STUDIES ON THE ONTOGENY OF IMMUNITY IN AN ANURAN AMPHIBIAN, *XENOPUS LAEVIS*, IN RELATION TO THE FUNCTIONAL DIFFERENTIATION OF THE THYMUS

(With 12 Tables, 13 Text-Figures, and 18 Plates)

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DOCTOR OF SCIENCE

By

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CONTENTS

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INTRODUCTION

Although Edward Jenner initiated the vaccination against small pox late in the 18th century, immunology as a science started in the late 19th century when Louis Pasteur, a famous French bacteriologist, demonstrated the occurrence of anti-bacterial substances or factors in the blood of animals which were infected with microorganisms. Through the efforts by many successors of immunology, the concept of *immunity* is now established as "the state of heightened responsiveness to non-self, in which non-self is bound or eliminated more rapidly than in the non-immune state". Early in the history of modern immunology during the 1930s, studies were concentrated on the chemical characterization of the resistant factors in the immunized mammals, *i.e.* antibody and complement. The field is now designated as immunochemistry.

Ever since around 1960, following the discovery of immunological function of the thymus in rodents (Miller, 1961) and of the bursa of Fabricius in chicken (Glick *et al.*, 1956), cellular immunobiology has become one of the mainstreams of modern immunology. Based on the well-established concept about the dichotomy of T- and B-lymphocytes, recent efforts in the studies of immunology have been directed

toward the elucidation of the mechanisms by which recognition of antigens, immune differentiation, and immune effector functions are regulated. And these researches have been made mainly in mammals. However, in order to understand the ontogenic differentiation of the immune system of vertebrates, mammals are not the best model because they develop *in utero* and, therefore it is not easy to handle the fetuses in experiments.

During the last decade, in parallel with the ontogenic emergence of immunity, phylogenetic emergence of immunity has also increasingly attracted many investigators' attention. It has now been widely accepted that cell-mediated immunity by T-cells and antibody production by B-cells are general properties of vertebrate species. Studies with lower vertebrates would provide a firm basis for the view that ectothermic vertebrates are invaluable models for studying the fundamental make-up of immune system, and would facilitate investigations which are extremely difficult to carry out with avian or mammalian systems. In particular, amphibians may provide an important model for the examination of basic aspects of the development of immunity (see reviews, Du Pasquier, 1973; Cooper, 1973; Cohen, 1976, 1977).

Among several amphibians, *Xenopus laevis*, a toad

phylogenetically belonging to a primitive group of anurans, seems to provide the most suitable material in this respect (see a review, Katagiri, 1978). Thus, detailed accounts have been accumulated of the histological aspects of lymphoid tissues and organs such as thymus, spleen, liver and mesonephros in larvae and adults, and ventral cavity bodies in the larval branchial region. Unlike other anurans, however, this species lacks lymph nodes (Sterba, 1950, 1951; Manning and Horton, 1969; Nagata, 1976, 1977; Rimmer, 1977). Toads and their tadpoles are known to reject skin allografts in acute or subacute fashion whereas urodelan amphibians reject them only chronically (Elek *et al.*, 1962; Simnett, 1964). In relation to this response, extensive immunogenetic studies carried out by Du Pasquier and his co-workers have suggested that in *Xenopus* there might be only one major histocompatibility locus which controls the alloimmune reactions (Du Pasquier and Miggiano, 1973; Du Pasquier *et al.*, 1975; Du Pasquier *et al.*, 1977). It has also been demonstrated that *Xenopus* belongs to the phylogenetically lowest group of vertebrates that produces not only high molecular weight immunoglobulins but also low molecular weight immunoglobulins which are comparable to mammalian immunoglobulin G (Hadji-Azimi, 1971; Jurd and Stevenson, 1974; Hadji-Azimi

and Michea-Hamzehpour, 1976). Immunocytochemical studies of lymphocytes from several lymphoid tissues of *Xenopus* showed that in contrast to the situation in mammals where thymocytes are surface-immunoglobulin-negative, surface immunoglobulins are detectable not only on lymphocytes from spleen and gut-associated lymphoid tissues but also on thymocytes (Du Pasquier *et al.*, 1972; Jurd and Stevenson, 1976; Michea-Hamzehpour, 1977; Hadji-Azimi, 1977; Nagata and Katagiri, 1978). In parallel with similar results obtained in urodelans and fishes (Charlemagne and Tournefier, 1975; Emmerich *et al.*, 1975), these findings raise an interesting question as to the phylogenetic origin of immune recongnition.

The most intriguing fact about *Xenopus* with respect to the study of ontogeny of immune system is its unusual feasibility of rudimental thymectomy, the situation which is inaccessible in any other animal species. The effects of thymectomy on the development of lymphoid tissues and immune responses have been extensively studied by an English school (Horton and Manning, 1972, 1974a, 1974b; Turner and Manning, 1974; Collie *et al.*, 1975; Du Pasquier and Horton, 1976). According to these investigators, the major result of thymectomy at stage 48 (7-8 days old) was the complete suppression of humoral response, whereas

the same operation only delayed but not abrogated the allograft rejection. In view of the fact that the thymus at stage 48 is in the initial stage of lymphoid differentiation, an interpretation was proposed that in contrast to the situation in the homoiotherms (*cf.* Miller and Osoba, 1967), animals at this phylogenetic level possess a compensatory, non-thymic origin of lymphocytes pertinent to the allograft rejection (Horton and Horton, 1975; Rimmer and Horton, 1977). The alternative possibility may still remain, however, that the thymus has performed the essential function in question before the larval stage 48. To answer this question, it seems extremely important to establish an experimental system in which thymectomy is made at much earlier stages than stage 48.

Another interesting question in relation to the ontogeny of immune system is concerned with the embryological origin of thymus lymphocytes. There has been a controversy among investigators as to whether thymic lymphocytes are extrinsic (mesodermal) or intrinsic (endodermal) in origin. The problem could not be solved by ordinary histological or electron microscopical observations on developing avian and mammalian thymuses (*cf.* Sanel, 1967; Tachibana *et al.*, 1974; Ackerman and Hostetler, 1970; Leene *et al.*, 1973). More recently, using heterospecific

combination between quail and chick thymic rudiments, Le Douarin and Jotereau (1975) provided unequivocal evidence for extrathymic origin of thymocyte stem cells. On the other hand, quite opposite view has been proposed for the anuran amphibian, *Rana pipiens*, in which thymic lymphocytes may be of intrinsic origin (Turpen and Cohen, 1976; Turpen *et al.*, 1973, 1975). The difference may be interpreted to show the two different origins of thymic lymphocytes between the two animals. Therefore, it would be worthwhile to investigate the origin of thymocyte stem cells in *Xenopus*.

Recently, the author succeeded in developing a technique to thymectomize the *Xenopus* larvae as early as stage 45 (4 days old). The success in obtaining metamorphosed animals with the least or no thymic influence prompted us to bring about a series of experiments on the problem of the functional differentiation of thymus during the ontogeny.

The first part of the present study was undertaken in an attempt to obtain a histological basis of the immune responses in *Xenopus*. Particular attention was paid to the development of lymphoid system in early-thymectomized animals. In the second part, the immune responses against skin

allografts, heterologous erythrocytes and a synthetic antigen, polyvinylpyrrolidone, in our thymectomized and intact control animals are described. In relation to these studies, a histocompatibly "syngeneic" *Xenopus* colony in our laboratory provided a means of carrying out cell- and organ-transfer experiments to understand the role played by lymphocytes in the immune reactions mentioned above. Finally, the origin of thymus lymphcoytes was explored by grafting diploid thymus rudiments at very early stages into histo-compatible triploid tadpoles. Overall results to be described in the present paper will provide an aspect that the establishment of immune system occurs at the larval stage much earlier than previously supposed.

MATERIALS AND METHODS

ANIMALS

The material used was the South African clawed toad, *Xenopus laevis* (Daudin). Most experiments were carried out using siblings from a colony which had been bred and maintained in our laboratory for more than 10 years. These animals, referred to as the "G group" in the present study, share histocompatibility gene(s) as defined by their lack of allograft rejection among individuals. Another group of toads (designated in this paper as "HD group") was supplied to us by courtesy of Prof. Dr. J. L. Hedrick, the University of California at Davis, California. It should be noted that the HD group toad is not genetically defined, but only known to differ histocompatibly from the G group toad.

Spawning was induced by injection of chorionic gonadotropin (Gonatropin; Teikoku Zoki, Tokyo) into mature male and female toads. Developmental stages were determined according to the Normal Table of Nieuwkoop and Faber (1956). Larvae and toads were reared in aquaria containing dechlorinated tap water. After stage 45, larvae were fed boiled alfalfa leaf powder; water and food were changed every other day. Metamorphosed animals were fed chopped pork liver

twice a week.

Triploid Animals

Triploid individuals were obtained by a brief treatment of eggs with low temperature (2°C) for 15 min immediately after fertilization (Kawahara, 1978). When the larvae from such refrigerated eggs developed to stages 49-50, small pieces of tail tip epithelium were removed to determine the number of nucleoli per nucleus. Since one genome has one nucleolar organizer in this species, counting the number of nucleoli per cell provided a convenient measure of ploidy, three nucleoli being counted in the triploid larvae.

THYMECTOMY

Stage 45 larvae were anesthetized with 1:5,000 MS222 (Sandoz), washed with Steinberg's solution, and positioned ventral side downward on a slide glass, which was placed on a Petri dish containing ice-chilled water. The excess water surrounding the larva was removed with tissue paper. The animal was fixed in position with a soft wooden stick held by one hand, and under the stereomicroscope a sting was made at the thymus rudiment several times with a fine steel needle, so that the thymus rudiment

together with the surrounding connective tissue was broken. A glass capillary of about 100 µm in diameter was pierced into the broken area. Through the tip of the capillary, broken tissues were sucked up and, thus, the thymuses were bilaterally removed. The sham operation consisted of inserting the needle to break the connective tissue surrounding the thymic rudiment. The operated larvae were transferred without suture to Steinberg's solution containing antibiotics (100 IU/ml penicillin G and 100 µg/ml streptomycin sulphate). From the next day onward, the larvae were raised under usual conditions.

Stage 51 larvae were thymectomized by cautery with a blunt steel needle. Anesthetization and all the cares following the operation were done in the same manner as for the surgically thymectomized larvae operated at stage 45.

When animals attained stages 56-58, the success or failure of the operation was judged under a stereomicroscope. Furthermore, the absence of the thymus in thymectomized animals was ascertained histologically at the end of each experiment.

THYMUS GRAFTING

Adult Thymuses into Adult Hosts

Donor and host toads were anesthetized with

1:1,000 MS222, and washed in sterilized De Boer's solution (DB) containing antibiotics (1,000 IU/ml penicillin G and 1 mg/ml streptomycin sulphate). A pair of thymuses with a small amount of surrounding tissues were removed with fine forceps and Wecker's scissors, care being taken not to give any injuries to the organ. Dissected thymuses were washed three times in the sterilized DB, and transferred to the ventral lymph sac of the thymusless host through a small incision made in the posterior abdominal skin. The thymuses were pushed as far anteriorly as possible to be settled well in the lymph sac. Without subsequent suturing, the host animals were placed in the shallow sterilized DB overnight. From the next day onward, the animals were reared in dechlorinated tap water containing antibiotics at a concentration of 100 IU/ml penicillin G and 100 µg/ml streptomycin sulphate.

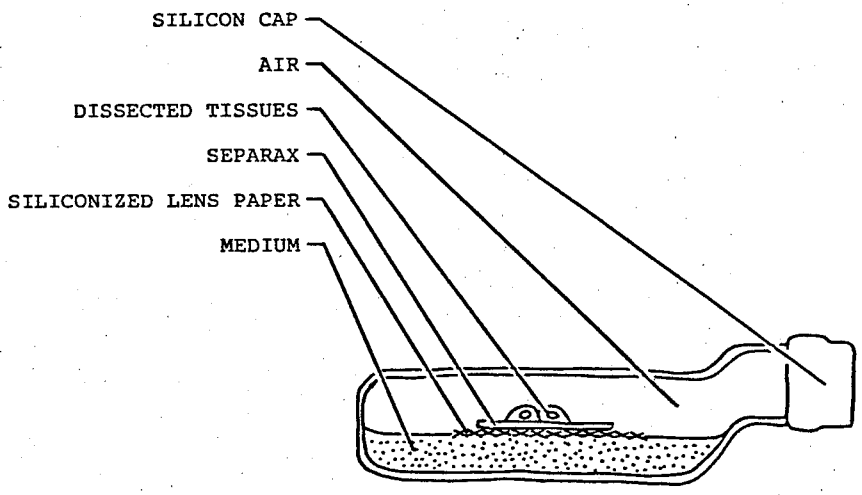
Thymus Rudiments into Larval Hosts

A pair of thymus rudiments were removed from stage 35/36-40 larvae (2-11 days after fertilization), and were grafted into triploid tadpoles (stages 56-58), beneath the tail epithelium near the hind limb. Before the grafting, donor larvae were sterilized by transferring into 0.001 % $KMnO_4$ for 30 min, then

anesthetized with sterilized MS222 solution (1:5,000) and rinsed briefly in 70 % ethanol. A pair of thymus rudiments were dissected out togehter with the surrounding tissue in sterilized Steinberg's solution. The size of dissected fragments was approximately 0.5 × 0.5 × 0.5 mm, so that the relative amount of contamination by surrounding tissue was larger when rudiments were derived from younger larvae. Dissected tissues were again rinsed in 70 % ethanol and sterilized Steinberg's solution. Since the thymus rudiments in the 2-day larvae (stage 35/36) are not detached from the pharyngeal epithelium and are too small to be manipulated well, grafting of the rudiments at this stage was made by implanting the whole head region. Host tadpoles were reared in sterilized Steinberg's solution containing antibiotics at 23°C. Seven to 16 days later, when the control larvae (siblings of donors) reached stage 51 (18 days after fertilization), grafted tissues were processed for microspectrophotometry of thymic lymphocytes or for histological examinations.

ORGAN CULTURE OF THYMUS RUDIMENTS

The thymus rudiemnts from stage 42-49 larvae (3-13 days after fertilization) were organ cultured for 10-14 days. The rudiments were removed from



Text-Fig. 1. Schematic illustration of experimental set-up for culturing thymus rudiments. For details, see text.

larvae by the same way as described for grafting thymus rudiments, and were cultured according to the modified "raft" method (*cf.* Paul, 1975). The whole culture system is diagrammatically illustrated in Text-Fig. 1; *viz*, a pair of tissue fragments were placed on a cellulose acetate membrane (Separax; Fuji Film Co., Tokyo) which was placed on a siliconized lens paper floating on the culture medium. The medium consisted of 7 parts of medium 199, 2 parts of glass distilled water, and 1 part of decomplemented adult rabbit serum, with 100 units of penicillin G, 100 µg of streptomycin sulphate, and 2.5 µg of Fungizone (Gibco) per ml (pH 7.2-7.4 with 30 m*M* HEPES buffer). The cultures were maintained in a closed atmosphere at 23°C and the medium renewed every other day, and they were processed for histological examinations at the end of the culture.

