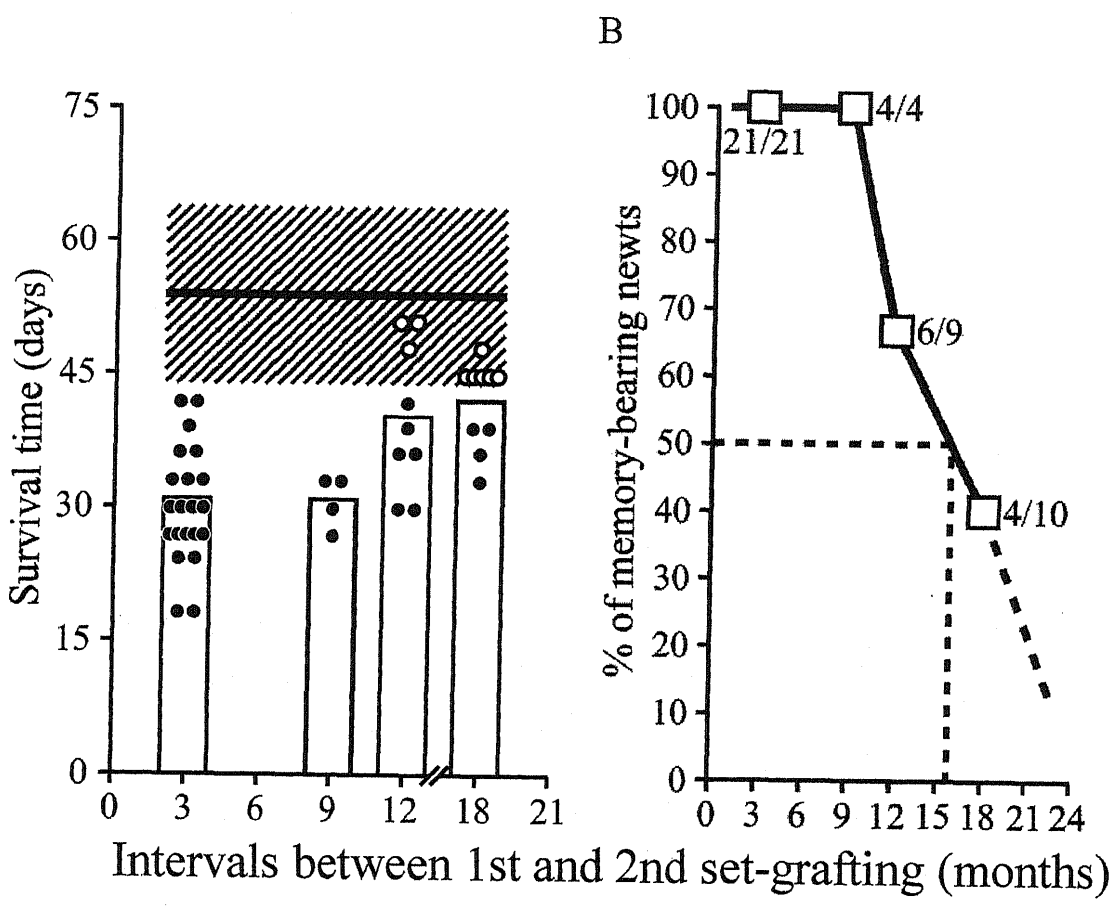

Figure 4. Immune memory for secondary graft lasts more than nine months: 16 months for memory loss in half of graft recipients. After adequate intervals, immune-primed newts were re-transplanted with skin grafts from identical donors. (A) Each graft's survival time was plotted by interval time length, i.e., three, nine, 12, and 18 months, and secondary responses were judged by graft rejection without latent stages (●). Grafts rejected after showing latent stages were referred as primary response-like rejection; in another words, memory decayed (○). The horizontal bar (▨) indicates MST of 54.6 ± 9.7 days of SD (25 newts) in primary responses under identical temperature conditions (23℃). (B) Frequency shift of memory-bearing newts. Figures beside square symbols are number of newts with memory per all newts tested.

Length of graft survival time reflected by that of target-cell destroying phase

The strength of immune activities in ectothermic vertebrates is reflected by the temperature shift in the primary and secondary allograft rejections. In the primary responses, MST gradually shortened from 77.5 to 40.4 days under the experiment condition from 18 to 27℃ (Fig. 5). On the other hand, in the secondary responses, MST sharply shortened from 18 to 23℃ and reached a level of 25 days of MST at 25℃; it wasn't shortened any more, even though the temperature was increased to 27℃. Note that the level is still much longer than that of other species of ectothermic vertebrates, such as fishes (Boryesnko and Hildemann, 1969), and toads and frogs (Cohen and Borysenko, 1970; Hildemann et al., 1981). The secondary grafts did not show typical latent stages within the tested temperature ranges.

Next, I tested whether there is a special stage that shows high susceptibility to the temperature shift on the relationship between the length of each stage and graft survival time in individuals. Figure 6 clearly shows good correlation in the length between the rejection stage and the graft survival time; the best is at 25 and 27℃, where no further shortening of MST was observed in the secondary grafts (Fig. 5).

To verify the above issue, I tested the effect of temperature shift on the length of a given stage. The results are shown in Fig. 7A. The stage showing the biggest change of MST due to the temperature shift was the rejection stage, as expected, and the stages before the latent one, which is the stage for the graft healing, were <u>not</u> categorized as acquired immune responses (Fig. 2). Note that the latent stage is not influenced at all by the temperature shift.

Finally I analyzed the rejection stages in the secondary grafts, resulting in almost the same pattern as that of MST (compare Figs.7B and 5), which is coincident with a lack of latent stage in the secondary grafts.

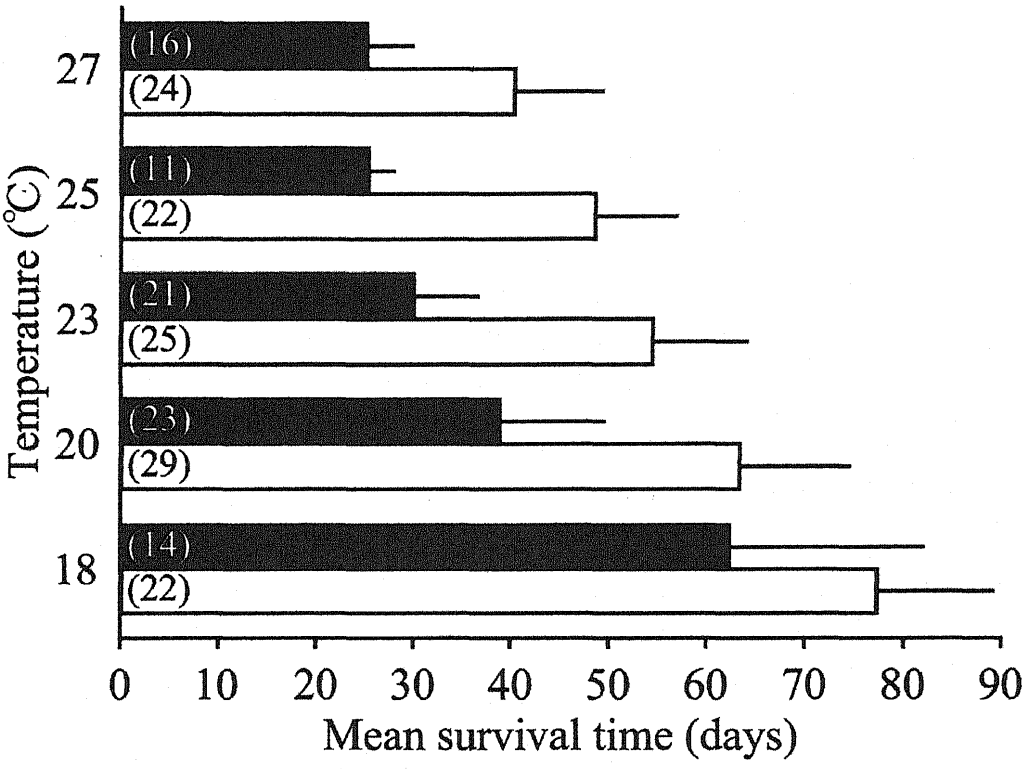

Figure 5. Effect of temperature shift on primary and secondary responses. Primary (open columns) and secondary (closed columns) transplantations were performed at different temperature conditions, as indicated. Figures in parentheses indicate numbers of newts used. Each column indicates MST with a bar of SD.