ADMINISTRATION OF LYMPHOCYTES

Thymocyte suspensions in 70 % Hanks' balanced salt solution were prepared by the method described by Nagata and Katagiri (1978): Thymuses from 6-8 months old toadlets were teased in ice-chilled 70 % Hanks' medium, collected in test tubes, and the cells freed from tissue debris were obtained by decantation. Free cells were washed 3 times with the

same medium by centrifugation at 1,000 rpm for 5 min each. To obtain splenic lymphocytes, cell suspensions from spleens were layered on a Ficoll-sodium iotaramate (Conray 400; Daiichi Seiyaku Co., Tokyo) mixture and centrifuged at 1,500 rpm for 15 min (Bøyum, 1968). Peripheral blood lymphocytes were obtained from heparinized blood using Ficoll-sodium iotaramate mixture as described above. In all cell suspensions, the proportion of lymphocytes was more than 90 %, with slight contamination of erythrocytes. Viability of lymphocytes was always higher than 97 % as determined by trypan blue dye exclusion test. Cells were counted using a hemocytometer, suspended in 0.6 % NaCl at desired concentrations, and introduced into dorsal lymph sac in 0.1 ml for each toad (6-8 g body weight).

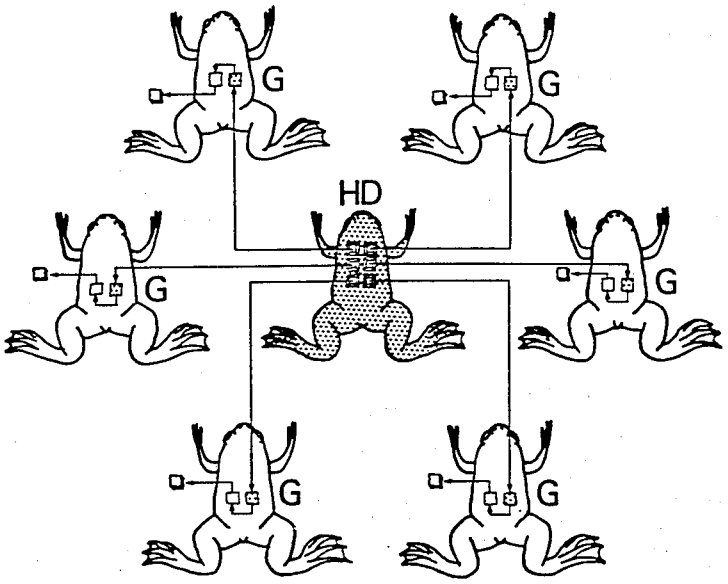
ASSAY OF IMMUNE RESPONSES

Skin Graft Rejection

Skin grafting was performed according to the technique described by previous workers (Hildemann and Haas, 1959; Simnett, 1964; Horton, 1969). Experimental and control G group toads were anesthetized in 1:1,000 MS222. On their dorsal trunk region was grafted a square piece of full-thickness skin (approximately 1.5-2.0 mm on a side)

from the HD group animals of about the same age (3-12 months old). To minimize the genetic variations between the host and the donor animals, 4-10 host animals in one experimental group received grafts from the same donor. Beside an allograft, each host received an autograft which was transplanted from one side of the trunk to the other (Text-Fig. 2). Since the toads thymectomized earlier tended to be infected with microorganisms during the grafting procedure, all the allografted animals, including controls, were kept in jars containing antibiotics for periods after the skin grafting. The fate of grafted skins was observed under a stereomicroscope every day without anesthetization.

The process of allograft rejection was divided into the following 4 phases, on the basis of both external appearance and histological examinations: Phase I, a firm adherence of the graft followed by a dilation of blood capillaries (primary vasodilation); phase II, restoration of blood circulation followed by a dilation of blood vessels (secondary vasodilation); phase III, cessation of the blood flow (hemostasis); and phase IV, progressive breakdown of the graft, leading to a duller and opaque appearance of the entire graft. Destruction of the pigments over the entire graft was taken as the completion of the graft



Text-Fig. 2. Schematic illustration showing the relationship between the donor and the hosts used for skin graft experiments. A single HD group donor provided skin grafts to 4-10 of recipients of the G group animals.

rejection (see RESULTS II.1.1.).

Humoral Antibody Response

Rabbit red blood cells (RRBC) were suspended in phosphate buffered mammalian saline (PBS) in a concentration of 3×10^9 cells/ml. Toads received 3 injections of the antigen at 2-day intervals *via* the dorsal lymph sac, at a dose of 5 µl/g body weight for each injection.

Polyvinylpyrrolidone (PVP, molecular weight 360,000; Tokyo Kasei, Tokyo) in a concentration of 100 mg/ml sterilized DB (Tris-buffered at pH 7.2) was used for immunization. Toads received 3 injections of the antigens at weekly intervals *via* the dorsal lymph sac, at a dose of 5 µl/g body weight for each injection.

After receiving the antigen, the animals were reared in tap water containing antibiotics at 23°C until they were sacrificed. To collect sera, animals were anesthetized in 1:1,000 MS222, and blood was collected from the exposed ventricle with a micro-pipette. Sera were obtained by removing a clot by centrifugation, followed by the addition of merthiolate (Thimerosal sol.; Maruishi, Osaka) to a concentration of 0.01 % (W/V) for storage at 4°C. Tests on serum antibody activities were conducted using only fresh

sera.

A microhemagglutination technique was used to determine the antibody titers against RRBC (*cf.* Turner and Manning, 1973). Test antigens were obtained from the same rabbit that had provided the immunization antigens. Since the ordinary decomplementation technique of heating sera at 56°C for 30 min was found to reduce the antibody activity by 50-75 %, complement was blocked by adding ethylene diaminetetraacetic acid (EDTA) to a final concentration of 0.03-0.04 *M* (*cf.* Du Pasquier *et al.*, 1972).

Serum antibody against PVP was determined by the passive hemagglutination technique; tanned rabbit erythrocytes coated with PVP (molecular weight, 40,000) were stabilized with 0.4 % gelatine and suspended at a concentration of 1 % in 0.15 *M* NaCl (pH 7.2 with phosphate buffer) containing 0.4 % gelatine and 0.04 *M* EDTA to block the complement.

In both assays, two-fold serial dilutions of the sera were titrated on microtitration trays. Non-immunized toad sera and buffered saline were used as controls.

DETECTION OF IMMUNOGLOBULINS

Electrophoresis

Sera were electrophoresed on a strip of cellulose

acetate membrane (Separax) in veronal buffer (μ=0.07, pH 8.6), for 40 min at 0.8 mA/cm strip width. After the run the strips were stained with Ponceau 3R and destained in 2 % acetic acid. The strips were then dried and placed in liquid paraffin, to be scanned for densitometric tracings by the OZUMOR-82 spectrophotometer (Asuka Mfg. Co. Ltd., Tokyo).

Immunoelectrophoresis

For immunoelectrophoretic analysis of toad sera, antisera were produced in rabbits against *Xenopus* whole serum or *Xenopus* immunoglobulins in Freund's complete adjuvant. The anti-*Xenopus* immunoglobulin sera were obtained by injecting rabbits with RRBC coated with the toad anti-RRBC antibodies. Toad sera were electrophoresed on 1 % agar gel plates (35 × 80 mm) for 60 min at 1.4 mA/cm plate width, in sodium veronal-HCl buffer (μ=0.05, pH 8.6). The electrophoresed sera were then reacted with the rabbit antisera described above. The plates were washed with PBS and deionized water, and stained with Amido Black 10B.

Gel Filtration

Two milliliters of pooled sera were gel-filtrated on Sephadex G-200 column (1.5 × 95 cm) at a flow

rate of 5 ml/hr at 1°C, using 0.1 *M* Tris-HCl buffered 0.15 *M* NaCl as eluant. Each 3 ml fraction was collected, and both its abosorbance at 280 nm and antibody activity were determined.

HISTOLOGICAL AND CYTOCHEMICAL TECHNIQUES

Microspectrophotometry for DNA

Triploid tadpoles with grafted diploid thymus rudiments were sacrificed 7-16 days after the grafting. Grafted thymuses were removed and placed on cover slips and aquashed to smear dissociated lymphocytes. After fixation with a mixture of acetic acid and ethanol (3:7), cells were stained with Feulgen's nuclear reaction to determine the relative quantities of DNA per lymphocyte. One hundred five lymphocytes (less than 13 μm in diameter) were randomly selected from each grafted thymus for determination of light absorbancy at 560 nm, using a microspectrophotometer (Olympus Optical Co., Ltd., Tokyo).

Routine Histological Techniques

Materials were fixed in Bouin's solution, embedded in paraffin by methyl benzoate-paraffin method, serially sectioned at 6-8 μm in thickness, and stained with Delafield's hematoxylin and eosine. Some specimens were stained with PAS method.

For a phase contrast microscopic observation, Carnoy-fixed and paraffin embedded sections were used.

In order to make thin sections for light microscopy, specimens were fixed in 5 % glutaraldehyde in 0.2 *M* cacodylate buffer (pH 7.4), and embedded in glycol-methacrylate (Kushida *et al.*, 1977). They were sectioned at 1-2 μm with glass knives, and stained with toluidine blue.

RESULTS

I. Histological Aspects of the Lymphoid Tissue Development

1. Non-Thymectomized Animals

In an attempt to obtain a morphological basis for the immunocompetence of the non-thymectomized and the thymectomized animals, histological studies were performed on the lymphoid tissues of the intact and the operated larvae and toads. The types of free cells found in the observed tissues were determined according to the criteria given by Fey (1962). In intact animals, the histology of the thymus and spleen confirmed the results obtained in earlier observations by Sterba (1950, 1951), and Manning and Horton (1969). However, the lymphoid accumulations heretofore undescribed by the earlier workers were found in the larval branchial region and beneath the epithelium along the whole length of both larval and adult alimentary tracts.

A preliminary observation showed that the larvae at stage 56 can be taken as representative samples for describing the larval lymphoid organs. Therefore, unless otherwise stated, the following descriptions on larvae will be concerned with the stage 56 larvae. Similarly, most observations on adults were made

by chiefly using the toadlets 3-12 months after metamorphosis.

1.1. Thymus

The fully differentiated thymus is composed of the lymphoid cortex and the essentially non-lymphoid medulla (Figs. 1 and 2), and is encapsulated by a delicate connective tissue layer containing a large number of melanophores (Fig. 3). The cortex is divided into several lobules by trabeculae extending from a collagenous capsule (Fig. 2). The outer part of the cortex, predominantly composed of medium-sized lymphocytes, is more loosely packed than the inner part where small lymphocytes predominate (Figs. 3 and 4). It is in the outer part that the mitotic figures are more frequent (Fig. 3). In the medulla predominate the epithelial cells of various shapes and sizes including the myoid cells, the cells possessing intracellular and intercellular cysts, and those containing either eosinophilic or basophilic granules, and PAS-positive inclusions (Figs. 5-11). These cells are present either separately or forming a multicellular complex (*cf.* Sterba, 1950). A number of blood vessels of various sizes are distributed throughout the thymus. The larger ones are associated with the invading trabeculae which are easily recognizable by accompanying

melanophores (Figs. 2 and 3). Reticular cells are distributed over the whole organ. They are not conspicuous in the Bouin-fixed materials, but are readily distinguished by a phase contrast microscope in the Carnoy-fixed materials (Fig. 12).

The thymic rudiments first appear at stage 40 (2.5 days of age) as an epithelial proliferation of the dorsal surface of the second visceral pouch. The outgrowth of the bud is detached from the branchial epithelium during stage 45, and takes a position between the eye and the ear. By placing the larva at this stage under a stereomicroscope, the thymic rudiment *in situ* can be seen through transmission light (Fig. 13). Free basophilic cells first appear in and around the thymic bud at stage 43 (Fig. 82), and they increase to a definite number at stages 45-46 (Figs. 14, 15 and 76). It should be stressed that virtually no lymphoid tissue is present in the stage 45 larvae. A considerable number of large and medium-sized lymphocytes and intracellular and intercellular cystic spaces are present at stage 48 (Figs. 17 and 78). Encapsulation of thymic rudiments by mesenchymal cells and vascularization of the rudiments also occur at this stage. At stage 49, the cortico-medullary differentiation of the thymus is evident (Fig. 18). Melanophores first appear

in the thymus at stage 48 (Fig. 18). Rapid growth and differentiation of both the cortex and medulla take place in larvae at stage 50, resulting in the appearance of above-mentioned types of epithelial cells in the medulla. By stage 51 the thymus is fully differentiated: From this stage onward, the thymus retains its essential feature as an lymphoid organ throughout their life (see Nagata, 1976), although the size of the organ and the proportion of its component cells vary according to the ages and physiological conditions of the animals as well. During metamorphosis the thymus changes its position to be localized subcutaneously behind the ear. About 1 year after metamorphosis, the cortex as well as the thymus as a whole begin to decrease their size, thus changing the ratio of the cortex to the medulla.

1.2. Spleen

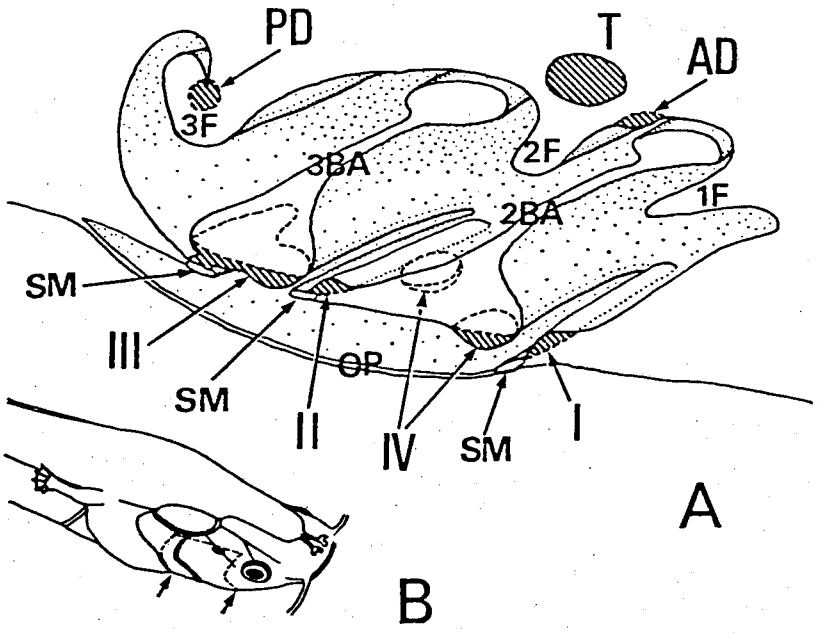
The spleen is a round or ovoid organ attached to the mesentery. It is encapsulated by a thick connective tissue layer without a trabecular invasion (Fig. 20). The organ is composed of the white and the red pulps, each being separated by the reticular boundary cells (Figs. 19 and 21). In the white pulp, the central arteriole is surrounded by the lymphoid aggregation which comprises many small and medium-sized

lymphocytes, as well as paler-staining cells with lobed nuclei and prominent nucleoli (Fig. 21). In the red pulp, there are two sorts of lymphoid aggregations. One aggregation, occurring more frequently and being situated immediately outside the boundary layer, is continuous with the white pulp lymphoid aggregation. The other lymphoid aggregation in the red pulp is present independently from the white pulp follicles (Fig. 22). In the red pulp, the erythrocytic series cells are also found. The melanophores are occasionally seen in the red pulp, but they rarely occur in the white pulp (Fig. 22).

The spleen commences its histogenesis at stage 45 as an accumulation of mesenchymal cells in the mesogastrium (Fig. 23). Both the red pulp and the white pulp of spleen differentiate at stage 48. At stage 50, the spleen acquires its fundamental structure; the red pulp, the white pulp, the boundary layer between the two, and lymphoid accumulation (Fig. 24).

1.3. Larval Gill-Associated Lympho-Epithelial Tissue (LET) Bodies

On gross examination of the Bouin-fixed larval branchial region, lymphoid microorgans were detected as white bodies (Fig. 25). Text-Fig. 3 illustrates the location of whole lympho-epithelial tissue (LET)



Text-Fig. 3. A. Schematic illustration of the right branchial region of a stage 56 larva, which is shown by a dotted line and arrows in B. The locations of 4 groups of ventral cavity bodies (VCBs; I, II, III and IV), anterior (AD) and posterior (PD) dorsal cavity bodies (DCBs) are shown. Gill rakers are not included in A. Anterior to the right. aBA, 3BA, the second and the third branchial arch; 1F, 2F, 3F, the irst, the second, and the third dorso-pharyngeal fold; OP, operculum; SM, subarcual muscle; T, thymus. A, ca. × 50. B, ca. × 2.

bodies found in the branchial region. For convenience' sake, two parts of these LET bodies are designated as "ventral cavity bodies (VCBs)" and "dorsal cavity bodies (DCBs)", respectively, according to their location.

VCBs are 4 pairs of grouped lymphoid microorgans situated in the ventral region of the branchial chamber near the opening to the opercular chamber (Text-Fig. 3 and Fig. 25). Of these, the first to the third bodies lie at the sites described by Manning and Horton (1969). Each of the first to the third VCBs either consists of 2-3 smaller sub-bodies or fused to form one larger body. Occasionally, the third pair extends caudally to the subarcual muscle, which is situated at the opening of the third branchial chamber. Besides these 3 pairs, the fourth VCBs were consistently found at the base of the second branchial arch. These bodies differ from the above-mentioned ones in that they appear at separate positions and never fuse into one, although sometimes each separate body has 2-3 sub-bodies.

Other lymphoid microorgans found in the branchial region are 2 pairs of DCBs, which occur on the ceiling of the branchial chamber (Text-Fig. 3); the anteriorly located ones (anterior DCBs) lie anterior to the level of the thymus on each side,

whereas the posterior DCBs lie in the depth of the hollows formed by the third dorsal pharyngeal folds. Anterior DCBs, not consistently found in all larvae, are likely to correspond to the "extra-thymic lymphocytes" of Sterba (1950) and Manning and Horton (1969). The posterior DCBs were present in all the larvae examined.

Histologically, both VCBs and DCBs were characterized by a densely packed lymphoid accumulation immediately beneath the epithelium (Figs. 26-30). Although histological features of the epithelium were different in these bodies, all the epithelia covering the lymphoid accumulations were always infiltrated heavily by lymphoid cells, forming a "dome epithelium" (Fichtelius *et al.*, 1968). At the base of such "dome epithelium", no basement membrane was visible (Fig. 26). Basal to the lymphoid mass neither particular capsules nor sacs were present, but blood vessels were always seen (Fig. 27). The lymphoid accunulations in these LET bodies were predominantly composed of small and medium-sized lymphocytes, and other leukocyte series cells and macrophages were only occasionally seen. Occurrence of a particular "giant inclusion" which possesses large and small particles stainable with hematoxylin (Figs. 29 and 30) was another feature seen in these LET bodies. It was spherical in shape, measuring

more than 30 μm in diameter, and its unusually large size and apparent lack of nucleus suggest that this inclusion is non-cullular in nature, possibly some fused body of disintegrated cells, although it looks like a macrophage.

1.4. LET Bodies along the Alimentary Tract

Gross examination of the isolated larval alimentary tract revealed no indication of lymphoid accumulations. Histological examinations, however, revealed numerous lymphoid accumulations in several portions along the whole length of the alimentary tract, from the pharyngo-esophagus to the rectum. These lymphoid accumulations were always present in close relationship with the epithelium. The number of the LET bodies distributed along the tract was fairly variable, ranging from 30 to 50 depending on the larvae. In an extreme case, as many as 66 such bodies were counted in one specimen.

The alimentary tract of the larva is long and coiled, making it difficult to specify the exact locations of its LET bodies in the histological sections. Most of the gut-associated LET bodies do not seem to occupy definite sites, except for a pair of LET bodies which occur consistently on the ventral side of the tract between the pharynx and the esophagus (Fig. 31). Besides those just beneath the gastric

epithelium, lymphoid aggregations were occasionally seen among the gastric glands in the stomach (Fig. 32). Since gastric gland cells are also epithelial in nature, these accumulations may well be included in the LET bodies.

The degree of lymphoid invasion into the epithelium varies among gut-associated LET bodies even in the same individual. Thus, in one pair of the bodies in the pharyngo-esophageal region and some in the ileum, the lymphoid invasion was so extensive that they formed the "dome epithelium" consisted of extremely deformed epithelial cells (Figs. 31 and 48A). In other gut-associated bodies, the lymphoid invasion was less extensive so that epithelial cell showed no changes in shape (Figs. 33-35). The gut-associated LET bodies are predominated by small and medium-sized lymphocytes. Usually the basement membrane was not discernible and the blood capillaries were always associated with the assemblage of lymphocytes.

Metamorphosed animals were found to possess a number of separate LET bodies of relatively large size along the whole length of the alimentary tract. In particular, the lymphoid accumulations located in the esophagus, stomach and ileum (Fig. 49B) were very large in size. In contrast with that in the larva, a "dome epithelium" with a heavy lymphoid

infiltration was confined to a pair of esophageal bodies in metamorphosed animals.

The small lymphoid aggregations closely associated with the gut epithelium make their first appearance at stage 48, and they grow to definite LET bodies by stage 49 (Fig. 36). During succeeding stages, the gut-associated LET bodies increase in number and size as the elongation and differentiation of the alimentary tract proceed, attaining their maximal size in larval life at stages 55-57.

1.5. Lymphoid Aggregations in Other Organs

In the larval and adult kidney (mesonephros), small and medium-sized lymphocytes make several aggregations among the renal tubules (Fig. 39). Under the ventro-medial wall of the kidney, there is a myeloid tissue which is composed of the predominant neutrophilic granulocytes having polymorphic nuclei (Figs. 37 and 38). In the pronephros of the larva, however, no lymphoid cells could be detected.

Both the larval and adult livers possess accumulations of lymphocytes and granulocytes in their subcapsular regions (Figs. 40 and 41). Cellular composition of this lympho-myeloid tissue includes the predominant neutrophilic granulocytes and other types of leukocytic series cells (Fig. 40). Small

lymphocytes are occasionally seen to form aggregations in the subcapsular region and around the central vein as well of the liver (Figs. 41 and 42).

The bone marrow is absent in larvae. Lymphoid tissues are rarely observable in the adult bone marrow.

2. Thymectomized Animals

Thymectomy in stage 45 larvae did not affect the growth rate of the animals, and they completed metamorphosis normally (Fig. 43). Since incomplete extirpation of the thymus at this stage always resulted in regeneration of the organ prior to stages 56-58, all the successful operation as shown in Table 1 was based on the observation of the animals at stages 56-58. The mortality of thymectomized as well as of sham-operated animals was relatively low, although it was fairly variable according to the batches used. It was found that thymectomy at stage 51 was highly successful and resulted in virtually not a single failure of the operation (Table 1).

It should be mentioned, however, that the early-thymectomized *toadlets* that had previously been thymectomized when they were stage 45 larvae could be affected easily by infectious diseases. Therefore, addition of antibiotics to the rearing water was

Table 1. Percentages of successful thymectomy in stage 45 (4-day-old) and stage 51 (18-day-old) *Xenopus* larvae as judged by examining them at stages 56-58. In parentheses are given the percentages of either survived or successfully operated.

Experiment	Stage at Thymectomy	Number of Animals		
		Operated on	Survived	Thymusless
1	45	30	15(50)	9(30)
2	45	40	34(85)	19(48)
3	45	65	49(75)	24(35)
4	45	53	26(49)	18(34)
5	51	40	40(100)	40(100)

necessary to keep the thymusless animals healthy.

Thymectomy of young larvae (stage 45) produced different degrees of effects on the lymphoid tissues depending on the organs and ages of animals, as follows.

2.1. Spleen

During the larval stages, the spleen increases in size and branching of the white pulp proceeds. Because of the relative immaturity and the paucity of lymphoid population of the spleen in the unoperated larvae, the effect of thymectomy on the larval spleen was not clearly discernible.

In the toads, the size of the spleen was determined by measuring the diameters of the organs at their broadest portion in histological sections (Table 2). As is indicated in the Table, overall size of the spleen varied greatly even among the non-immunized intact animals, apparently reflecting the variability in myelopoietic and lymphopoietic activities of this organ. A comparison of the values between thymectomized and non-thymectomized toads, both before and after the antigenic challenge, revealed that neither the early-thymectomy nor the antigenic stimulations had a significant effect on size of the spleen.

However, the effect of theymectomy was evident

Table 2. The sizes (mm) of spleens in thymectomized and non-thymectomized control toads, both before and after injection of antigens. RRBC, rabbit red blood cells; PVP, polyvinylpyrrolidone.

Antigen	Non-thymectomized toads		Early-thymectomized toads	
	No. of animals	MEAN±S.D. (Range of values)	No. of animals	MEAN±S.D. (Range of values)
None (control)	10	1.95±0.42 (1.31−2.93)	7	1.81±0.43 (1.29−2.71)
RRBC	10	2.22±0.59 (1.62−3.32)	8	1.97±0.50 (1.55−2.81)
PVP	9	1.89±0.45 (1.33−2.86)	6	1.67±0.20 (1.27−1.91)

Student t-test revealed that there is no significant difference between these values.

in the histological features of the toads' spleens. In the thymectomized toads, the depletion of small lymphocytes was significant in the white pulp, leading to a reduction of the proportion of the white pulp to the red pulp areas in sections. It should be noted that a certain amount of small lymphocytes were still present in the white pulp. In the red pulp of thymusless toads, the depletion of lymphocytes was severer than in the white pulp so that lymphocytic aggregations were hardly observable. The effect of thymectomy was more pronounced when spleens of the early-thymectomized toads were compared with those of the intact controls after they had received antigens (Figs. 44 and 45). Histology of the spleen in the antigen-stimulated animals will be described later in detail.

2.2. Larval Gill-Associated LET Bodies

Of the 4 pairs of VCBs and 2 pairs of DCBs in the larval branchial region, the anterior DCBs suffered most severely by thymectomy with the animals possessing, in fact, none of these bodies. The effect was also evident in VCBs (Fig. 46) and the posterior DCBs (Fig. 47), although small lymphoid aggregations were occasionally discernible, particularly in the latter.

2.3. LET Bodies along the Alimentary Tract

The larval gut-associated LET bodies were severely affected by thymectomy, consistently decreasing both in number and size, with their lymphoid invasion into the gut epithelium greatly reduced (Fig. 48). In contrast to the situation in the larvae, the LET bodies along the alimentary tract in the thymectomized toads were not very different both in their number and histological appearance from those in the non-thymectomized controls (Fig. 49).

2.4. Lymphoid Aggregations in Other Organs

Lymphoid aggregations in the kidney and liver of both larvae and adults were fairly variable in number and size, even among non-thymectomized control animals. Nevertheless, it was evident that in the thymectomized animals the aggregations were usually reduced. As was shown in the observation by Horton and Manning (1974b), bone marrow in thymectomized toads developed in a quite normal fashion.

2.5. Notes on a Disease Associated with Thymectomy

Besides the above-mentioned lymphoid aplasia, some comments should be given of the histological

aspects of the thymusless toads which died of a particular syndrome. Lesions in the liver and lung were evident in these toads at autopsy. In the liver, areas of focal necrosis with conspicuous melanin granules and basophilic debris were found, together with the invasion of many monocytic cells (Figs. 50-52). The lesions found in the liver are thus similar to those observed in the "wasting" *Alytes* (Du Pasquier, 1968) and *Pleurodeles* (Charlemagne, 1974) as a results of thymectomy. Besides the liver, the alveoli of the attacked area of the lung were filled with blood including a large number of monocytic cells (Figs. 53-56). Quite unexpectedly, lymphoid aggregations were observed in the septa of necrotic lungs (Fig. 55). Another characteristic of this syndrome was an increase in the volume of the spleen; this was caused primarily by an increase of the red pulp area which contained an extraordinarily large number of monocytes as well as the cells of the erythrocytic series (Fig. 57). This syndrome, although occurring not so frequently as in thymusless mammals (Azar, 1964), may be taken as an indication of the failure in immunological activity of our thymectomized toads against microorganisms.

II. Role of Thymus in Establishment of Immunity

1. Immune Responses in Relation to Thymectomy

1.1. Response to Skin Allografts

Non-Thymectomized Toads

When the non-thymectomized G group toads received skin allografts from HD group toads, all the grafts were rejected. With respect to the events of rejection, the following 4 phases were recognized on the basis of both external and histological examinations.

Phase I

In 15 hr after grafting, the grafted skin adheres firmly to the graft bed. By the next day of the operation, a narrow space of exposed bed between the contracted edges of both graft and host skins is covered by the epidermis. The graft looks paler than the surrounding host skin, because melanin in the melanophores of the graft is usually aggregated. At the end of this phase, a dilation of the blood capillaries is evident for 1-2 days in the grafted skin. This leads to the re-establishment of the vascularization in the next phase. Histologically, the area between the graft and the host skin is completely covered with the expanding epidermis (Fig. 58). There is no indication of the histological change in the graft (Fig. 60). Toward the end of this phase, leukocytes in and around the graft become phagocytic in appearance

(Figs. 59 and 61).

Phase II

Restoration of the blood flow in the graft makes it indistinguishable from the surrounding host skin, although the scars along the boundary between the two persist evidently. A marked vasodilation in the allograft and the host skin is the first sign of the histoincompatibility occurring toward the end of this phase, which leads to the hemostasis in the next phase. At the end of this phase, lymphocyte aggregations are seen in and around the dilated blood capillaries of both the graft and the host tissues (Fig. 62). Both melanophores and the skin glands of the graft look undamaged. A massive connective tissue layer is formed under the graft.

Phase III

Following the hemostasis, the gross appearance of the graft remains unaltered while it continues to protrude outwards. Histologically, an invasion of lymphocytes is highly extensive, particularly into the stratum spongiosum and the massive connective tissue layer underlying the graft (Fig. 63).