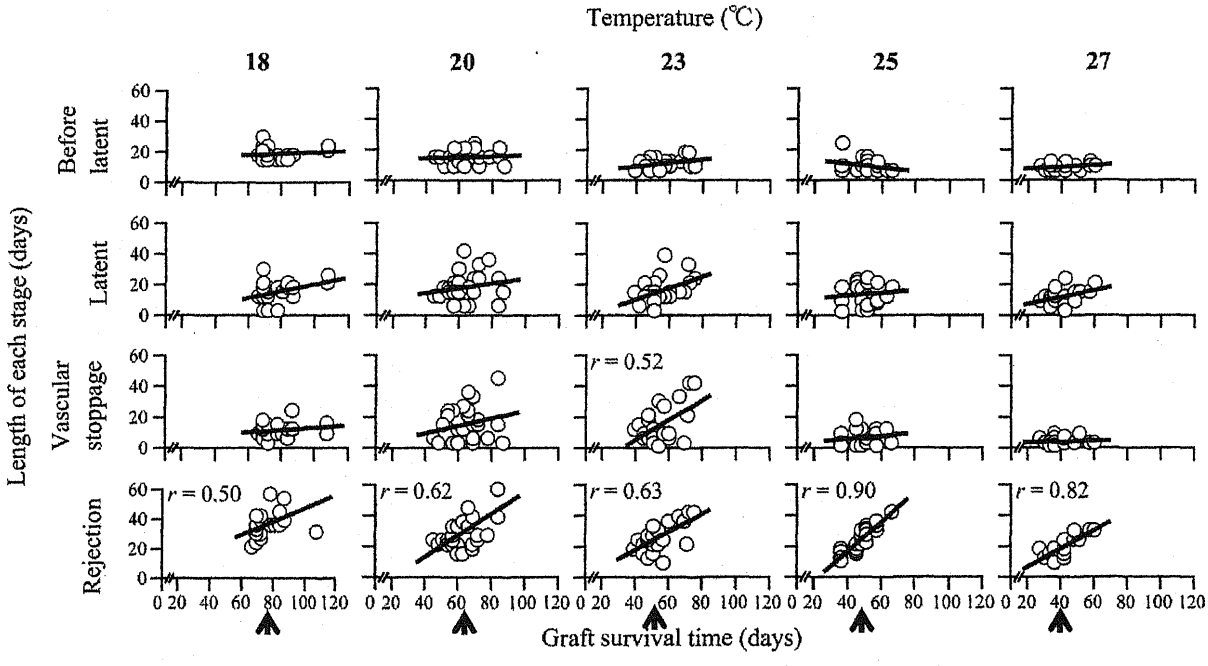

Figure 6. Degree of length of MST is reflected by rejection stages. To verify restricted stages involved in the length of MST, I individually investigated the relationships between MST and the length of each stage. The value of Pearson's correlation efficiency, r, was indicated in each test, and $r \geqq 0.5$ was considered significant and $r < 0.5$ non-significant correlations. The arrows indicate MSTs of skin allograft at the different temperature conditions.

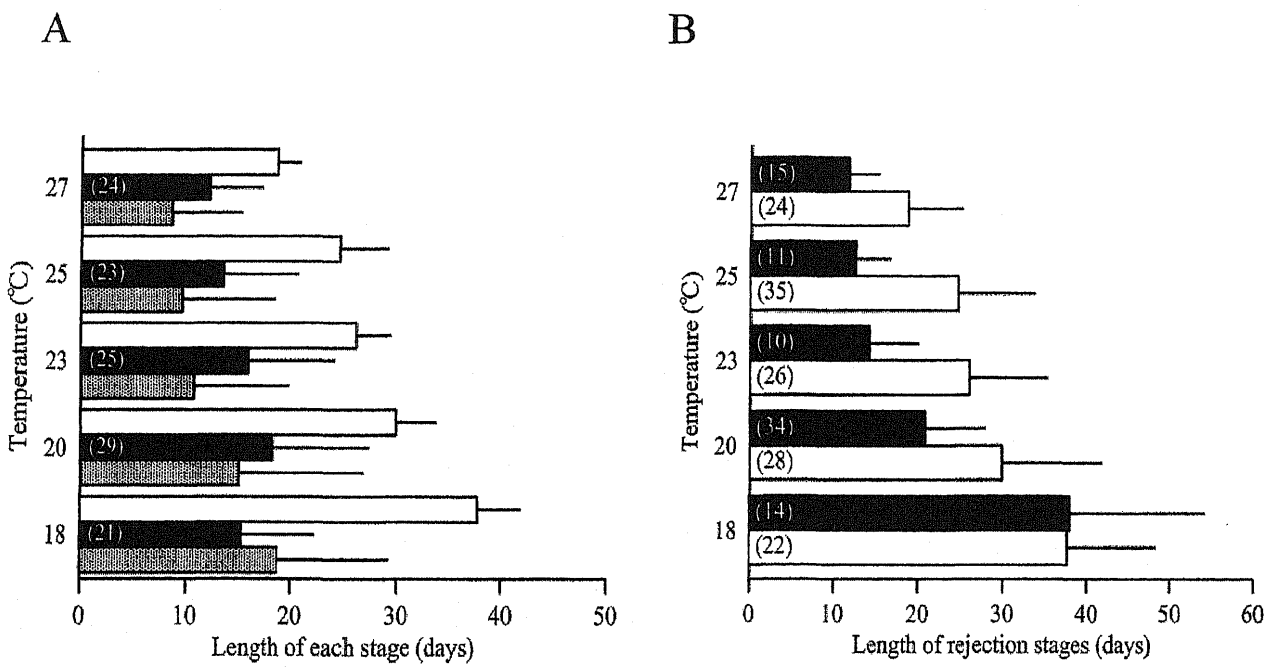

Figure 7. Rejection stages, but not latent stages, are susceptible for temperature shift. How susceptible for temperature shift is the latent stage (closed columns) as well as the rejection stage (open columns) of the acquired immune responses of transplantation immunity was explored by comparing length of each stage (A). The stages earlier than the latent stage, which probably correspond to natural immunity, as seen in the autograft responses (cf. Fig. 3B) are also compared (shaded columns). (B) the effect of temperature shift on the rejection stage in the primary (open columns) and the secondary (closed columns) responses was compared. Each column indicates mean length of the stage with a bar of SD. Figures in parentheses indicate number of newts used.

Mitosis inhibitors retarded graft rejection by prolongation of latent stages

Acquired immune responses depend deeply on the proliferation of immune competent cells, so I explored whether the stage, which is sensitive to the temperature shift, is closely related to immune cell proliferation. The graft recipients were treated with mitosis inhibitor of cyclophosphamide (CY), on three different stages of the rejection process, i.e., the latent, vascular stoppage and rejection, by three CY inoculations every other day. These treatments were selectively effective on the latent stages by slow entry into the vascular stoppage stage, significantly prolonging MST (Fig. 8). No affect was seen in the other two stages. No affect by the CY-treatment was seen in the secondary responses without a latent stage, either (data not shown).

Thus, considering the high susceptibility to CY of the latent stages, this stage may proliferate immune cells (induction phase), while the temperature sensitivity of the rejection stages (effector phase) is completely resistant to the mitosis inhibitor.

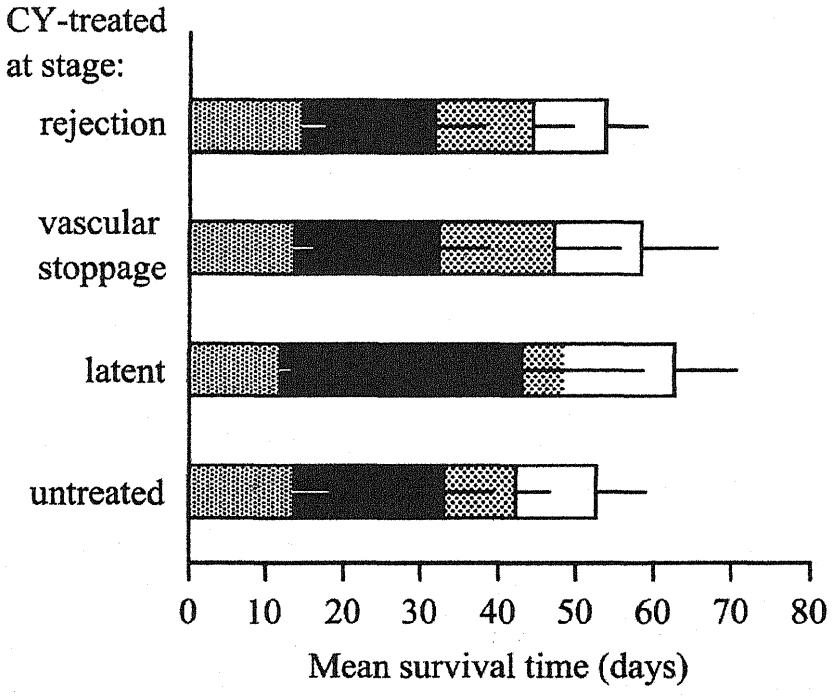

Figure 8. Mitosis inhibitor (cyclophosphamide) elongates the latent stage, but not the rejection stage. 40 allo-skin-grafted newts were divided into four groups of 10 and received intraperitoneally the mitosis inhibitor of cyclophosphamide (CY), as described in Materials and Methods; CY-treatments were carried out at the latent, vascular stoppage, and rejection stage and one group was left for PBS control. When treated at the latent stage, MST was significantly prolonged ($p < 0.01$), although the vascular stoppage stage entering the rejection stage was not clearly determined (▨). Each column indicates MST with a bar of SD. ▩; before latent stage, ■; latent stage, ▨; vascular stoppage stage, □; rejection stage (in cooperation with Masato Johnouchi)

Effect of very low temperature on the graft rejection: prevention of both generation and function of effector cells

The ranges of the temperature shifts from 18 to 27℃ in the above experiments are the ranges of field life seasons of newts and their chronic primary responses for a few months could not be ended by the time of hibernation. Therefore, the effects of very low temperature in hibernation on the graft rejection process and on the maintenance of immunological memory once induced are very interesting and remain to be tested, because early experiments using frogs with allograft, which remained more than two months at 5℃, showed secondary response of accelerated acute rejections when the recipients were shifted to 25℃ (Macela and Romanovsky, 1969). This might indicate that in frogs the immune response proceeds during hibernation.