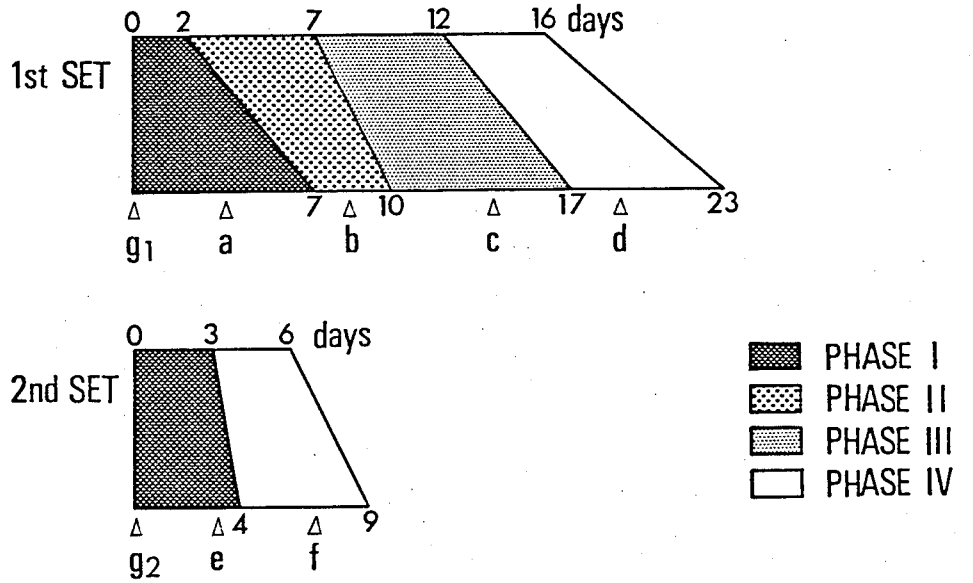
Phase IV

The destruction of capillary walls, pigment cells, and skin glands commences almost simultaneously at the graft's edges, then proceeds toward the center

of the graft, making its periphery progressively duller and opaque centripetally in appearance. During the process of this destruction, secondary vascularization becomes apparent, presumably due to the invasion of the host capillaries into degenerating graft area. Histological sections of the allograft clearly demonstrate that the tissue of the graft are under destruction. The lymphocytic invasion is most extensive at the beginning of this phase; it looks as if the whole grafted area is occupied by the lymphocytes (Figs. 64 and 65). Near the end of this phase, aggregated lymphocytes begin to decrease in number (Fig. 66), and remarkable is the accumulation of a large number of phagocytes which engulf conspicuous melanin granules and other amorphous cellular debris (Fig. 67).

Based on these observations the depigmentation over the entire graft was designated for convenience' sake as the termination or the "end point" of the graft rejection. As illustrated in Text-Fig. 4, the median survival time (MST) for the first-set graft was 18.9 ± 1.4 days.

After the "end point" of the allograft rejection, the whole graft including its stratum compactum undergoes more destruction (Fig. 68). Although macrophages are still encountered in and around the



Text-Fig. 4. Diagrammatic presentation of the four phases of first-set and second-set allograft rejections in 3-11-month-old toads. The range of duration of each phase is based on the observation in which the grafts from the HD group toads were placed on seventeen G group toads. a-f indicate the average time in days of the terminals of each phase; a, 3.6 ± 0.8; b, 8.2 ± 0.8; c, 14.0 ± 1.1; d, 18.9 ± 1.4; e, 3.2 ± 0.3; f, 6.9 ± 1.0 (mean ± S.D.) days; g1, first-set grafting; g2, second-set grafting. For details, see text.

remnant of the graft (Fig. 69), no lymphocytic aggregation is found in the 30th post-operative day graft (Fig. 70).

All the second-set allografts were rejected in much more acute fashion, giving an MST of 6.9 ± 1.0 days (Text-Fig. 4). The blood circulation was never restored in these grafts. Thus, the process of rejection was divided into 2 phases, corresponding to the phases I and IV of the first-set graft rejection. Histologically, the lymphocyte invasion was highly extensive when it was first detectable in 2-3 post-operative days, as contrasted with the case of first-set graft where the first lymphoid invasion occurs on about the 8th post-operative day. Other histological aspects of the graft rejection were essentially similar to those described for the phases I and IV of the first-set graft rejection.

Thymectomized Toads

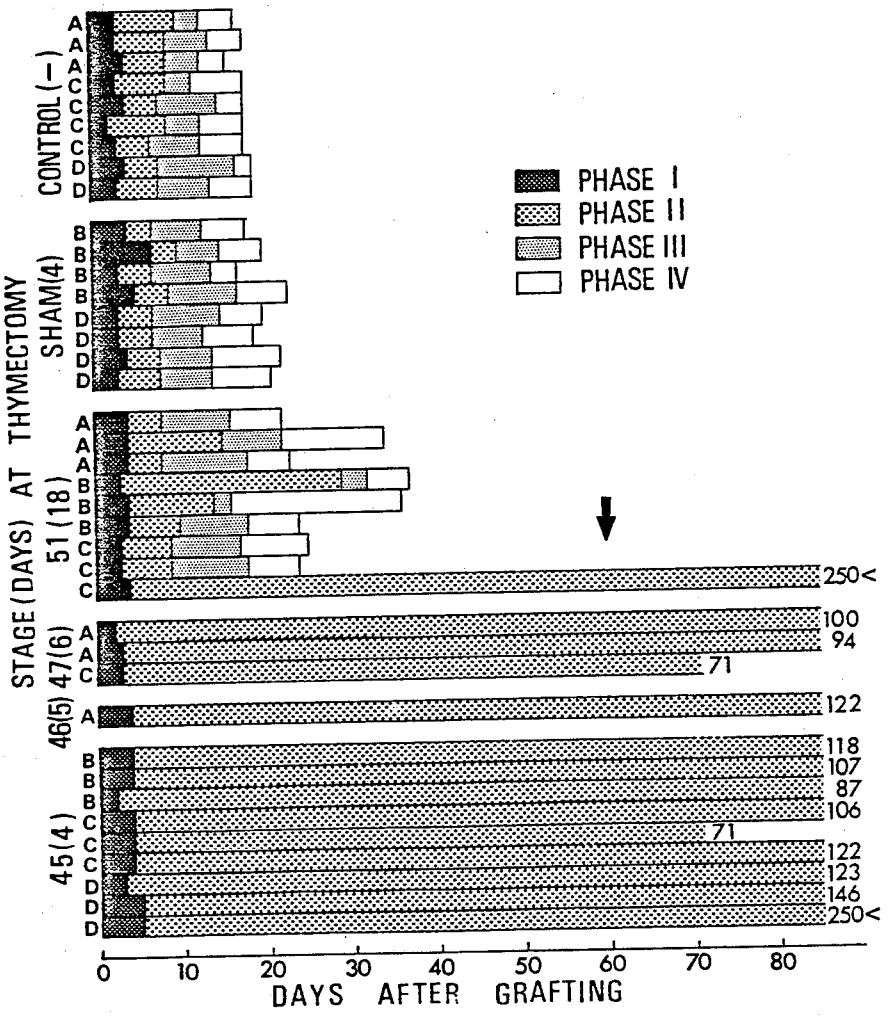
The thymectomized, the sham-thymectomized, and the unoperated G group sibling toads received skin allografts from HD donors. Autopsy and histological inspection at the end of the experiment confirmed the absence of thymus in all the thymectomized animals. As summarized in Text-Fig. 5, all the 9 unoperated and the 8 sham-operated toads rejected grafts in a

fairly constant fashion, with MSTs being 17.9 ± 0.9 days and 20.0 ± 1.9 days, respectively.

By contrast, all 13 toads thymectomized at stages 45-47 (4-6 days after fertilization) retained the grafts as long as the toads survived, without showing incompatibility. It was noticed that transfer of the thymectomized-and-allografted toads to ordinary (antibiotics-less) rearing water (arrow in Text-Fig. 5) caused a severe effect on their survival. Thus, 12 out of 13 toads thymectomized at stages 45-47 were dead within 146 post-operative days. Necropsy and histological observations on these toads showed readily appreciable lesions and monocytic infiltrations occurring preferentially in their livers and lungs. The grafts, however, looked viable at the moment of death (Figs. 71 and 72B, *cf.* Figs 70 and 72A).

The fate of allografts in the toads that had been thymectomized at stage 51 (18 days of age) was variable; 5 out of 9 toads rejected the grafts in a way comparable to that of intact or sham-operated controls, but rejection was delayed in 3 toads, and in a toad the viable graft remained attached for more than 250 days.

It is clear from these results that thymectomy of the larvae at stages 45-47 induced a complete abrogation of the rejection capacity to allografted



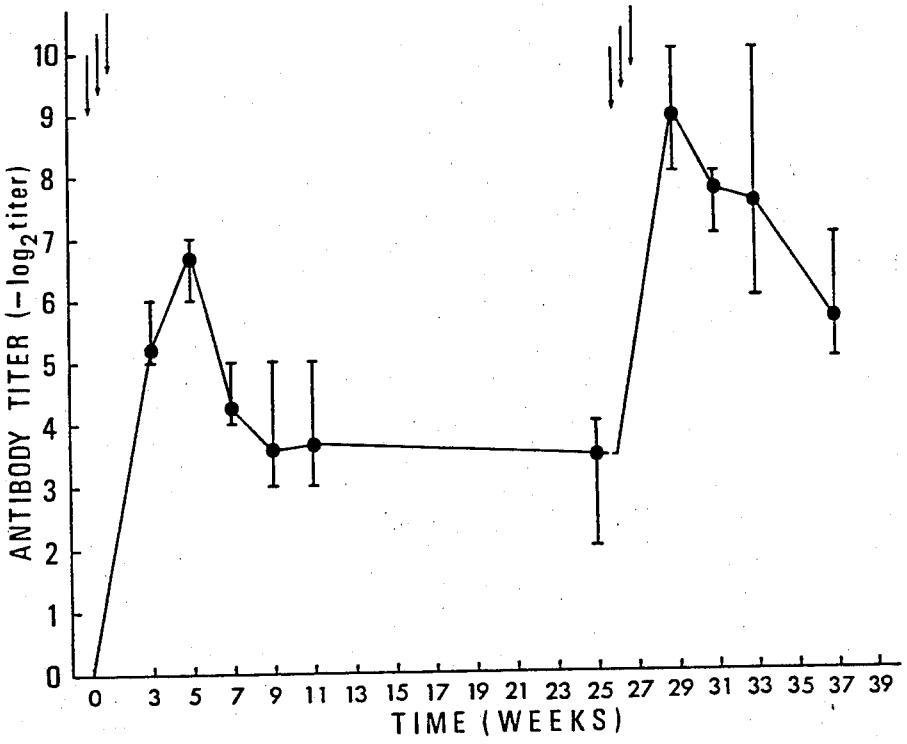
Text-Fig. 5. Thymectomy in G group larvae (stages 45-51) resulted in various degrees of rejection of allografts supplied by four HD group toads (A-D) after the former metamorphosed. The animals (3-12-month old) spent 60 days in the water (arrow) containing antibiotics after receiving grafts, and then transferred to tap water. Numbers to right of some bars indicate termination of the observations due to animals' death. For details of the phases of the graft rejection process (phases I-IV), see text II. 1.1.

skins. The results may also indicate that the stages 47-51 are the critical period with respect to the maturity of the transplantation immunity of these animals.

1.2. Response to Rabbit Red Blood Cells (RRBC)

Preliminary tests showed that the sera from 2- to 3-year-old toads that have not been immunized occasionally possess antibodies against rabbit red blood cells (RRBC) in a titer of up to 1:16, indicating the occurrence of natural antibodies. This situation is similar to that of *Alytes* (Du Pasquier, 1973), in which natural antibodies of similar titers against sheep and human red blood cells have been recorded. *Xenopus* 3-12 months after metamorphosis, however, did not possess naturally occurring antibodies in this respect so far as examined here.

Text-Fig. 6 depicts the kinetics of antibody production in the toads which received 3 successive injections of RRBC. It is apparent that the antibody titer attains its highest level (up to 1:128) 5 weeks after the first antigen injection, and retains a certain level for several weeks. It is also shown that the toads respond more quickly to the secondary injection series than to the primary ones, giving rise to a titer up to 1:1,024 in 3 weeks.



Text-Fig. 6. Kinetics of antibody production against rabbit red blood cells (RRBC) in the toads, as determined by microhemagglutination technique. Arrows indicate injections of antigen. Each point indicates the mean titer of antibody in sera collected from 3-4 toads. Vertical bars indicate range of titers. Antibody titer ($-\log_2$ titer), antibody titer expressed as $-\log_2$.

To determine the role of the thymus in the humoral immune responsiveness, the early-thymectomized, the sham-operated, and the unoperated siblings, each 3-12 months after metamorphosis, were given RRBC according to the schedule presented in Text-Fig. 6. Toads were sacrificed to provide sera for antibody titrations 5 weeks after the first antigen injection. The results are summarized in Table 3. The toads thymectomized at stage 51 produced antibodies of a titer similar to that of the control animals, although 3 out of 10 showed lower titers.

The effect of thymectomy at stages 45-46, however, was significant; except for one which showed a titer of 1:16, all the thymusless toads failed to show any antibody response. Similarly, those thymectomized at stage 47 gave a very low, almost undetectable, titers. Autopsy and histological examinations indicated that there was no thymic tissue in these thymectomized toads. It should be added that the 4 toads thymectomized at stages 45-46 were dead before bleeding for antibody assay.

These results thus indicate that, like in the case of allograft rejection, the humoral response of the toads against RRBC is dependent on the thymus of early larval stages. The importance of the larval stages 45-51 with respect to the acquisition of

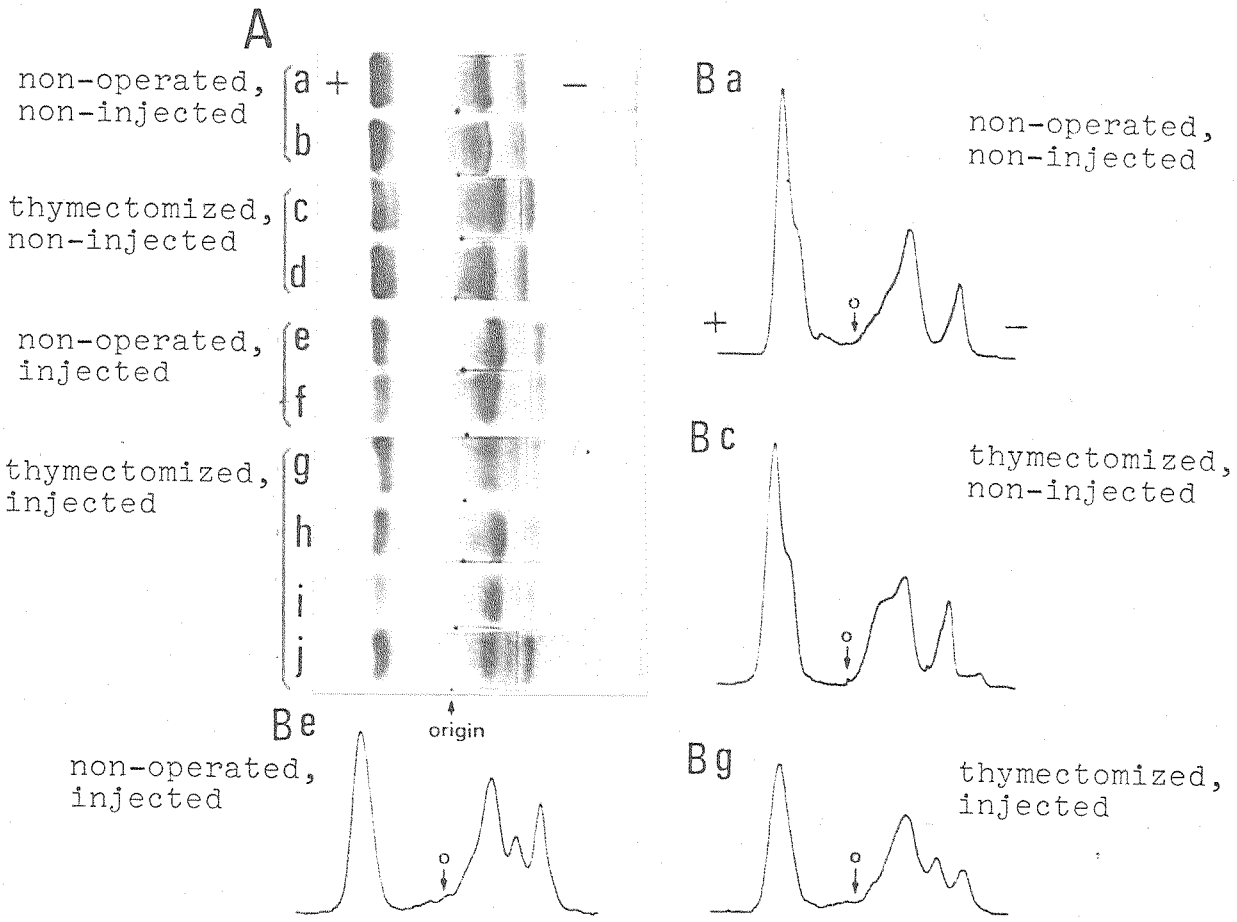
Table 3. Effect of larval thymectomy on production of antibodies against RRBC in adult toads, as determined by microhemagglutination technique. Determinations were made 28 days after the third administration of antigen. Antibody titer ($-\log_2$), antibody titer expressed as $-\log_2$.