Newts with allografts at either the latent or rejection stages at 25℃ were transferred, after a few days of temperature-shifting, in an enclosure of an artificial field for hibernation for 3.5 months of winter (Fig. 9) The temperature in the enclosure was shifted within a narrow range of 5 to 10℃, and 10 to 15% weight loss during hibernation was recorded. After winter, these newts were moved to 25℃ after one week, meanwhile the temperature was shifted from 13 to 21℃, and the situation of each graft was routinely observed under stereomicroscope.

As shown in Fig. 10, each graft, regardless of its stages when it entered hibernation, progressed further based on the categories for the skin graft rejection, resulting in almost the same survival time as the grafts on the newts entirely kept at 25℃. Thus low temperature at hibernation neither advances nor delays the immune response at all; it just freezes whole process. The case for the maintenance of immunological memory may be similar, because the MST (27.5 ± 3.5 days, $n = 6$) in the secondary grafts operated after winter was not affected by hibernation. This result

is comparable to the MST observed in the newts kept completely at 23℃ without hibernation (30.1 ± 6.6 days, n = 21).

Figure 9. An enclosure for the artificial field for hibernation (A), and the skin graft-bearing newts just awaking from hibernation (B). For details, see text.

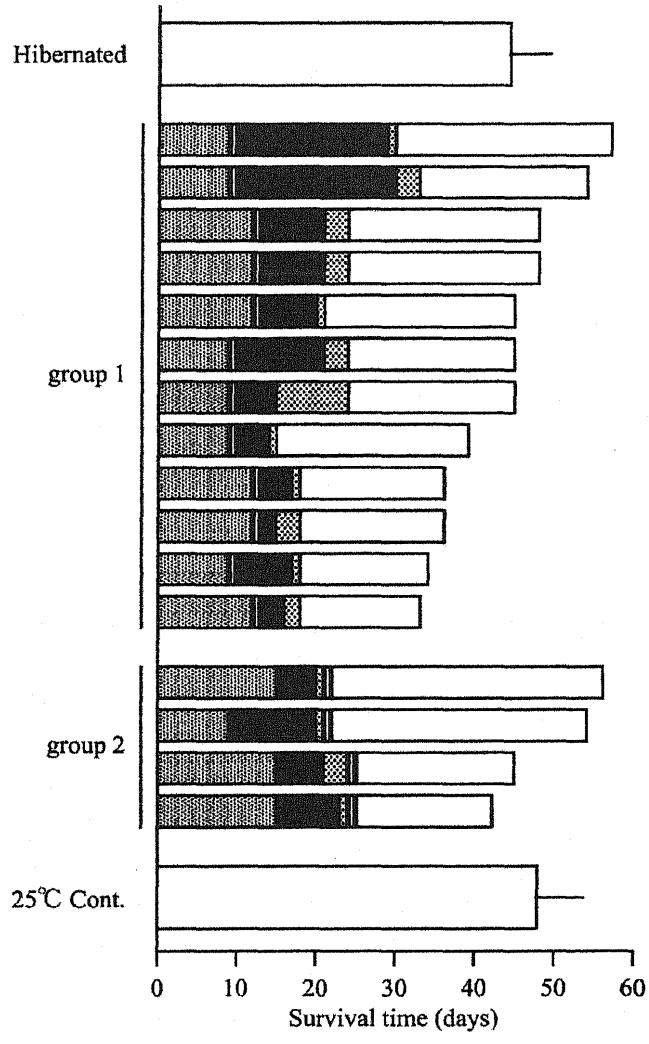

Figure 10. Very low temperature of 5 to 10℃ in hibernation froze every stage of the immune responses. Newts with allografts at either latent stage (group 1, n = 12) or rejection stage (group 2, n = 4) were placed in the enclosure for hibernation (for detailed procedures and conditions, see Materials and Methods). At end of hibernation period, newts were gradually temperature-shifted to the 25℃ water tank within a week. This shifting period (expressed by ‖ in this figure) equaled four days at 25℃, and the exact survival time in days were shortened by four days when evaluated as data. There is no significant difference between MST for hibernation (groups 1 and 2 were combined, n = 16) and MST under the 25℃ water tank without hibernation (n = 22). ▒; before latent stage, ■; latent stage, ▩; vascular stoppage stage, □; rejection stage

Discussion

The large cells and the slow immune reaction in the newts allowed us to categorize the external changes of the skin graft into five stages: healing, vascular reaction, latent, vascular stoppage, and rejection (Fig. 2). This is primarily based on the pioneering work by Murakawa (1965) who called the vascular stoppage stage the rejection stage: Similar events on the grafted skins are generally observed in different species of newts (Cohen, 1966a, 1966b). Although earlier works judged the vascular stoppage (Murakawa, 1965, 1968) or hemostasis (Cohen, 1966b) as the end point of graft rejection, I refer to the day of the complete graft destruction as the end of the graft survival, since the completion of graft destruction or pigment cell death requires an additional one-third to half length of the graft survival time, and this stage is the most sensitive to temperature shift (Fig. 7A). These five categorizations are not applicable for animals that show acute response around one week of graft survival time, such as fishes, toads, frogs, and other higher vertebrates including mammals. Furthermore, newts are suitable for the external observation of blood circulation and the dynamics of epithelial pigment cells in the skin. The general process of graft rejection was confirmed by once healing the transplanted graft, followed by blood circulation at the latent stage, the vascular stoppage, and the destruction of the target pigment cells, although all stages took extremely longer periods than acute responses.

In humans, the secondary set of skin grafts is rejected without blood circulation, so the graft is called a "white graft" and resembles the cases of newts because no latent stage exists. This result, which is an excellent biological sign of the memory responses in newts, is meaningful especially for chronic responses that show very large variation in individual's skin-graft survival time; the presence or absence of immune memory in newts could not be simply expressed by the length of the graft's

survival time. In mammals, because they lack vascularization in secondary grafts, white grafts are thought to be due to immune attacks by antibodies against skin grafts that were produced during primary responses (Festenstein and Démant, 1978; Brent, 1997). However, in newts the significance of antibodies as primary or entire factors for preventing vascularization remains to be clarified, because I do not have evidence that these newts produce anti-graft antibodies.

The whole data, in which the latent stage is insusceptible for the temperature-shift effect (Fig. 7A) and the CY-treatment at this stage causes a significant delay to enter the next stage of vascular stoppage (Fig. 8), provide a conclusion that the latent stage involves the induction of the effector cells to cause graft damage, where immune cell proliferation has taken place. A well-known phenomenon in the mammals of the CY-augmentation of delayed type hypersensitivity due to the removal of suppressor cells by CY may not exist in newts, since the latent stage is not observed in the second set graft under CY-resistant memory responses, as mentioned above.

In contrast, the effector phase of the vascular stoppage and rejection stages is severely affected by temperature shift, but not at all by the CY-treatment (Fig. 8). Note here that neither temperature elevation from 18 to 27℃ (Fig. 5), confirming the previous report (Cohen, 1966c), nor repeated grafting from the same donors (K.K. unpublished observations) shorten the graft's survival time of around 20 days; this is still much longer than the graft-survival time of around five days or the acute responses in bony fishes and frogs (Cohen and Borysenko, 1970; Hildemann et al., 1981). This may indicate that direct effector cells are cells with quantitatively limited efficacy and probably not proliferative cells such as immune T cells; if effector cells resembled lymphocytes, the graft-survival time would become much shorter and reach

the level of acute responses.

The acute transplantation response in the higher classes of vertebrates is concerned with the cellular cooperations of several types of cells, such as antigen-presenting, $CD4^+$ helper T, and $CD8^+$ effector, or cytotoxic T cells. However, when $CD8^+$ T cells were removed from *Xenopus*, the anti-allo-transplantation response progressed chronically (Rau et al., 2001), where $CD4^+$ cells may have been engaged in the activation of macrophages becoming effector cells as categorized for delayed hypersensitivity in transplantation immunity. If similar situations exist for newts, they may lack the second type of T cells of cytotoxic ones that should be engaged in acute responses even in the lower classes of vertebrates, although CD-phenotypes on T cells have not been identified yet (Nakanishi et al., 2002). This is quite likely when I consider the very poor architecture of the thymus without medulla and of the spleen without discrimination between red and white pulp (Fig. 2F, G) and the dynamic changes of the graft-infiltrating cells from lymphocytes showing a peak at the vascular stoppage stages (Fig. 2N) to the monocytes/macrophages accumulating through the rejection process (Figs. 2O, and 3).

An alternative possibility for chronic responsiveness in anti-allo-responsiveness may be unusual expression of functional major histocompatibility complex (MHC) antigens. Indeed, it was once proposed that urodela may lack MHC (Hildemann et al., 1981), but recent genetical analyses using axolotl, which are Mexican salamanders, with immunodeficiency indicate that urodela's MHC is functionally limited (Kaufman et al., 1995; Tournefier et al., 1998). But other groups using terrestrial adult tiger salamanders reported the presence of a functional MHC system that compares to that of mammals (Bos and DeWoody, 2005). Therefore, the MHC features in Japanese newts with chronic immune activities (Murakawa, 1968), as confirmed here, are

interesting and remained unclarified.