Stage (days) at thymectomy	Number of animals examined	Number of animals									
		Antibody titer ($-\log_2$)									
		0	1	2	3	4	5	6	7	8	9
45 (4)	8	7				1					
46 (5)	3	3									
47 (6)	2		1	1							
47 (7)	2			1	1						
51 (18)	10			1		1		1	7		
45 (4) (sham-operated)	5								4	1	
unoperated	10								2	5	3
unoperated (non-immunized)	5	5									

immune responsiveness is again stressed.

Electrophoresis on cellulose acetate membrane of the sera from unoperated and non-immunized taods showed 3-4 major peaks, as described by Hadji-Azimi (1969) and Yamaguchi *et al.* (1973). Except for the most cathodally or anodally migrating bands, the intermediate bands varied in their relative levels according to the serum samples (Text-Fig. 7). The most anodally migrating peak represents albumin, while the most cathodally migrating one evidently represents immunoglobulins because of its increase upon immunization with RRBC as well as its hemagglutinating activity against the same antigen. A comparison of the ratio of immunoglobulins to albumin in the sera of the non-immunized and the immunized toads is given in Table 4, showing a significantly higher value in the latter.

The early-thymectomy did not have a significant effect on the electrophoretic pattern of the serum components. It should be noted that the sera from early-thymectomized toads consistently possessed a certain level of immunoglobulins comparable to that found in immunized controls, although the ratio of immunoglobulins to albumin did not increase after the RRBC administrations (Table 4). The occurrence of



Text-Fig. 7. A. Electrophoretic patterns on cellulose acetate membrane, of the sera from thymectomized and non-thymectomized toads, with or without administration of RRBC. a and b, non-operated-and-non-injected; c and d, early-thymectomized-and-non-injected; e and f, non-operated-and-RRBC-injected; g, h, i, and j, early-thymectomized-and-RRBC-injected. Sera were electrophoresed after antibody titers were determined. B. Densitometric patterns of some electrophoresed strips shwon in A.

Table 4. Comparison of the ratio of the levels of immunoglobulins to albumin in sera from early-thymectomized and non-operated toads, before and after administration of RRBC. Densitometric determinations were made after electrophoresis on cellulose acetate membrane. See Text-Fig. 7.

Sera from	Number of animals examined	Immunoglobulin/Albumin (mean ± S.D.)
unoperated uninjected toads	10	0.30 ± 0.10*
unoperated injected toads	11	0.43 ± 0.06*
thymectomized uninjected toads	4	0.42 ± 0.06
thymectomized injected toads	13	0.39 ± 0.10

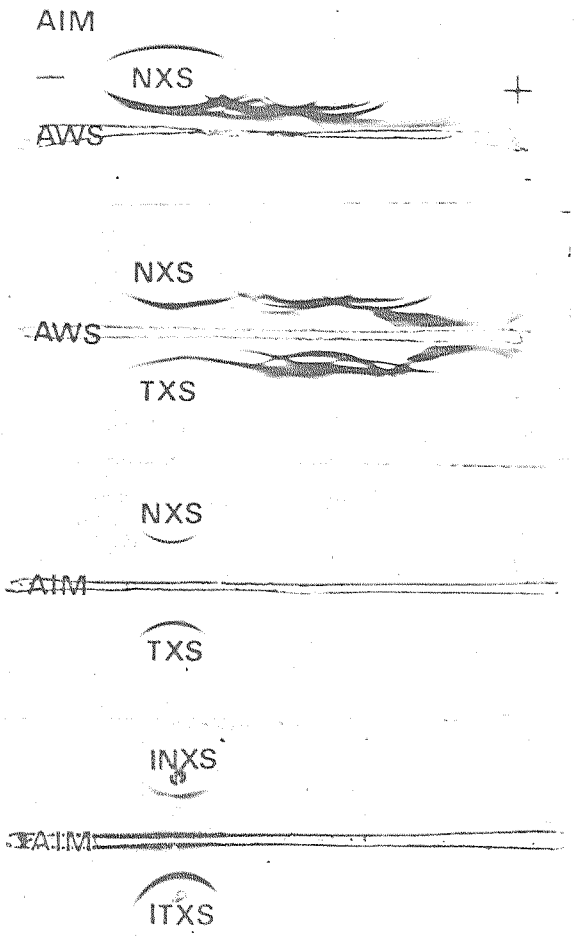
* STUDENT's t-test reveals that the significance of difference at 95% level is valid only between these values.

immunoglobulins in the early-thymectomized toads was further confirmed by immunoelectrophoretic analysis, in which the sera from the thymusless and the intact toads reacted to rabbit antisera against *Xenopus* immunoglobulins (Text-Fig. 8).

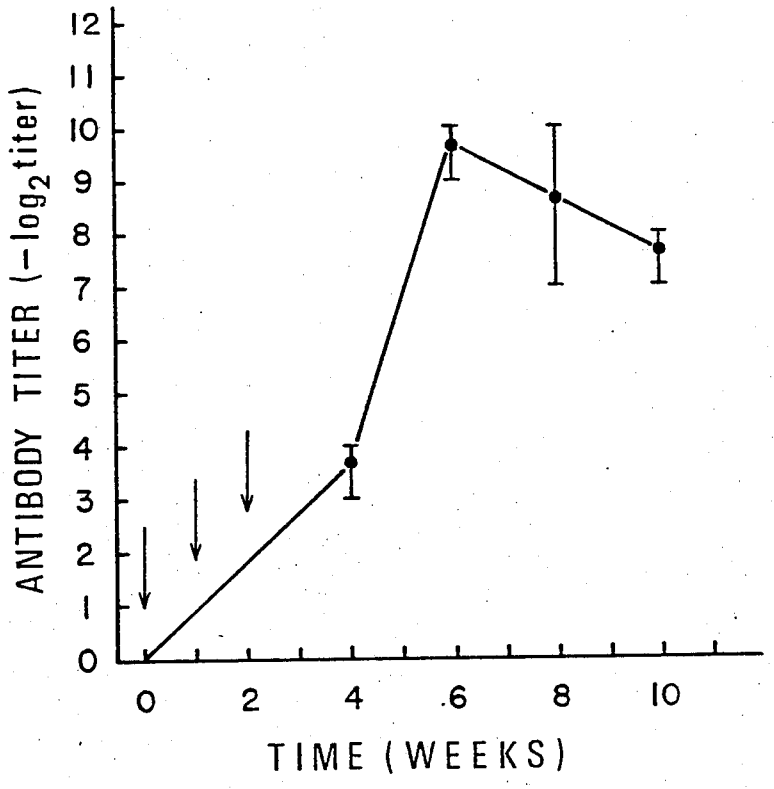
Histological examinations of the spleen showed that, after receiving RRBC, the lymphocytic population increased remarkably in both red and white pulps of unoperated control toads (Figs. 44A and 45A). As stated previously (I.2.1), the lymphocytes in splenic red and white pulps of thymectomized toads were extremely poor in number. Even after RRBC injections, these toads showed virtually no change in red and white pulps (Figs. 44B and 45B). The results provide a histological support for the results that the early-thymectomized toads failed to produce specific antibodies against RRBC.

1.3. Response to Polyvinylpyrrolidone (PVP)

When non-thymectomized toads received polyvinylpyrrolidone (PVP), they produced the antibody against it in the manner illustrated in Text-Fig. 9. The antibody titer attained its highest level 4 weeks after the last antigen injection, then decreased gradually. On the basis of the results given in



Text-Fig. 8. Immunoelectrophoretic patterns of the sera from the non-operated toads and the toads thymectomized at larval stage 45, each with or without RRBC administration. Stained with amido-black. NXS, serum from a non-operated toad; INTX, serum from a non-operated toad injected with RRBC; TXS, serum from an early-thymectomized toad; ITXS, serum from an early-thymectomized toad injected with RRBC; AWS, rabbit antiserum against toad whole serum; AIM, rabbit antiserum against toad immuno-globulins.



Text-Fig. 9. Kinetics of antibody production in toads against polyvinylpyrrolidone (PVP). Arrows indicate the days of antigen injection. Each point shows the mean titer of the sera from three to four toads. Vertical bars indicate the range of titers.

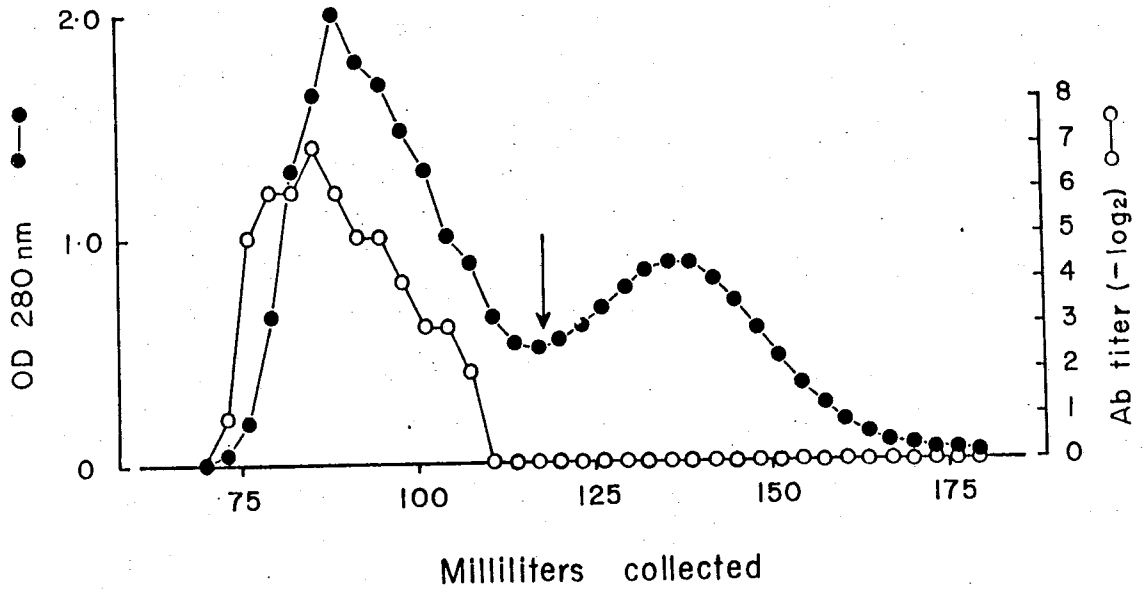
Text-Fig. 9, the effect of thymectomy was studied by giving PVP into the early-thymectomized and the non-thymectomized siblings, and by bleeding them 6 weeks after the first antigen injection.

The results of antibody titrations (Table 5) clearly showed that the thymectomized toads produce the same titers of antibodies as the control animals do. None of the non-injected toads tested possessed any antibodies against PVP. The autopsy and histological examination revealed no thymic tissue left in any of the thymectomized toads.

In an attempt to characterize the antibody produced against PVP, pooled sera from the PVP-injected animals were gel-filtrated on Sephadex G-200. The antibody activities were found only in the break-through fractions (Text-Fig. 10), indicating that the produced antibodies consisted exclusively of 19S Immunoglobulins (Hadji-Azimi, 1975). Treatment of the gel-filtrated sera with 2-mercaptoethanol (0.1 *M* at 37°C for 1 hr) destroyed antibody activities completely, whereas heating the sera at 56°C for 30 min did not affect them (*cf.* Yamaguchi *et al.*, 1973). These properties of the antibodies were true for antisera from both thymectomized and unoperated toads.

Table 5. Antibody titers against polyvinyl-pyrrolidone (PVP) in sera from thymectomized and control toads.

Sera from	Number of animals examined	Number of animals											
		Antibody titer ($-\log_2$)											
		0	1	2	3	4	5	6	7	8	9	10	11
thymectomized injected toads	6									2	2	2	
unoperated injected toads	7										5	2	
unoperated uninjected toads	5	5											



Text-Fig. 10. Sephadex G-200 fractionation of sera from toads immunized with PVP. The arrow indicates the peak of elution of human IgG under the same conditions. OD 280 nm, optical density at 280 nm; Ab titer ($-\log_2$), antibody titer expressed as $-\log_2$.

Histological examination of the spleen of thymectomized-and-PVP-injected toads revealed the enlargement of the white pulp in association with the lymphocyte accumulation, to a degree similar to that found in non-thymectomized counterparts. In the splenic red pulp of thymectomized toads, however, lymphoid aggregations were hardly observable, in contrast with those highly developed ones in the non-thymectomized-and-PVP-injected ones (Fig. 73).

2. Restoration of Immune Reactivities in Thymectomized Toads

2.1. Restoration by Adult Thymus Implants

The 12-18 months old G group toads which had been thymectomized at stages 45-46 received implantation of a pair of thymuses from histocompatible toads. Despite the use of antibiotics, 9 out of total 31 thymusless toads receiving thymus implants were dead within 4 weeks of the operation. Necropsy of these animals (6/9) showed the histological characteristics which can be referred to as the "wasting syndrome" described before (I.2.5.). The possibility of the graft-versus-host reaction as a cause of the mortality of host animals may be well ruled out on the basis of the histocompatibility

of donors and hosts as evidenced in non-thymectomized toads, as well as the normal histological features of the grafted thymuses and their surrounding host tissues. Thus, the high mortality of thymusless toads is most likely ascribable to their high susceptibility to microorganisms which invade them during procedure of grafting.

Response to Skin Grafts

Four weeks after the thymus grafting, the 11 toads received skin allografts from HD donors. The control toads thymectomized at stage 45 were unable to reject allografted skins until the termination of the experiment (more than 250 days after the grafting), without any indication of incompatibility. Other intact control rejected skin allografts within 18-19 days (Table 6).

It was found that the thymus implants induced a remarkable restoration of immunoreactivity in the thymectomized toads. Thus, 10 out of 11 thymectomized- and thymus-implanted toads eventually rejected allografts from third party donors after variable duration of their survival, 25-66 days (table 6). Sixty-eight days after the first-set skin grafting, 10 toads which had rejected the skins received the second-set skin grafting from the first-set donors. As shown in

Table 6. Restoration of skin allograft rejection capacity in the toads thymectomized at larval stage 45 after implantation of adult thymuses.