Under the concept for chronic responses, the following steps exist: 1) lymphocytes specifically proliferate in the latent stage, 2) these lymphocytes activate the effector cells of macrophages, finally 3) the effector cells kill the target cells at the rejection stage. The most susceptible step for the temperature shift may be that of effector-macrophage activation by lymphocytes, because the length of the latent stages hardly fluctuated (Fig. 7A). I exclude the last step as a candidate because rejection stages in the secondary response were around 12 days at 23 to 27℃ (Fig. 7B). Activation was probably done by something like macrophage-activating factors, although very low temperature prevents this step (Fig. 10). Interestingly in frogs, which show acute responses by other types of effector cells of T cells, the effector activating step still advances even under very low temperature (Simnett, 1965; reviewed in Marchalonis, 1977). Activation signal acceptance by effector T cells, may be much more effective than in effector macrophages. Although available data at present are quantitatively and qualitatively quite limited and poor, the lack of acute responses and their relation to the special steps of the temperature effect in newts contain significant meaning for understanding immune evolution.

Part 2:

Lack of acute responses even to xenografts

Abstract

Urodele amphibians, which possess a high potency of tissue and organ regeneration, are unique in their inferior immune responsiveness to evolutionally lower classes of fishes. To explore the physio-cytological basis for their poor immune responsiveness, I surveyed the dynamism of the graft rejection process in transplantation immunity using Japanese newts and Asiatic salamanders in comparison with several species of frogs. The mean survival times (MSTs) of the allografts in the primary responses at 20℃ are around 60 and 20 days in urodeles and anuras, respectively, and MST was significantly shortened in the secondary responses to 40 and 10 days in urodeles and anuras, respectively. However, MSTs in the anamnestic secondary responses were not further shortened even when repeatedly grafted from identical donors, suggesting that no major histocompatibility complex (MHC)-mediated responses by lymphocytes are engaged in. Responsiveness against xenogeneic grafts was also investigated in amphibians, where in mammals, xenografts are generally destroyed and rejected by natural killer (NK) cells in an accelerated acute manner under the MHC-class I molecule's control. The obtained results were prominent with a strong contrast of accelerated acute responses in frogs to chronic responses in urodeles. Thus, urodeles show quite poor immune system of MHC-mediated responses: lack of acute responsiveness in both primary and secondary sets of allografts, even xenografts, and of allo-aggression. Finally I estimated the numbers of conventional or weak histocompatibility antigens on the allografts to be five, and surprisingly part is shared with those on the xenografts. The uniqueness in the inferiority of the immunological natures of newts was discussed with respect to their very low tumorigenesis and strong regeneration properties.

Introduction

The evolution of immune systems in vertebrates accompanies the development of more fascinating, well-defined and more sophisticated body controls. However, urodele amphibians are quite inferior in immune activities to lower classes of fishes, especially in cell-mediated immunity for anti-allotransplantation antigens, where cytotoxic cell functions have not been demonstrated (Cohen and Borysenko, 1970; reviewed in Murakawa, 1971; Hildemann et al., 1981; Kinefuchi et al., 2011). Therefore, graft rejection proceeds in a chronic manner in strong contrast to that in bony fishes, which possess cytotoxic cells and reject allografts in an acute manner, as seen in anuras and higher classes of animal (Borysenko and Hildemann, 1969: Horton, 2001; Nakanishi et al., 2002).

Because acute responses are functionally controlled by major histocompatibility complex (MHC) gene expression, it was once thought that urodeles may lack a MHC-system due to chronic responses even in anamnestic responses (Hildemann et al., 1981). However, recently much progress in molecular and genetic studies has demonstrated that they really do have genetically defined T cell receptors with normal diversification (Charlemagne et al., 1998) and normal properties of MHC diversity in tiger salamanders, as seen in evolutionally higher classes of vertebrates (Bos and DeWoody, 2005), even though they were reported to show chronic responses to allografts (Cohen, 1968; Cohen and Hildemann, 1968). In contrast, the accumulated data on the Mexican salamander, axolotl, show that they bear limited diversification and deficient architecture of MHC molecules speculated to be the direct cause of the lack of acute responses (Tournefier et al., 1998). So, the reasons for the chronic manner of the graft rejection in urodeles remain under dispute.

Although molecular and genetic data are not sufficiently available yet, Japanese

newts are also known to show chronic responses in allotransplantation immunity, as seen in other species of urodeles (Murakawa, 1968; reviewed in Murakawa, 1971). Recent studies, which have demonstrated cyto-physiological dynamics during allograft rejection in these animals, suggest that the chronic responses reflect their lack of cytotoxic lymphocytes whose functions should be controlled under the MHC system (Janeway Jr. et al., 2001). In other words, the chronic responses are probably due to no engagement of MHC-class I molecules, as usually seen in general acute responses; this suggests that urodeles do not develop an immune system associated with MHC-class I molecules. If this is the case, urodeles would also lack accelerated responses even to xenografts, to which other MHC-class I-controlled effector cells of natural killer (NK) cells are known to participate in destroying xenografts, because xenogeneic but not allogeneic, MHC-class I molecules on the target cells could not provide suppressing signals for the NK functions (Auchincloss Jr. et al., 1999). Indeed, *Xenopus* NK cells are known to kill tumor cells that lack allogeneic MHC-class I antigen expressions (Horton et al., 2003).

In this report, I analyzed the dynamism in the xenograft rejection in urodeles, Japanese newts and Asiatic salamanders, comparing with frogs, and obtained very interesting results where the urodeles rejected xenografts in a chronic manner, as seen in allografts. The frogs did reject the xenografts by accelerated acute response, as expected. Newts showed no discrimination between allo and xenotransplantation responsiveness, or they may possess no functional MHC-class I-mediated immune activities. If cytotoxic T or NK cell activity, both of which are thought to be developed and work in the immune surveillance on the generation of malignant neoplasm, are not engaged in newts, how do they prevent tumor development? The immune surveillance mechanism in newts was discussed in correlation with their

strong regeneration capacity.

Materials and methods

Animals

The following animals were used in this report. The prefectures, from which they were collected other than Niigata, are indicated in parentheses.

For urodela, newts; Japanese newts: *Cynops pyrrhogaster* and *Cynops ensicauda* (Okinawa), and Asiatic salamanders: *Hynobius lichenatus, Hynobius nigrescens,* and *Onychodactylus japonicus* (Fukushima). For anura, tree frogs; Japanese tree frog: *Hyla japonica*, green tree frog: *Rhacophorus arboreus,* and true frogs; brown frogs: *Rana japonica, Rana ornativentris,* and *Rana tagoi tagoi,* pond frogs: *Rana nigromaculata,* and *Rana porosa porosa* (Miyagi), and bull frog: *Rana catesbeiana,* and *Rana rugosa.*

Urodeles and anuras were given pig liver chops every five days and every week, respectively.

Titration of natural antibodies

The titration of natural antibodies was performed by a microtitration technique using a microtitration device (8 × 12 V-type depression in each tray) (Hosono and Muramatsu, 1972). Briefly, 25 μl of sera were serially prepared into two-fold dilutions, and 25 μl of 0.2% blood cells as target indicator cell suspensions was pipetted into each depression. After incubation for 1 hr at 37℃, the end points of the hemagglutinin were read and the amounts of the natural antibodies were expressed by Log_2 titer.

Skin transplantation

Skin transplantation in the urodeles was performed as described previously (Kinefuchi et al., 2011). After complete anesthetization of the recipient newts in one-third saturated ethyl *p*-aminobenzoate (Wako, Osaka), a graft bed was prepared

on the dorsal site, and the graft of the ventral skin was carefully placed on and expanded over the graft bed. The newts were kept on a plastic tray with a wet sheet. The experimental temperature was controlled at 25℃, unless otherwise mentioned.

In the case of frogs, skin-grafting was performed based on the method of Plytycz et al. (1993) with minor modification. The frogs were anesthetized by immersion in a 0.001% solution of MS-222 (meta aminobenzoic acid ethylester methanesulfonate; Sankyo, Tokyo), and around 5-mm piece square of dorsal skin was carefully peeled off to prepare the graft bed. Meanwhile, as a graft, dorsal skin was also carefully peeled off and trimmed to around 4-mm square with small scissors. The obtained graft was put on and expanded over the graft bed. The graft-bearing frogs were put separately into a plastic box with shallow water to avoid soaking the graft. They were kept under a 20℃ atmosphere, and the graft and body surface were carefully cleaned every day with fresh tap water.

Observation of skin grafts

After the animals were anesthetized, external observation of the grafted skin was carried out under a stereomicroscope every three days in the urodeles and every two or three days in the anuras. The grafts of the urodeles and the anuras were considered to be rejected when all pigment cells were destroyed.

Histological and immunohistological observations were performed based on the methods in a previous report (Kinefuchi et al., 2011), where paraffin sections were stained with hematoxylin and eosin (HE), and cryostat sections were analyzed for CD3ε-bearing cell distribution

Criteria for anamnestic responses

Especially for urodele's transplantation experiments, the length of the graft survival time (ST) is not always a good indicator of anamnestic responses because of

the large variation of graft STs in chronic responses. As a criteria for anamnestic responses, I used the fact that normal blood-circulation does not appear in the secondary set of grafts, as described in a previous report (Kinefuchi et al., 2011).

Statistic analyses

The significance of the differences among the experimental groups was determined by Student's *t* and Fisher's exact probability tests. A *p* value less than 0.05 was considered to indicate a significant difference.

Results

Urodeles show chronic responses and anuras show acute responses in allograft rejection

In anti-allograft transplantation immunity, urodeles respond in a chronic manner (Cohen and Borysenko, 1970; Hildemann et al., 1981) and this is also accepted in Japanese newts (Murakawa, 1968), as I recently confirmed (Kinefuchi et al., 2011). To see if this is the case for other species of urodela and to find, whether anura controversially show acute responses in anti-allo responses (Cohen and Borysenko,1970; Hildemann et al.,1981; Jozkowicz and Plytycz, 1998), transplantation experiments were performed in two species of two families of urodela, Japanese newts: *Cynops pyrrhogaster* and Asiatic salamanders: *Hynobius nigrescens*, and eight species of three families of anura, i.e., one species of tree frogs, six species of true frogs, and one species of green tree frogs.

As shown in Fig. 1, clearly demonstrating the above prediction, the MSTs of the grafts in the urodeles were three to five times as long as those in the anura. Although all species of animals responded very severely against the secondary set of grafts from identical donors with a similar effective factor of one-third, the frogs responded in an accelerated acute fashion and newts in a chronic fashion (Fig. 2).

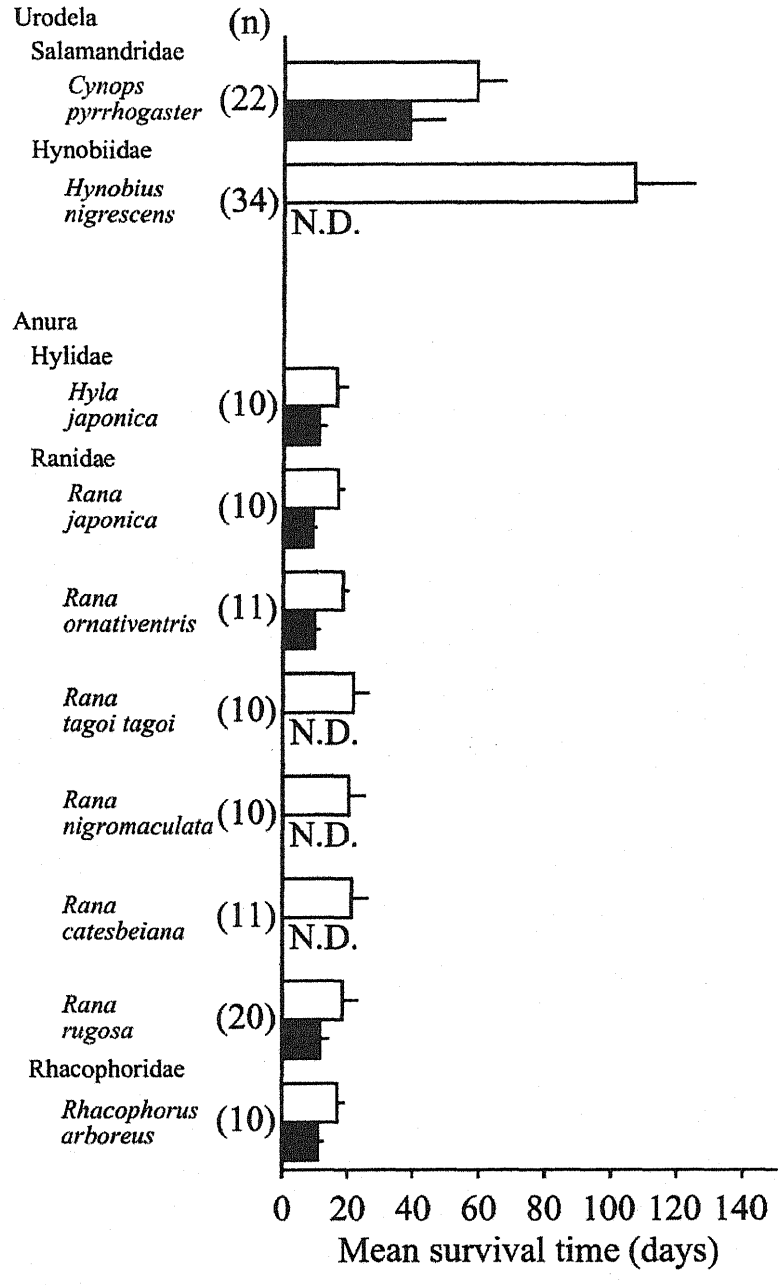

Figure 1. Comparison of allotransplantation immunity between urodela and anura. Each column indicates MST with a horizontal bar of ± SD. Open bars: primary responses, and closed bars: secondary responses. Figures in parentheses indicate number of animals used. N.D. = not done. Experiments were carried out at 20 ± 1℃.

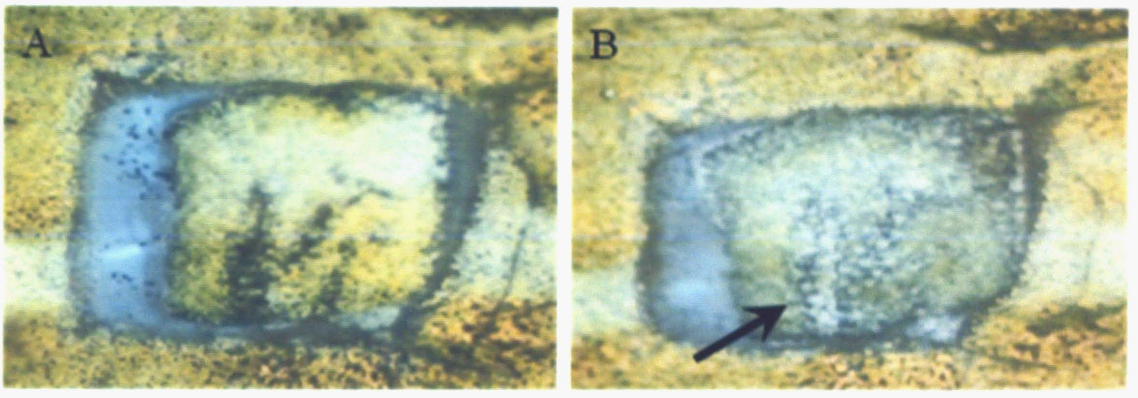

Figure 2. Externals of the allograft in the primary transplantation in the tree frog, *Hyla japonica*. A) At the early latent stage or 7 days after the grafting, equivalent to around 20th day of allotransplantation in newts. B) At the rejection stage or 14 days after the grafting, equivalent to around 40th day of allotransplantation in newts (cf. Fig. 2 in the Part 1, p 16). Note an arrow indicating the melanophores becoming

Large variation of graft STs in newts may not be due to differential immunogenic strength of histocompatibility antigens

Since the chronic responsiveness of transplantation immunity in newts shows a very large variation in graft STs from 30 to 60 days in the primary responses, either of the following two possibilities may be considered. One is that the animals, which were collected from a given field of one valley water are of a closed colony genetically related, especially in the case of the longer STs of the grafts, and the other is that the shorter graft STs means stronger immunogenicity of the transplantation antigens involved, indicating the presence of a well demonstrable, functional MHC system.

To see which possibility is more likely, I provided two groups of newts from two different valley waters 15 km apart and across five mountains and four rivers. The newts received allo-skin grafts from the same field on one dorsal side, and allo-skin grafts from the other field on the contralateral site. The STs of each graft from the same valley waters were plotted against those from the other valley waters. The results are shown in Fig. 3. Either the individual degree of strength of the rejection activity to one allo-graft closely resembled that of the other allo-graft on a line of $y = 0.79 x + 0.99$ or no significant difference existed from $y = x$, indicating that there may be no direct effects mediated by the MHC system. Furthermore, these results guarantee that the animals are genetically well-randomized.

In this condition, note that the strength in the primary response was not correlated with the strength in the secondary response, where the strength of the target graft-damaging activity or the length of the rejection stages of the graft-rejection process was not correlated between the primary and secondary responses at all (date not shown). This may indicate again that the MHC-associated

events in the target cell-killing by lymphocytes, as is well known in higher animals, are not engaged in newts.

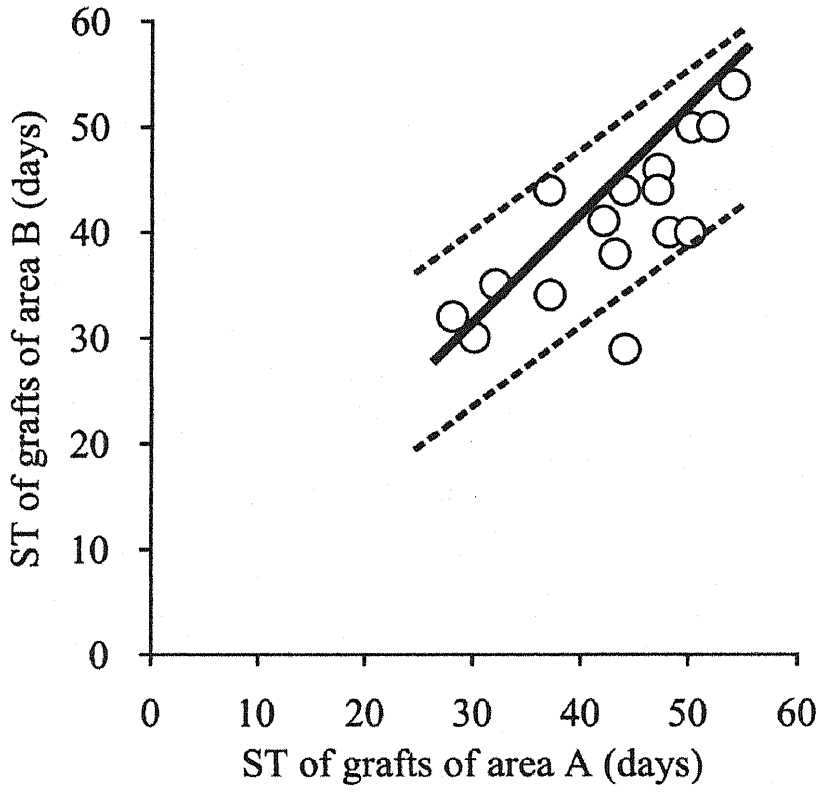

Figure 3. Large variation of graft survival time in allotransplantation immunity in newts may not be differential immunogenetic strength among allotransplantation antigens. The zone between the dotted lines indicates 95% confidence limits on the regression line, $y = 0.79\ x + 0.99$, and the straight line indicates $y = x$. For details, see text.