Recipient Type	No.	Skin donor	Survival time (days) 1st-set graft	2nd-set graft
4-day-thymectomized, thymus-implanted	1	A	40	ND[a]
	2	A	36	16[b]
	3	A	25	17[b]
	4	B	66	16
	5	B	47	18
	6	B	44	27
	7	B	>100	>35[c]
5-day-thymectomized, thymus-implanted	1	C	39	16
	2	C	30	16
	3	C	29	22
	4	C	29	15
4-day-thymectomized (control)	1	D	>100	ND
	2	D	>100	ND
	3	D	>100	ND
	4	D	>100	ND
Nonthymectomized (control)	1	E	18	6[d]
	2	E	18	8[d]
	3	E	18	9[d]
	4	E	19	8[d]
	5	E	19	7[d]

a) ND, not done.
b) Grafts were transferred from a sibling of the first-set graft donor. Apart from these two cases, the second-set grafts were always taken from the first-set donors.
c) Intact at time of killing (35 days after the second-set grafting).
d) Second-set grafts were given 25 days after first-set grafting.

Table 6, all the second-set grafts were rejected in an apparently accelerated fashion (15-27 days). An exceptional thymectomized-and-thymus-implanted toad, which retained viable skin allografts (first and second) for over 100 days (for the first-set graft), was found to have a healthy implanted thymus at autopsy. The sections of the grafted skins from this particular individual revealed small aggregations of lymphocytes around the dilated blood vessels (Fig. 74), suggesting that the failure of the animal to combat the grafted skins was due to the paucity of the lymphocytes.

Response to RRBC

Four weeks after thymus grafting, the 11 early-thymectomized toads received 3 sucessive injections of RRBC, according to the immunization schedule described before. The results presented in Table 7 clearly show that the reactivity to the injected RRBC was restored by implanting adult thymuses. As mentioned previously, non-immunized toads occasionally possessed "natural" antibody against RRBC in a titer of 1:16, at most. The thymectomized-and-thymus-implanted toads produced an apparently higher titer of antibody. Although the titers obtained tended to be lower than those in non-thymectomized controls,

Table 7. Restoration of antibody producing capacity against RRBC in toads previously thymectomized at larval stage 45 after implantation of adult thymuses.

Animals	No. of animals	Number of animals Antibody titer ($-\log_2$)									
		0	1	2	3	4	5	6	7	8	9
4-day-thymectomized, thymus-implanted	4				1			3			
5-day-thymectomized, thymus-implanted	7					1	2	2	2		
4-day-thymectomized (control)	8	7				1					
Nonthymectomized (control)	10								2	5	3

the difference between the thymus-implanted and the non-thymectomized toads was statistically insignificant.

Histological Observations

Histological studies showed that there was no thymic remnant in the "thymic" region of any thymectomized toads, while the implanted thymuses were found in the area between the dermis and the muscle layer. These histologically healthy implants were seen highly vascularized by host blood vessels (Fig. 75). In the animals with implanted thymus, lymphocyte aggregations were evident both in the red and the white pulps of the spleen, in contrast with the situation in thymectomized animals (see I.2.1.).

2.2. Restoration by Lymphocyte Suspensions

The immunity-restorative capacity of implanted thymuses, described in the above section, may be attributed either to the thymic lymphocytes or to the humoral factors emitted by reticulo-epithelial cells, both from the grafted organs. In an attempt to delineate more precisely the factor(s) involved in the immunity-restorative activity of the thymus, isolated thymic lymphocytes were injected into early-thymectomized toads and their immune

responsiveness was studied. Along with this experiments, effects of injected splenic and peripheral blood lymphocytes on toads were also studied.

Two thymectomized toads that received histo-incompatible thymocytes and three thymectomized but non-injected toads died 10 days after skin grafting. Necropsies revealed no indications of graft-versus-host reaction measured by splenomegaly or "wasting syndrome". These 5 toads will not be referred to in the following description.

Effects of Histocompatible and Histoincompatible Thymocytes

Summarized in Table 8 are allografts' survival durations in early-thymectomized toads after receiving 1×10^7 thymocytes from either histocompatible or histoincompatible toads. Non-thymectomized control toads rejected skin allografts in 18-21 days, whereas thymectomized toads developed no signs of incompatibility to skin allografts until the death (70 and 135 days) due to the characteristic "wasting syndrome" or the termination of experiment (150 days after grafting). Injection of thymocytes, however, restored allograft responses in thymectomized toads, and the restoration was more pronounced when histocompatible rather than incompatible thymocytes were given; the grafts survived

Table 8. Response of thymusless toads to skin allografts after receiving 1×10^7 histocompatible or histoincompatible thymocytes.

Recipient			Graft survival time in days	(mean ± SD)
TX[a)]	Injected with	No. of toads		
+	Histocompatible thymocytes	8	19,20,21,23 23,24,26,35	(23.9 ± 4.7)
+	Histoincompatible thymocytes	4	67,84,91,126	(92.0 ± 21.3)
+	None	7	70<[b)],135<[b)],150< 150<,150<,150<,150<	(-)
-	None	4	18,19,19,21	(19.3 ± 1.1)

a) Thymectomy at stage 45 (4 days of age).
b) Recipient toads died on these days after skin grafting. Grafts were healthy at the time of toads' death.

19-35 days in the animals that received histocompatible thymocytes, whereas they lived for 67-126 days in the animals that received histoincompatible thymocytes. In an additional experiment, in which the HD toads which had supplied histoincompatible thymocytes received skin grafts from the HD toads that had supplied skin allografts in the previous experiment, all the grafts (HD grafts in HD hosts) were rejected in 19-23 days. Thus, the lower efficiency of histoincompatible thymocytes in restoring immunity does not seem to be due to the little difference in histocompatibility between the applied HD thymocytes and grafted HD skins.

In order to determine the extent of restoration of allograft immunity, the early-thymectomized toads received different numbers of histocompatible thymocytes. Allografted skins remained on hosts' bodies for different periods of time, evidently depending upon numbers of injected thymocytes (Table 9). Thus, if thymectomized toads received 1×10^7 cells it took only 23.9 days on average to reject allografts, the duration being almost comparable to that when intact toads reject allografts. Similar animals that had received less numbers of thymocytes needed longer periods of time for complete rejection of allografts (2×10^6 cells in 33.5 days; 4×10^5

Table 9. Restoration of the skin allograft rejection capacity in early-thymectomized toads, depending on the number of histocompatible thymocytes administrated.

Thymectomized recipient		Graft survival time in days	(mean ± SD)
No. of thymocytes injected	No. of toads		
1×10^7	8	19,20,21,23 23,24,26,35	(23.9 ± 4.7)
2×10^6	4	24,29,34,47	(33.5 ± 7.8)
4×10^5	4	41,45,55,73	(53.5 ± 12.4)

cells in 53.5 days).

Effects of Lymphocytes from Spleen or Peripheral Blood

Histocompatible lymphocytes from spleen and peripheral blood also showed strong restorative capacities with respect to allograft immunity (Table 10). When equivalent number of lymphocytes (2×10^6) was introduced, restorative capacity was most pronounced when splenic lymphocytes were used, the grafts surviving for 20-27 days only. Blood lymphocytes were as effective as thymocytes in restoration, both allowing the grafts to survive for about 33 days.

Histological Observations

Consistent with the previous descriptions, early-thymectomy resulted in immense reduction of lymphocyte population in the spleen. Restoration of this lymphocyte population was obtained concomitantly with that of allograft immunity after introducing lymphocytes. The introduction of thymocytes restored lymphocyte population in the white pulp but not in the red pulp (*cf.* Fig. 73B). In contrast, introduction of splenic or peripheral blood lymphocytes restored lymphocytes both in red and white pulps. Histological

Table 10. Restoration of skin allograft rejection capacity in early-thymectomized toads, after receiving 2×10^6 histocompatible lymphocytes from thymus, spleen or peripheral blood.

Thymectomized recipient		Graft survival time in days	(mean ± SD)
Source of injected lymphocytes	No. of toads		
Thymus	4	24,29,34,47	(33.5 ± 7.8)
Spleen	4	20,21,23,27	(22.8 ± 2.7)
Peripheral blood	4	28,31,33,42	(33.5 ± 5.2)

examination of allografted skins at the end point of their survival revealed that in all the thymectomized-and-lymphocyte-injected toads had their skin grafts as heavily infiltrated by lymphoid cells as in non-thymectomized control toads.

III. Demonstration of the Inflow of Thymocyte Stem Cells into Early Thymic Rudiments

All the above experiments provided the firm evidence that the thymus is the first lymphoid organ to commence its essential function of establishing the immunological activities. The experiments were then performed to delineate when and how the lymphocytes differentiate in thymic rudiments.

1. Organ Culture of Thymic Rudiments

The first series of experiments were performed to determine whether or not lymphocyte can differentiate in thymus rudiments *in vitro*. Tissue fragments containing thymic region were explanted from 3-13-day (stage 42-49) larvae, and organ-cultured.

Thymuses from the larvae at any developmental stages examined increased their cell number in the culture, indicating that cell proliferation proceeded well under the *in vitro* conditions. However, the direction of lymphocyte differentiation in each culture differed according to the stages at which the rudiments were transferred *in vitro*, as summarized in Table 11.

As described previously (I.1.1.), the thymic rudiments of 8-9-day (stage 48) larvae already possess

Table 11. Differentiation of lymphocytes in *in vitro* thymus from the larvae at different steges of development. Serial sections were made with the thymus after 10-14 days of organ-culture. Only when a certain number of lymphocytes were counted, the lymphocyte differentiation was judged as positive.

Age in Days (Stage) at the Start of Culture	Lymphocyte Differentiation	
	negative	positive
3(42)	4	0
4(45)	5	0
5(46)	24	0
6(47)	6	0
7(47)	19	0
8(48)	1	15
9(48)	0	17
11(48-49)	0	6
13(49)	0	6

a number of large and medium-sized lymphocytes (Figs. 18 and 80). When the thymuses taken from 8-9-day old or more advanced stage larvae were cultured *in vitro* for 10-14 days, lymphoid differentiation proceeded well to yield aggregates of small lymphocytes (Fig. 81). Large intercellular cysts and some myoid cells also developed. In none of these cultures, however, did cortico-medullary differentiation occur.

Large lymphocytes are the chief lymphoid component in thymic rudiments of 7-day (stages 47-48) larvae (Fig. 78). Culture for 10-14 days of such rudiments, however, yielded very little or no lymphoid differentiation (Fig. 77). Small lymphocytes, even if appeared, were very few in number and formed no aggregate. In contrast, epithelial cell proliferation proceeded well in these cultures, accompanied by the appearance of epithelial cysts and myoid cells. Histological examination of these rudiments maintained *in vitro* for 4-5 days revealed a large number of pycnotic cells, suggesting that selective death of lymphcoytes occurred.

Thymic rudiments in 5-6-day (stage 47) larvae are already in possession of a population of large lymphocytes (Figs. 16 and 76). After 10-14 days of culture, neither lymphoid cell nor myoid cell was observed. Only epithelial cysts differentiated

well.

Thymic rudiments of 3-4-day (stages 41-46) larvae are composed of two types of cells (Figs. 14, 15 and 82). Although cell proliferation proceeded in these rudiments *in vitro*, differentiation of both epithelial and lymphoid cells did not occur. Results of the culture of thymic rudiments just described thus proved that thymuses taken from the 8-day-old or even older larvae consistently come to contain lymphocytes, indicating that the whole cellular population in thymic rudiments at these stages have laready been determined to differentiate into small lymphocytes.

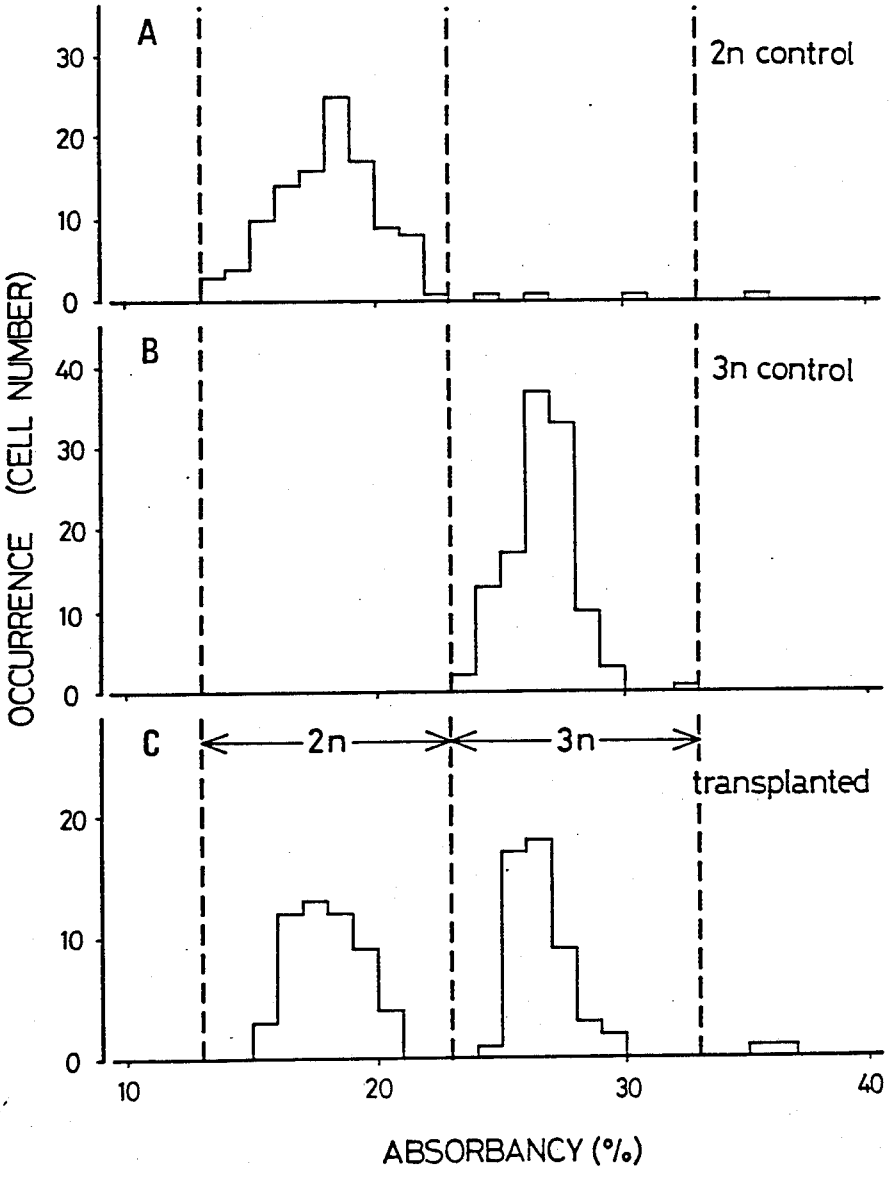
2. Inflow of Thymocyte Stem Cells into Grafted Thymic Rudiments

The experiments described in the previous section showed that the thymic rudiments from larvae younger than 6-7 days of age fail to differentiate small lymphocytes *in vitro*. However, the differentiation of small lymphocytes was observed when the same early rudiments were grafted into histocompatible tadpoles. Thus, in the rudiments even from 3-day larvae (stage 42) epithelial as well as lymphoid components differentiated during 10 days' culture *in vivo*, accompanied by hosts' vascularization (Fig. 83).