Repeated grafting from identical donors does not strengthen anamnestic response

Repeated the antigenic stimulations would cause clonal expansion to result in more shortened STs of the graft toward the level seen in frogs. In newts, although repeated stimulations with skin grafts from identical donors shortened STs within the first three grafts, no further extension of the rejection effect was observed, and MST was settled at 25 days, which remains chronic and almost two or three times of the MST in frogs (Fig. 4 and compared with Fig. 1)

Interestingly, the goal of this experiment is similar to that seen in temperature-shift experiments, where a temperature increase to physiologically up-regulate the immune situation shortened skin STs to 25 days but not smaller (Kinefuchi et al., 2011). These results suggest that the graft was not damaged through the clonal expansion of the cells under the MHC-mediated strong target-killing procedure.

Note here that in the third party skin grafts, which were employed as controls for the primary responses, MSTs seemed to shorten around 10% of every control-grafting (Fig. 4). This may indicate that the numbers of allotransplantation antigens in newts are relatively small and that they share part of them.

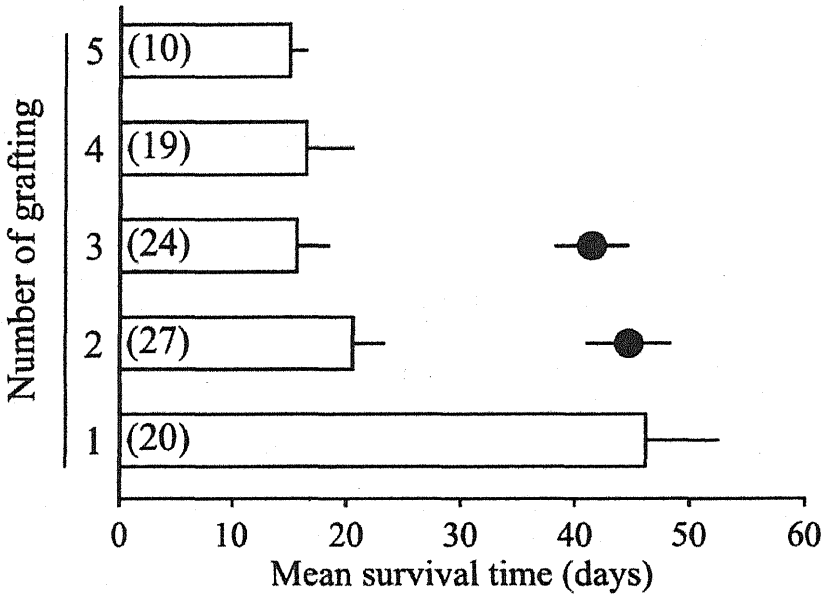

Figure 4. Effects of repeated sensitization with allografts from identical donors on strength of anamnestic responses. Each recipient received allografts repeatedly up to fifth set of grafting. Figures in parentheses indicate number of animals used. Closed circles indicate MST with bars of ± SD of grafts from unrelated donors for controls; numbers of grafts at second and third graftings are 5 and 10, respectively. (in cooperation with M. Johnouchi)

Estimation of possible numbers of weak histocompatibility antigens in newts

The newts were divided into five groups of around 14 individuals. Each received one, three, six, nine or 12 allografts from donors unrelated to each other at once. Three months later when the allografts were completely rejected, each recipient was re-transplanted on one dorsal side with a secondary set of grafts from the donor of the first set of grafts with STs near the MST of the multiple grafts. The secondary grafts were expected to be smoothly rejected due to the anamnestic responses. The same recipient received a graft on the other side from unrelated, fresh donors and the frequencies of the recipients expressing anamnestic responses to the unrelated skin graft were calculated.

The results in Fig. 5 clearly show that regardless of the increasing numbers of the first grafts, the MSTs of the secondary set of grafts from identical donors were almost the same, around 32 days, although the ST deviation became smaller and smaller. On the other hand, for the grafts from unrelated donors, increasing the numbers of the first set of grafts shortened the graft STs to the level of the secondary set of grafts, 32 days, from the same donors for the primary responses, especially when the number of the primary grafts exceed nine (Fig. 5A). To see the precise anamnestic responses among the secondary set of grafts, the frequencies of the grafts rejected without the latent stages were plotted against the numbers of the first set of grafts in Fig. 4B, indicating that more than 10 allografts from unrelated donors are required to provide cross-sensitization against allografts from any possible donors. When the possible numbers of transplantation antigens (n) are calculated from the following formula,

$$n! / 2! (n - 2)! = N,$$

where N = graft numbers used at the primary response, 5 for n is obtained after 10 for

N is put into the formula.

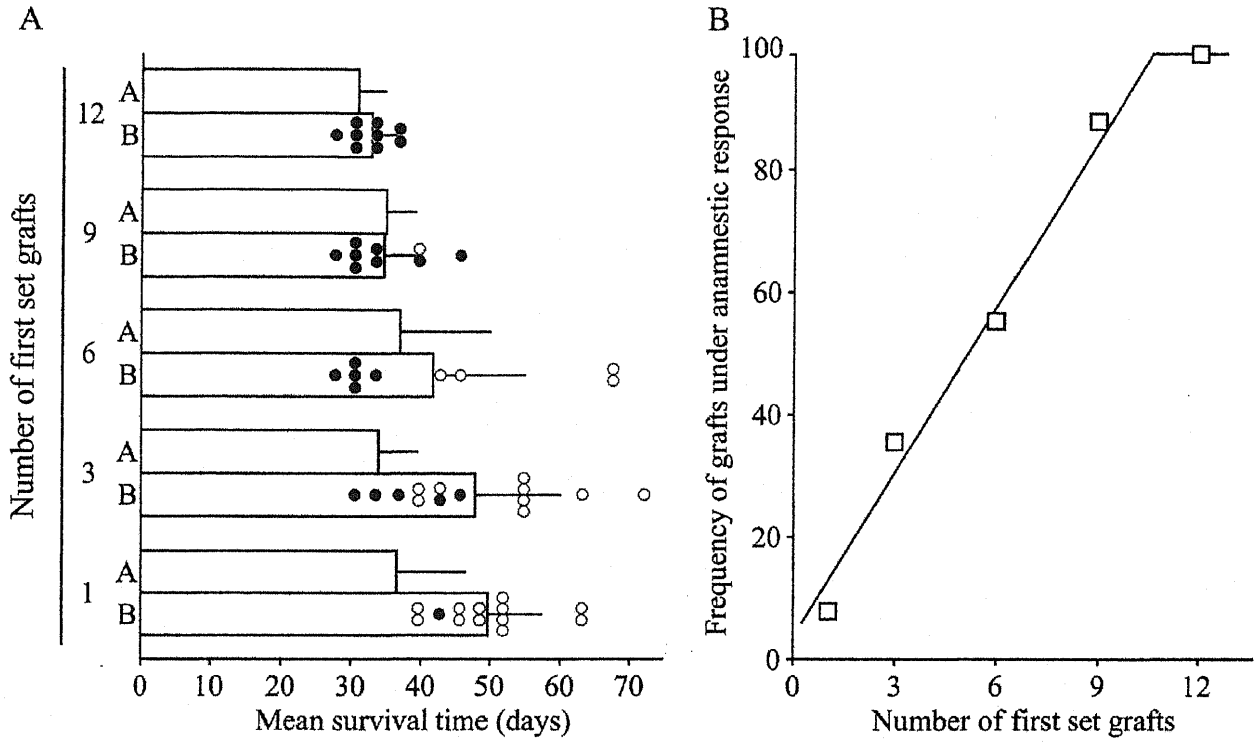

Figure 5. Estimation of number of allotransplantation antigens in newts. Experiments were carried out at 23 ± 1℃. A) Allo-cross reactivity by multi-grafting. Column A indicates MST with a horizontal bar of SD of grafts from identical donors used for primary response. Column B indicates MSTs of each graft from donors unrelated to first set graft; open circles show grafts under primary response and closed ones under anamnestic response. B) Increase of incidence of cross-priming for allo-antigens on third party of donor due to increasing number of primary allografts from different donors. Any grafts from third party donors would be rejected by anamnestic responses, when number of primary grafts exceeds 10, as indicated.

Urodela shows chronic responses even to xenogeneic skin grafts, while anura accelerates acute responses

In anura, as seen in the higher classes of animals, transplantation immunity is well characterized by the profound involvement of the MHC system in the acute responses by the activation of $CD8^+$ cytotoxic T cells and in the accelerated acute responses by NK cells against MHC-non bearing target and xenogeneic cells. Here, I investigated xenotransplantation to see the significance of the possible involvement of MHC in newts in comparison with frogs.

In the case brown frogs and green tree frogs, the MSTs of the allografts in the secondary responses were significantly shortened in an accelerated fashion with 17 and 19 days of MST, respectively (Table 1). These accelerated acute responses were equally observed against xenografts under species-disparity and family-disparity conditions as well. The anti-xenograft responses were probably not mediated by anti-xeno antibodies, which are sometimes naturally present in the hosts (Brent 1997), because the mean titer of the natural antibodies against the graft donors of brown frogs was less than 1 and only 2, in the case of species-disparity and family-disparity, respectively. Note that accumulation of many CD3ε-positive lymphocytes was prominent in the xenografts at 14 days in the graft rejection (Fig. 6). In allografts, no cellular responses were observed at this time of transplantation.

For newts, on the other hand, the primary responses against xenogeneic grafts were just slightly stronger in the species-disparity but not in family-disparity in this case (Table 2). In the secondary responses, the rejection process to the allografts was still chronic, even though MST became significantly reduced, i.e., 47 days, resembling that in the species-disparity situation. When xenografts were from family-disparity donors, the ST of the xenografts was reduced to 27 days. On the

natural antibodies against xenografts in the responders of Japanese newts, the mean titers were less than 1 in both species- and family-disparate graft donors. Therefore, in all the cases of xenotransplantation, the newts showed totally chronic responses, although weak but significant acceleration was observed for xenografts from phylogenically more distant donor.

From all the above experiment data, my tentative conclusion is that there may not be present strong histo-incompatibility, or a functional MHC system, and the longer the phylogenic distance is, the stronger the antigenic disparity may be, resulting in shorter MSTs of the xenografts. Indeed, this was the case when I tested Asiatic salamanders: *Hynobius nigrescens* and Japanese newts: *Cynops pyrrphogaster*, as the recipients of xenografts (Fig. 7). Here, perhaps some allotransplantation antigens may be shared with xenoantigens, and this is strongly suggested by the fact that newts, which were once primed and sensitized with multigrafts from several allogeneic donors, rejected a significant proportion of both allografts (7 of 9) and xenografts (4 of 8) from unrelated donors under the anamnestic response.

Table 1. Accelerated acute responses in xenotransplantation in frogs[#]

Hosts	Grafting	MST in days			p
		Allograft	Xenograft*: Disparity in		
			Species	Family	(Allo. vs Xeno.)
Brown frogs: *Rana porosa porosa*	1°	22.8 ± 2.4 (16)[†]	19.1 ± 1.8 (14)	N.D.	$p < 0.001$
	2°	17.0 ± 2.2 (6)	17.4 ± 3.1 (9)	N.D.	N.S.
	p: 1° vs 2°	$p < 0.001$	N.S.	-	
Tree frogs: *Hyla japonica*	1°	28.6 ± 4.9 (17)	N.D.	18.9 ± 3.1 (13)	$p < 0.001$
	2°	19.0 ± 4.1 (9)	N.D.	18.0 ± 2.8 (6)	N.S.
	p: 1° vs 2°	$p < 0.001$	-	N.S.	

[#]: This experiment was carried out at 19 ± 1℃. N.D.: not done

*: The graft donor is *Rana japonica*. N.S.: non significant difference

[†]: Figures in parentheses are number of animals used.

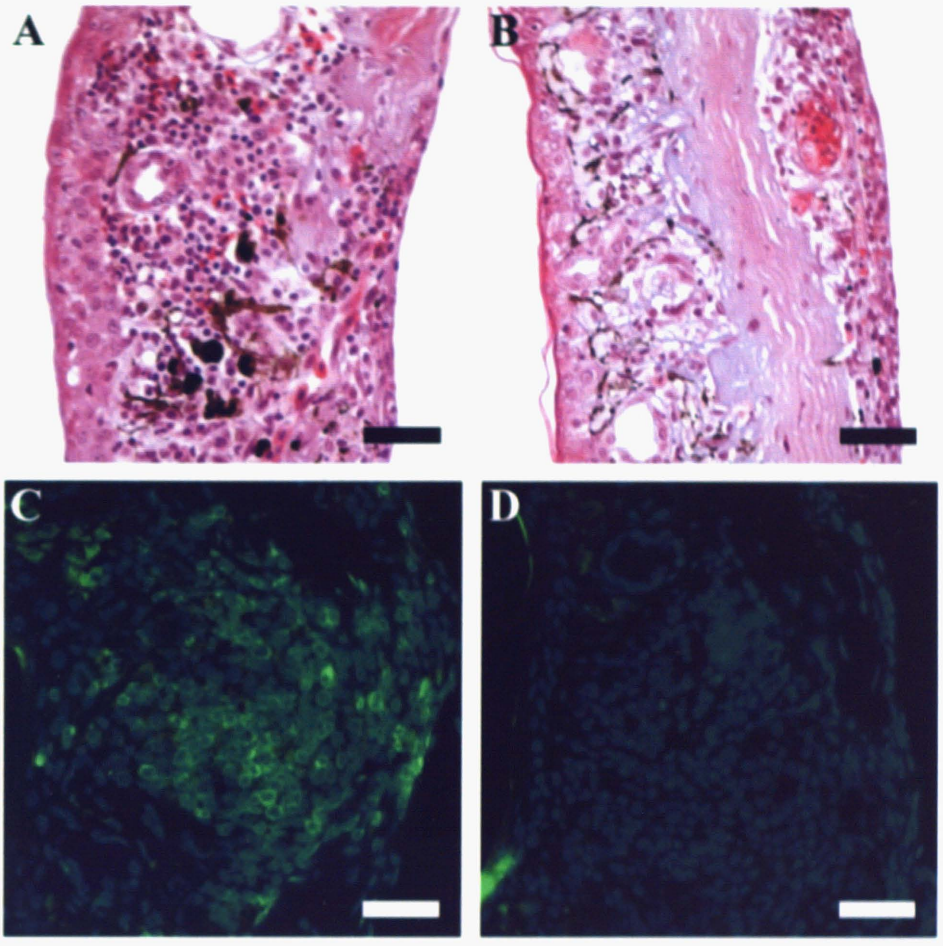

Figure 6. Graft-infiltrating cells positive for CD3ε molecules in early phase of xenotranplantation in frogs. Tree frogs; *Hyla japonica*, received xenograft of donor brown frogs, *Rana japonica* (A, C, D) and allograft (B). Xenografts on day 14 were assessed by histological observation of HE-staining (A), indicating cluster of lymphoid cells in the graft and immunofluorescent staining shows that the accumulating cells are CD3ε-positive. D: control for C, without primary antibody of anti-CD3ε Ab. HE-staining of 21 days allograft (B) indicates relatively dispersed distribution of lymphocytes. Scale bar: A-D, 50 μm. For details, see text. (in cooperation with Y. Kushida)

Table 2. Chronic responses in both primary and secondary responses in xenotransplantation in newts[#]

Hosts: *Cynops pyrrphogaster*	Grafting	MST in days: Allograft	MST in days: Xenograft: Disparity in Species*	MST in days: Xenograft: Disparity in Family**	*p* (Allo. vs Xeno.)
Exp. 1	1°	63.8 ± 7.6 (24)[†]	55.4 ± 10.4 (23)	N.D.	$p < 0.05$
	2°	47.3 ± 9.8 (14)	43.1 ± 10.1 (8)	N.D.	N.S.
	p: 1° vs 2°	$p < 0.001$	$p < 0.001$	-	
Exp. 2	1°	69.8 ± 9.7 (24)	N.D.	66.1 ± 9.8 (21)	N.S.
	2°	46.8 ± 8.7 (22)	N.D.	26.9 ± 6.1 (15)	$p < 0.001$
	p: 1° vs 2°	$p < 0.001$	-	$p < 0.001$	

[#]: These experiments were carried out at 19 ± 1℃.

[†]: Figures in parentheses are number of animals used.

*: The graft donor is *Cynops ensicauda*.