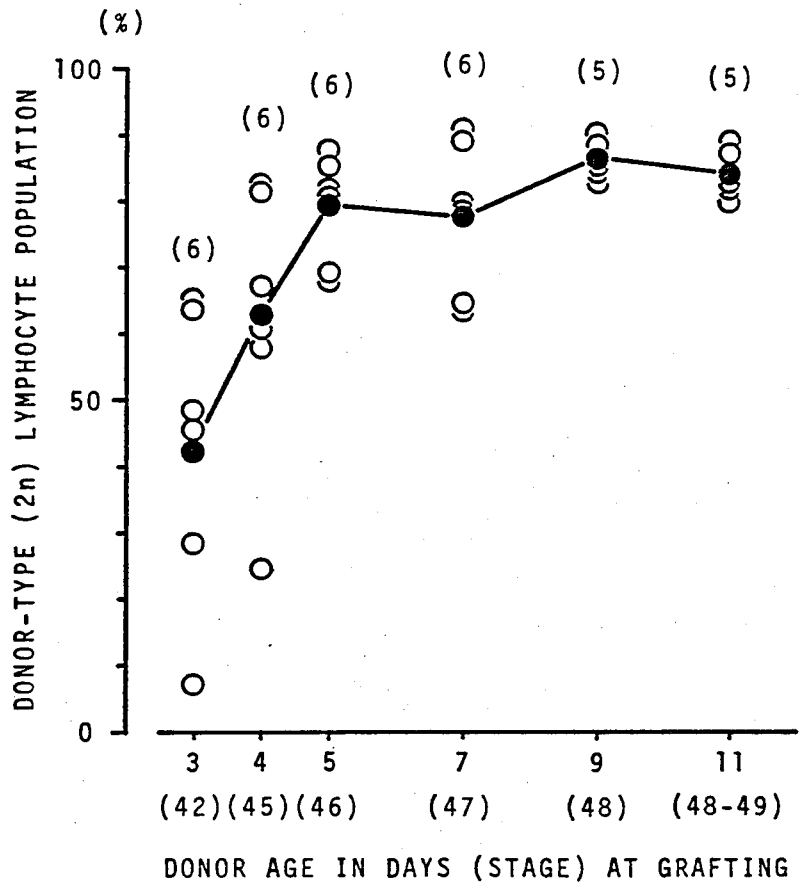
In order to determine whether these lymphocytes

in the grafted thymus differentiated intrinsically from the rudiment itself or not, tissue fragments from 3-11-day (stages 42-49) larvae were grafted into histocompatible, 40-day triploid (stages 56-58) tadpoles. When the grafted thymus attained a total age of 18 days, its lymphocytes were assessed by microspectrophotometric measurements of their DNA content. The results obtained from the measurements show that the cells representing the optical absorbancy of 13.1-23.0 % are diploid and that of 23.1-33.0 % are triploid (Text-Fig. 11). Furthermore, diploid and triploid cells were clearly distinguished in a thymus which was removed from 3-day-old diploid larva and allowed to develop in a triploid tadpole for 15 days (Text-Fig. 11).

Text-Fig. 12 summarizes the results of a series of experiments, expressed in terms of the percentages of graft-derived lymphocyte population as a function of stages at which rudiments were grafted. Grafted thymuses from 5-11-day-old larvae always possessed consistently high percentages of graft-derived lymphocytes, although a small but definite population of host-derived lymphocytes was also encountered with. In a grafted thymus derived from 3-4-day-old larvae, the ratio of graft-derived to host-derived lymphocytes was highly variable, but a tendency of sharply



Text-Fig. 11. Distribution of Feulgen-stained lymphoid cells having different optical absorbancies. Cells were harvested from a diploid thymus (A), a triploid thymus (B), and a thymus rudiment taken from a 3-day-old diploid larva and grafted in a triploid host for 15 days (C).



Text-Fig. 12. Percentages of graft-derived diploid lymphocytes in the thymuses that were grafted into triploid hosts at different developmental stages. Dots, mean values; circles, observed values. In parentheses are given the numbers of grafted thymuses examined.

increased rate of graft-derived lymphocytes was evident during these particular stages. In an extreme case of rudiment derived from a 3-day larva, more than 90 % of lymphocytes were of host-origin.

These results strongly indicate that lymphoid precursor cells of thymus are of extrinsic origin, immigrating into rudiments 3-4 days after fertilization. Sufficient number of precursor cells may have been in the thymus before 5 days of age.

3. Immigration of Stem Cells: Observation on Fresh Materials

The above experiment proved that the lymphoid stem cells migrate into thymus rudiments in 3-4 day larvae. Attempts were then made to observe in fresh materials the cellular immigration into thymic rudiments. Small pieces of live tissues containing a thymus rudiment were removed from 3-5-day larvae with watchmaker's forceps, and transferred to a shallow depression slide which was filled with the medium containing L-15, glass-distilled water and fetal calf serum (5:4:1). Specimens were then sealed with a cover glass for microscopic observation at room temperature (20°-30°C).

Under these conditions, the tissues from the 3-day larvae were too opaque to be observed through

transmission light, because of the contamination by yolk granules in the constituent cells. In contrast, thymic regions of 4-day larvae can be readily observed without giving significant alteration in the tissue integrity. Particularly noticeable were a number of large amoeboid cells in the mesenchyme surrounding the ovoid thymic rudiment. By protruding a broad pseudopod (lobopodium) in the direction of locomotion, these cells actively migrated toward the thymic rudiment through mesenchyme. As evidenced by time-lapse photomicrographs (Figs. 84-86), the rate of their migration was in the order of 10-35 μm/10 min, so that the cell migration could be readily observed under the microscope. The migration was by no means at random. On the surface of thymic rudiment, the amoeboid cells assumed the shape of a cone (Figs. 84C, 85C and 86G), as if to make a firm contact to the surface, and finally entered into the rudiment. Sometimes, the migrating amoeboid cells strayed away from the ordinary pathway that is perpendicular to the surface of thymic rudiment, but they always came to keep the right way toward the rudiment later to eventually enter it. In more than 20 thymic rudiments from 4-day larvae examined, the amoeboid cells in the course of migration were always found to behave in the manner described above. In the preparation

from the 5-day larvae, there were no longer amoeboid cells wandering toward the rudiment.

Examination of squash preparations of the thymic rudiments from 4-day larvae revealed two types of cells, the reticulo-epithelial cells and the lymphoid cells (Fig. 15). Examination of similar preparations of extrathymic mesenchyme (Fig. 87) revealed that above-mentioned cells are quite similar in appearance to one type of intrathymic cells, the lymphoid cells. In histological preparations, the latter amoeboid cells were characterized by a strongly basophilic cytoplasm, indicating that they are the lymphoid precursor cells of the thymus (*cf.* Fig. 82).

DISCUSSION

Prior to discussing the significance of the results obtained above, the two experimental conditions that are unique to the present studies should be pointed out.

First, the studies are concerned with development of the new technique of thymectomizing ealy larvae, which eventually leads to interpretation of the stage at which the thymus commences its function. Thymectomy of *Xenopus* larvae has been already performed by Horton and Manning (1972), who successfully employed a high frequency cautery apparatus for thymectomizing larvae as early as stages 47-48. As compared with their technique, our simple technique employed here yielded a relatively higher rate of thymic regeneration but a much lower mortality. This higher regenerative potency may be intrinsic to the extremely early larvae. Even the possibility of regrowth of the thymus from the pharyngeal epithelium in thymectomized *Rana pipiens* tadpoles has been suggested (Curtis and Volpe, 1971). But thanks to the transparent skin of the larvae, any incomplete thymectomy was never overlooked externally in *Xenopus*. Above all, it should be emphasized that the present technique offers the advantages of extirpating the organ at

the very initial stage of its histogenesis, enabling the study of the ontogeny of the immune system under conditions certainly free from the thymic influence.

Second, thanks to the availability of the histocompatible *Xenopus* colony, it was possible to carry out thymus grafting and injection of lymphocyte suspensions, as well as the grafting of thymus rudiments at any desired stages of both donor and host individuals. Although the genetic background of these toads is not well defined, a high degree of shared histocompatibility gene(s) among them has been evidenced repeatedly in our laboratory by their complete acceptance of a number of transplanted tissues such as skin, thymus, spleen, liver, and kidney. There are evidences to indicate that *Xenopus* may possess a relatively small number of alloimmune antigens (Du Pasquier and Miggiano, 1973; Du Pasquier, 1974; Du Pasquier *et al.*, 1977; Chardonnens, 1975). More recently, it was estimated that 95 % homozygosity can be expected in the 7th generation in *Xenopus* (Müller, 1977).

Genetically identical indivuals of *Xenopus* have already been obtained by nuclear transplantation technique (Simnett, 1964) and by interspecific hybridization technique (Kobel and Du Pasquier, 1975, 1977). Along with these efforts, our G colony *Xenopus*

will provide an important material for refined immunobiological studies and for equalizing the working conditions among various laboratories.

Lymphoid Histogenesis in Relation to Thymectomy

In their study on histogenesis of lymphoid organs in larval *Xenopus*, Manning and Horton (1969) described lymphoid tissues in the thymus, spleen, kidney (mesonephros), liver, and 3 pairs of lympho-epithelial tissues (LETs), or the ventral cavity bodies (VCBs), in the branchial region. The present observation added other LET bodies, the fourth VCBs and the posterior dorsal cavity bodies (DCBs), which consistently occur in the branchial region. Furthermore, contrary to the previous observation, a large number of scattered LET bodies were found along the whole length of the gastro-intestinal tract. A pair of LET bodies that are designated as anterior DCBs in the present study correspond to those previously referred to as "extra-thymic lymphocytes" by other workers (Sterba, 1950; Manning and Horton, 1969). The anterior DCBs might possibly differ from other LET bodies in the branchial region by their occasional absence as well as by their greater dependency on the thymus, as will be discussed below.

The location and the number of VCBs in larval

amphibians differ considerably in different species (*cf.* for *Rana pipiens*, Horton, 1971a; for *Rana catesbeiana*, Cooper, 1967a, 1967b). The differences are not surprising in view of the gross anatomical features of the branchial region among different animals. The variability might also reflect the function of these transient microorgans, although their exact function is still the subject of mere speculations (*cf.* Manning and Horton, 1969; Horton, 1971a, 1971b).

Despite the lack of both allograft rejection capacity and the anti-RRBC antibody response, the histogenesis of lymphoid tissues was not affected severely in the larvae and adults both thymectomized at stage 45 (4 days after fertilization). The thymectomy resulted in total lack of the anterior DCBs and the significant reduction in number of lymphocytes from the splenic red pulp of adult toads. This observation is essentially similar to that by Horton and Manning (1974b), who gave a full account of the lymphoid histogenesis after thymectomizing *Xenopus* larvae at stage 48 (8 days old). However, the extent of lymphoid depletion was apparently greater in thymectomized animals used in the present study, probably reflecting the degree of "seeding" or peripheralization of thymus-derived cells during the 3 day interval between stages 45 and 48.

Development of almost normal lymphoid tissues in early-thymectomized urodeles, *Triturus alpestris* (Tournefier, 1973) and *Pleurodeles waltlii* (Charlemagne, 1974) has been reported. In the anurans other than *Xenopus*, the histogenesis of lymphoid tissues and their immune activities are not well defined at present (*cf.* Du Pasquier, 1973). In any event, the subnormal level of lymphocyte population, no matter where it originates from, may suffice to explain the relative resistance of the thymectomized amphibians to antigenic challenges from the environment (*cf.* Du Pasquier, 1973; Manning, 1975).

The lympho-epithelial tissues associated with the digestive tract deserve attention because of their possible equivalency to the bursa of Fabricius in birds (*cf.* Cooper *et al.*, 1967). Thus, there are a number of observations in the poikilotherms in which the gut-associated LET bodies were found (Fichtelius *et al.*, 1968; larval *Alytes obstetricans*, Du Pasquier, 1968; larval and adult *Rana pipiens*, Horton, 1971a, 1971b). More recent observation by Goldstine *et al.* (1975) on several amphibian species showed that anurans possess well-organized gut-associated LET bodies while urodeles do not. The present observation is consistent with these studies in showing that the gut-associated LET bodies do occur

in both larval and adult *Xenopus*. Of particular interest in the present observations is that the gut-associated LET bodies showed an entirely normal histological features even when the thymus-dependent immune responses had been suppressed by early-larval-thymectomy. In view of the evidences presented in this study (Table 5) and Collie *et al.* (1975) that *Xenopus* is the lowest group of vertebrates in which dichotomy of the immune responses has been demonstrated, further experimental analyses are extremely important to determine the exact role of gut-associated LETs in humoral and cellular immunity as well as in the ontogeny of immunological diversity in this animal.

The spleen is the only lymphoid organ in *Xenopus* with any complexities of structural organization. Several lines of evidences have indicated that the spleen must be the site of the accumulation of cells which recognize the antigens and carry out responses in terms of humoral- or cell-mediated-way. Thus, the injected antigens are first trapped in the splenic red pulp and later in the white pulp (Collie, 1974); antigenic stimulations induce the marked cell production in the spleen, particularly in the white pulp (Manning and Turner, 1972; Turner and Manning, 1973); the formation of antibodies against heterologous

erythrocytes is detectable by immunocytoadherence or plaque-formation technique (Amirante, 1968; Kidder *et al.*, 1973; Ruben and Vaughn, 1974), as well as by the tissue culture technique (Auerbach and Ruben, 1970). Evidences are also available that grafting the spleen (Horton, Horton and Rimmer, 1977) or splenic lymphocytes (Table 10) can restore the allograft rejection capacity in thymectomized toads, and that the lymphocytes pertinent to allograft rejection are stored in this organ (Clark and Newth, 1972; Horton, Horton and Rimmer, 1977).

Upon early-thymectomy of stage 48 *Xenopus* larvae, reduction or diminution of either splenic antibody forming cells *in vitro* (Horton *et al.*, 1976; Horton, Rimmer and Horton, 1977; Ruben *et al.*, 1977) or the antigen trapping by the white pulp cells (Horton and Manning, 1974a) take place. Similarly the mixed leukocyte reaction, phytohemagglutinin- and concanavalin A-responsiveness manifested by splenocytes are all diminished (Manning *et al.*, 1976; Donnely *et al.*, 1976; Du Pasquier and Horton, 1976; Horton and Sherif, 1977). On the other hand, both the *in vitro* responses to *E. coli* lipopolysaccharides (LPS) and to purified protein derivative of tuberculin (PPD) have been shown to be thymus-independent (Manning *et al.*, 1976). That the spleen lymphocytes comprise distinct

thymus-dependent and thymus-independent populations is supported by the immunohistochemical studies employing surface immunoglobulins as markers (Weiss *et al.*, 1972; Nagata and Katagiri, 1978). In view of these facts, it is remarkable that splenectomy in *Xenopus* has so far barely reduced production of the antibodies to sheep erythrocytes (SRBC) and human gamma-globulins (HGG; Turner, 1973, 1974; Collie and Turner, 1975).

Although the function of the spleen is not the primary concern of the present study, histological changes of this organ observed during the course of the present study may provide helpful informations to its better understanding. Histological features of the spleens in various experimental conditions obtained in the present study are summarized in Table 12. It is readily seen that the extent of lymphocyte accumulation in the white pulp correlates well with the occurrence of specific antibody response. In contrast, situations in the red pulp are more complicated. The lymphocyte population in the red pulp always increases when intact toads were antigenically stimulated. In the thymectoized toads injected with polyvinylpyrrolidone (PVP), however, lymphocytes in the red pulp are extremely small in number in spite of a fine antibody response of the animals. The same

Table 12. Summary of the lymphocyte population in the red and the white pulps of the toads' spleens under various experimental conditions employed in the present study. The population is expressed on arbitrary standard, with those in intact, non-operated animals being assumed as "moderate". Asterisks, data from the personal communication by Nagata.