N.D.: not done

**: The graft donor is *Hynobius nigrescens*.

N.S.: non significant difference

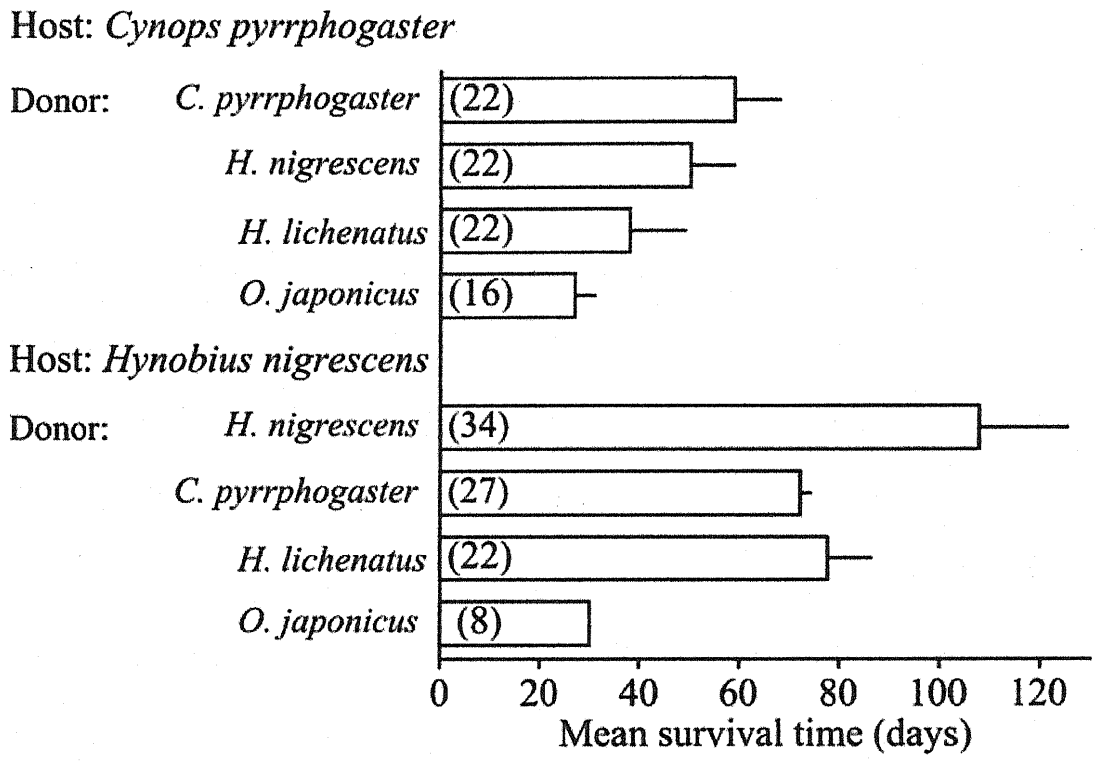

Figure 7. Strength of allo and xenotransplantation in Japanese newts, *Cynops pyrrhogaster* and Asiatic salamanders, *Hinobius nigrescens*. Figures in parentheses indicate number of animals used. Experiments were carried out at 20 ± 1℃. For details, see text.

Discussion

Experiments concerning allotransplantation immunity have been classically and widely employed for a long time to investigate the biological significance of acquired immune responses in several classes of animals. The degree of the strength of the allograft rejection is determined based on the phylogenical status of the graft recipients in animal evolution. Allotransplantation immune responses in vertebrates are generally categorized by the tempo of the graft rejection into four types: 1) hyper acute by specific antibodies and complements, 2) accelerated acute within days by anamnestic responses by memory T cells, 3) acute within days or weeks by T cells developed under the effects of the MHC system, a well developed immune system, and 4) chronic within months or years by unknown causes (Hutchinson, 2001).

Bony fishes are generally known to bear the MHC system with a function of immune T cells (Du Pasquier and Flajnik, 1999; Nakanishi et al., 2002) and to show an acute response in the primary allograft rejection and an accelerated acute response in the secondary set of allografts from identical donors, as seen in frogs and higher class animals (Cohen and Borysenko, 1970; Hildemann et al., 1981; Jozkowicz and Plytycz, 1998). However, in urodele amphibians, the immune activity is inferior to fishes: neither acute nor accelerated responses by cytotoxic T cells. They show only chronic forms of allotransplantation immunity (Cohen and Borysenko, 1970; reviewed in Murakawa, 1971; Hildemann et al., 1981; Kinefuchi et al., 2011). Therefore, the circumstantial evidence so far reported provides a possibility that there are no cytotoxic T cells in urodeles (Hildemann et al., 1981), although in the genome, they should have functionally well constructed T cell receptors (Charlemagne et al., 1998). If so, generation of cytotoxic T cells might be limited because of the functionally poor molecular construction of MHC molecules, as proposed in molecular and genetic

studies using a Mexican salamander, axolotl (Kaufman et.al., 1995; Tournefier et al., 1998). In contrast, Bos and DeWoody (2005) reported that tiger salamanders, which are known chronic responders in allotransplantation immunity (Cohen, 1972), bear normal and evolutionally well developed polymorphism of the MHC-system. Thus, contradictions on the nature of the chronic manner of immune activities seen in urodele amphibians is very interesting in evolutional studies, but unfortunately, information is quantitatively and qualitatively insufficient for understanding the precise dynamisms of the response.

For Japanese newts, probably because their cells are tremendously large, I could not handle them to investigate the in vitro nature of immune cells. In collecting single spleen cells, a cell washing procedure even by ~ 50 × G centrifugation killed them (Ohneda, Master's thesis, Niigata University, 2010). Therefore, at present I must conclude based on such circumstantial evidence on the in vivo approach for the activities of immune cell functions. All the data presented in this report showed clearly and more profoundly that newts have only chronic transplantation immunity, since the MSTs for allografts are not shorter than 25 days under immune-strengthened conditions, such by repeated sensitization (Fig. 4), and the engagement of cytotoxic T cells as target cell-killing effector cells does not seem likely. Such a chronic response was seen in the frogs from which $CD8^+$ T cells of cytotoxic T cells lineage were serologically removed by specific antibodies (Rau et al., 2001). In the case of newts, the effector cells themselves did not expand the T cell properties, because increased antigenic stimulation did not shorten the MST less than 25 days (Fig. 4). Similarly, multi-antigenic stimulation brought maturation of the effector cell function, and it made the graft STs converge on the MST of 32 days without shortening the MST (Fig. 5). Therefore, agreeing with my previous report (Kinefuchi et al., 2011), these

effector cells seem to be monocytes/macrophages.

Such the poor generation of cytotoxic T cell lineage might be related to the poor functional construction of the MHC system, considering that their generation and development are dependent on the presence of MHC molecules in the thymus and that the histological architecture of the thymus with no medulla in newts (Kinefuchi et al., 2011) shows a strong contrast to the thymus with medulla, where matured T cells are lodged in such bony fishes as zebrafish (Lam et al., 2002), lungfish (Mohammed et al., 2007), and sea bass (Picchietti et al., 2009). Preferential selection in the thymus of T cells with T cell-receptors corresponding to self-MHC molecules are thought to bring the generation of T cells direct to allogeneic MHCs. This phenomenon is known as allo-aggression with a peculiarity of a stronger immune attack in allogeneic cells than in xenogenic cells. However, I could not demonstrate allo-aggression in urodeles at all: no discrimination between allo and xenotransplantation antigens (Fig. 6, Table 2). Thus the presence of a functional MHC system in urodela seems unlikely. This may explain the very high frequency of successful parabiosis (20%) in newts (Murakawa, 1968).

Finally, in my survey of a functional MHC system in newts, xenotransplantation immune activities were compared with frogs that have NK cells (Horton et al., 2003), because NK cell function is also severely controlled by MHC molecules, especially by MHC-class I molecules under the discrimination of the molecular structures between allogeneic and non-allogeneic MHC, including xenogenic MHCs (Seebach et al., 1996; Inverardi et al., 1997; Auchincloss et al., 1999). Urodeles could not discriminate xenografts from allografts in the immune strength (Table 2). This is in strong contrast to the frog's perfect rejection of xenografts in an accelerated acute manner in the primary set of xenografts (Table 1). It is impressive that many

manner in the primary set of xenografts (Table 1). It is impressive that many lymphoid cells, which are positive in an anti-CD3ε antibody's survey, accumulated at xenografts as early as 14 days after transplantation (Fig. 6). Although I lack direct evidence, these accumulating cells are probably NK cells, since anura NK cells are known to have CD3ε molecules (Göbel and Bolliger, 2000).

Taken all together, these data emphasize that urodeles, especially in Japanese newts, show extremely poor or no functional activity of the MHC system, which is closely related to the generation and the function of cytotoxic T cells and the activation of NK cells. These target-killing activities were thought to be generated during the evolutional process as immune surveillance mechanisms (Robert and Cohen, 1998). If so, how urodela deal with the prevention of neoplasm development remains a very interesting question. It has been a long time since the rare occurrence of spontaneous tumors among amphibia, especially urodela (Effron et al., 1977) was thought to have a connection with their strong regeneration capacity (Waddington, 1935; Balls, 1962). Japanese newts rarely suffer from tumors in the field, and even if skin papillomas appear once, they spontaneously regress without metastasis (Asashima and Komazaki, 1980). Furthermore, newts are resistant to treatment with so-called carcinogens and respond by regeneration (Eguchi and Watanabe, 1973; Okamoto, 1997). These interesting phenomena have been widely discussed for the connection between tumorigenesis and regeneration (Harty et al., 2003; Oviedo and Beane, 2009). Indeed, regeneration to a given functional cell might be a strategy against tumorigenesis in newts. Because under the regeneration process in once differentiated and functional tissues, the self-markers of MHC-class I antigens disappear due to de-differentiation, they could escape from attack by cytotoxic T cells. However, they would soon become targets of NK cells. Easy de-differentiation

by urodeles under their evolution process.

In this scenario, data from frog tadpoles may provide a clue for understanding the urodeles' immune status, since tadpoles have unique immune systems for the following issues: 1) expression of MHC-class II , but not of MHC-class I antigens, 2) no expression of cytotoxtic function in $CD8^+$ T cells, although they are present, and 3) absence of functional NK cells (Robert and Cohen, 1998; Rollins-Smith, 1998; Robert and Ohta, 2009): These physiological potencies could be acceptable as the immune nature in newts through circumstantial evidence. Therefore, mechanisms for immune surveillance in frogs after metamorphosis appear in the generation of MHC-class I associated with immune functions of $CD8^+$ cytotoxcic T and NK cells, but de-generation and regeneration without MHC-class I engagement could be an escape mechanism from the generation of neoplasm in cellular stress in urodeles, where the highly controlled generation of immune cell-specificity may be insufficient, resulting in poor immune activities.

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