	Lymphocyte population in		Antibody response against injected antigen
	Red pulp	White pulp	
Control	moderate	moderate	
RRBC-injected	large	large	yes
PVP-injected	large	large	yes
Thymectomized	scarce	small	
Thymectomized, RRBC-injected	scarce	small	no
Thymectomized, PVP-injected	scarce	large	yes
Thymectomized, Thymus-grafted, RRBC-injected	large	large	yes
Thymectomized, Thymocyte-injected, RRBC-injected	small	large	yes*
Thymectomized, Splenocyte-injected, RRBC-injected	large	large	yes*

is true for the thymectomized-and-thymocyte-injected toads when challenged with rabbit red blood cells (RRBC). The reason for these seemingly contradictory results cannot be explained at present. Probably the white pulp is concerned more directly with the antibody production, whereas the red pulp may be involved more indirectly in the immune response such as the antigen recognition and/or its processing. Detailed histochemical analyses employing appropriate cellular and/or molecular markers to follow the whole process from antigen recognition to effective immune responses are necessary for a fuller understanding of the function of the spleen.

Ontogeny of Immune Responsiveness in Relation to Thymectomy

The major finding derived from the present thymectomy experiments is that the total lack of allograft rejection is induced in *Xenopus*, provided that the thymus is extirpated at the very initial stage of histogenesis. The results is in contrast with previous experiments with several species of anurans including *Xenopus* (*Alytes obstetricans*, Du Pasquier, 1965; *Rana catesbeiana*, Cooper and Hildemann, 1965; *Rana pipiens*, Curtis and Volpe, 1971; *Xenopus laevis*, Horton and Manning, 1972, Horton and

Horton, 1975, Rimmer and Horton, 1977), in which larval thymectomy was claimed to cause a delay, but not a total abrogation, of allograft rejection. To our knowledge, the definite loss of allograft rejection following thymectomy in amphibians has so far been recorded only in urodeles, *Triturus alpestris* (Tournefier, 1973) and *Pleurodeles waltlii* (Charlemagne, 1974), which with intact thymus display a chronic rejection of skin allografts. The evaluation of our results in the following discussion will be made chiefly in terms of the state of the thymus at the time of its extirpation, as well as the roles played by this organ when it is still only rudimentary during early stages of ontogeny.

In analyzing the role played by the thymus in the ontogeny of immune responsiveness, Horton and Manning (1972) found in the *Xenopus* larvae thymectomized at stage 48 (8 days old), 2-4 days later than in the present experiments, that the invasion of host lymphocytes into allografted skin was inhibited. These thymus-depleted animal after metamorphosis eventually rejected the transplanted skin after a prolonged median survival time (MST) of the graft, accompanied by a heavy invasion of lymphocytes from somewhere. A more recent experiment by Horton and Horton (1975) showed that even a typical second-set

allograft rejection was induced in the toads thymectomized at stages 46/47 (5 days old) and 48.

In interpreting these results, these authors postulate tha alternative source of lymphocytes other than thymus, which might function in supplying a population of lymphocytes pertinent to allograft rejection (Horton and Horton, 1975; Rimmer and Horton, 1977). They further argue that the lack of allograft rejection in our 4 day-thymectomized toads is ascribable not to a primary consequence of the absence of thymus but to the poor health of our thymusless toads (Rimmer and Horton, 1977). Nevertheless, the induction of a nearly complete restoration of allograft rejection capacity after introducing thymus grafts or lymphocytes to thymusless toads as well as the full dependence of restoration to the thymocyte population as demonstrated in the present study (Tables 6 and 9) would provide a definite answer to their question. It is worth to mention in this connection that the allografts' MSTs in our thymectomized-and-thymus-grafted toads (25-66days) was quite comparable in their 8-day-thymectomized toads (27-65 days, Horton and Manning, 1972). It is also noticed that in the experiment by Horton and Horton (1975), some toads which had been thymectomized at stage 46/47 (5 days old) retained intact skin

allografts when killed 57-153 days after the grafting. Thus, the most likely conclusion emerging from these considerations would be that the allografts' MSTs in thymectomized toads is the direct consequence of the number of the thymus-derived or influenced lymphocytes in the body.

The significant difference in the outcome of the alloimmune reactivity between the toads thymectomized at stages 45 and 47/48, respectively, thus warrants a comparison of the state of the thymus and related lymphoid tissues betwen the stage 45 and the 47/48 larvae. The spleen, which is no more than a small mesenchymal aggregation at stage 45/46 (Manning and Horton, 1969), already comprises up to 1,000 cells at stage 48, including large and medium-sized lymphocytes (Horton and Manning, 1972). At atage 45, the thymus comprises less than 100 cells, whereas at stage 48 it is shown to contain nearly 2,000 cells (Horton and Manning, 1972). The study of developing thymus by fluorescent antibody technique (Du Pasquier *et al.*, 1972) proved that the immunoglobulin-carrying lymphocytes first appear late in stage 46, and their number sharply increases during stages 47-48. Electron microscopic observations of differentiating thymus also revealed a pronounced increase of lymphocytes during stages 47-48 (Nagata, 1977). These results

may lead one to conceive that during stages 46-48, the thymic rudiment is a center for lymphoid differentiation, whereby a population of immunocompetent cells is determined to be capable of reacting with alloantigens in later life. Further studies are needed to decide that the stages 46-48 are the very crucial period for eatablishing immunity to allografts in *Xenopus*.

The loss of circulating antibody production against RRBC observed in the present study is in agreement with the results of Turner and Manning (1974) and Horton, Rimmer and Horton (1976, 1977), who found a lack of antibody response to SRBC and HGG in *Xenopus* thymectomized at stage 48. The latter authors also showed that in contrast to the early establishment of alloimmunity, thymectomy at considerably later stages can still suppress the response to SRBC; presence of the thymus appears to be necessary up to about stage 57 in order to establish and maintain proper anti-SRBC reactions. The present experiment indicates that the thymus had an effect on the establishment of anti-RRBC response before stage 51. Hence, under thymic influence some immune responses seem to mature earlier than others in ontogeny; the critical larval stage beyond which thymectomy can no longer diminish development of immune responses

may differ according to the nature (determinants) of challenged antigens (*cf.* Manning and Collie, 1977).

The restoration of immune activities in early-thymectomized, 1.5-year-old toads by implantation of adult (1-year-old) thymuses observed here is striking in view of the relatively insufficient restorative capacity of adult thymic lymphocytes in mammals (Miller, 1965). More recently, similar restoration of immune responses was reported in *Xenopus* which received implantation of larval thymuses 2 weeks after larval thymectomy (Horton and Horton, 1975). The success of restoration in the present studies by introducing isolated lymphocytes from the thymus, the spleen and the peripheral blood and the dependence of the extent of restoration on the number of lymphocytes further support the concept that the restorative capacity of the grafted thymus or spleen (Horton and Horton, 1975; Horton, Horton and Rimmer, 1977) is attributed primarily, if not solely, to their lymphocytes. The splenic lymphocytes had a more pronounced restorative activity than thymic lymphocytes, the results being similar to the situation reported for mice (Dalmasso *et al.*, 1963). Very recently, Ruben *et al.* (1977) demonstrated that the *in vitro* antibody response to SRBC was

restored in spleen of early-thymectomized *Xenopus* by co-cultivation of the spleen with isolated allogeneic thymocytes, indicating that the helper function was provided by the latter. At present, the question is open how the introduced lymphocytes behave in thymectomized hosts. Currently, experiments are being carried on to follow the fate of these lymphocytes, using a triploid cellular marker.

Another important finding in the present study is an unequivocal demonstration of thymus-independent immune response; the toads thymectomized at stage 45 were found to produce a high titer of specific antibodies against PVP (Table 5). Recently, Collie *et al.* (1975) reported that the *Xenopus* thymectomized at stage 48 produces antibodies against *E. coli* LPS, which are similar to the antibodies obtained in the present study in molecular weight, heat stability, and mercaptoethanol sensitivity. Presence of such thymus-independent immune responses may be supported further by our observations as follows. First, toads that had previously been thymectomized at stage 45 evidently possessed a certain amount of serum immunoglobulins (Text-Figs. 7 and 8, Table 4). Production of immunoglobulin even at an increased level has been documented in *Xenopus* that had been

thymectomized at stages 47-48 (Weiss *et al.*, 1972). Second, these thymusless toads possessed a significant number of lymphocytes in the gut-associated LETs. Similarly an increase in the number of splenic lymphocytes possessing larger amount of surface-associated immunoglobulins is reported (Weiss *et al.*, 1972). Recent experiment by Nagata and Katagiri (1978) clearly demonstrated by immunoperoxidase method that more than 90 % of splenic lymphocytes in toadlets that had been thymectomized at stage 45 bear surface-immunoglobulins as contrasted with 53 % in an average of those in intact toadlets. If the splenic lymphocytes without surface-immunoglobulins are derived from the thymus, their finding strongly suggests that the T-cells might have been completely eliminated in our toadlets thymectomized when they were 4-day-old, while antibody producing lymphocytes have remained intact in these animals.

It is interesting from the phylogenetic viewpoint that exactly the same antigens elicit a thymus-independent immune response in *Xenopus* and mice, the two unrelated species, and that all antibodies thus synthesized are of high-molecular-weight type (*cf.* Basten and Howard, 1973). These findings will furnish the basis to the view that *Xenopus*, with its much simpler lymphoid system than in mammals, provides

an excellent material for studying the immune mechanism.

Problems of Lymphocyte Differentiation in Early Thymus Rudiments

An unavoidable question to any kind of tissue or organ culture studies is whether the *in vitro* conditions used were appropriate enough to draw any conclusion from the results. Although no evidence is by no means available to argue that the organ culture technique used in the present study was the best one, the epithelial cysts differentiated well in the thymus rudiments taken from the 5-day-old larvae, indicating that the experimental conditions employed here were sufficient at least in helping their differentiation and maintenance.

On the other hand, the extent to which the cultured lymphocytes increase in number was dependent on the ages of the larvae from which the explants were removed. Thus, no lymphocytes differentiated in the culture of the rudiments taken from the 5-7 day larvae, whereas the differentiation of lymphocytes was made sometimes excellently in the culture of the rudiments taken from the 8-day-old or older larvae. Failure of *in vitro* lymphoid differentiation from the 5-7-day rudiments is interesting to note, because these rudiments have already been colonized by a

sufficient number of lymphoid precursor cells *in situ*. Therefore, it is surmised that a certain condition in the 5-7-day thymus rudiments affects the lymphoid precursor cells colonized there as to take their definite differentiation pathway, and that the condition is presumably lost in the culture of younger rudiments used in the present experiments.

In our preliminary culture of the 3-7-day rudiments in a diffusion chamber, it was found that lymphoid differentiation was hardly realized. In these experiments, alike in the cases of mice (Hays, 1967) and rats (Bellamy and Hinsull, 1975), lymphocyte differentiation has so far been unsuccessful, although epithelial components differentiated well in them.

The question is now open to ask exactly what occurred in the 5-8-day thymus rudiments of this anuran. At any rate, the question raised by these *in vitro* and diffusion chamber studies provides again the support to the conclusion presented in the foregoing section that the thymus commences its essential function at 7-8 days of age (stage 48).

The origin of thymic lymphocytes has been analyzed by either the orthotopic (*Rana pipiens*, Turpen *et al.*, 1973; *Pleurodeles waltlii.*, Deparis and Jaylet, 1976; *Xenopus laevis*, Volpe *et al.*, 1977) or

the heterotopic grafting of presumptive gill area (chick and quail, Le Douarin and Jotereau, 1975; *P. waltlii*, Deparis and Jaylet, 1976; *R. pipiens*, Turpen *et al.*, 1975). The culture of thymic rudiments of mice in a diffusion chamber placed on chick chorio-allantoic membrane was another technique employed for analysis (Owen and Ritter, 1969). The present study differs from these previous studies in that the diploid rudiments at various developmental stages were grafted to much older (stages 56-58) triploid larvae.

Because we used histocompatible individuals, the results exclude the possibility that alloimmune reactions by host lymphocytes may account for some of the findings. In our study the host animals were much older than the donors. Therefore, cells that would have entered the graft (whatever their appearance) may well have been in more advanced stage of cytogenesis than cells which would normally be there. For instance, the thymus from the 3-day-old animals would normally have only 3-day-old cells around it.

In the present experiment, significant number of host-type lymphocytes was seen in the thymuses derived from 3-4-day-old rudiments, apparently reflecting the stage-dependent immigration of lymphoid precursor cells. This implies, in turn, that as early as 5

days after fertilization, thymus rudiments have acquired a population of lymphoid precursor cells sufficient to support the differentiation of small lymphocytes *in situ*.

Volpe *et al.* (1977), by reciprocal transplantation of gill buds between diploid and triploid *Xenopus* embryos at stage 21, succeeded in showing the extrinsic origin of thymocyte stem cells, but they failed to demonstrate the exact time when the active inflow of lymphoid precursor cells takes place. The concept of extrathymic origin of stem cells has been strongly supported by recent electron microscopic observation by Nagata (1977). The situation in *Xenopus* is similar to that in birds in that thymic rudiments of both species receive an inflow of stem cells at a particular stage of their histogenesis (Le Douarin and Jotereau, 1975). In birds, it has been shown that the inflow of stem cell takes place in a relatively short period, *i.e.* 24 hr in quails and 36 hr in chicks, and that after that the inflow is extremely slow if not totally absent. On the contrary, the endodermal origin of thymocyte stem cells, instead of the inflow of lymphoid precursor cells from somewhere, has been repeatedly claimed in *Rana pipiens* (Turpen and Cohen, 1976; Turpen *et al.*, 1973, 1975). The discrepancy between the results in *Xenopus* and *Rana* may be explained as that

the stem cell immigration in *Rana* embryos takes place very early so that the thymus rudiments have already received inflow of stem cells when the rudiments were exchanged. By continued analysis of immigration patterns of precursor stem cells in different vertebrate species at different stages through several technical approaches, the problem will be resolved to an agreeable generalization.

The entrance of stem cells into the thymic rudiments has so far been claimed on either experimental evidences or histological observations on the fixed materials. In this respect, the present observation should be evaluated as the first direct demonstration of extrathymic stem cell immigration into the rudiments.

A surprisingly swift movement of migrating cells together with their migration in a straight pathway toward the rudiment suggests that these cells are attracted to some factor or factors from this epithelial rudiment. These migrating cells frequently occur around the thymus rudiment of 4-day-old larvae with apparently polarized structures and conspicuous pseudopodia. These facts may well explain that rapid inflow of the stem cells takes place only during the short period of thymus histogenesis. These migrating

cells with morphological characteristics such as the large size, conspicuous nucleoli and cytoplasmic hyperbasophilia as well as their pseudopodia are quite similar to the so-called "thymic stem cells" of mouse (Moore and Owen, 1967), rabbit (Ackerman and Hostetler, 1970), chick (Le Douarin and Jotereau, 1975; Leene *et al.*, 1973), lizard (Pitchappan and Muthukkaruppan, 1977) and urodelan amphibian (Charlemagne, 1977).

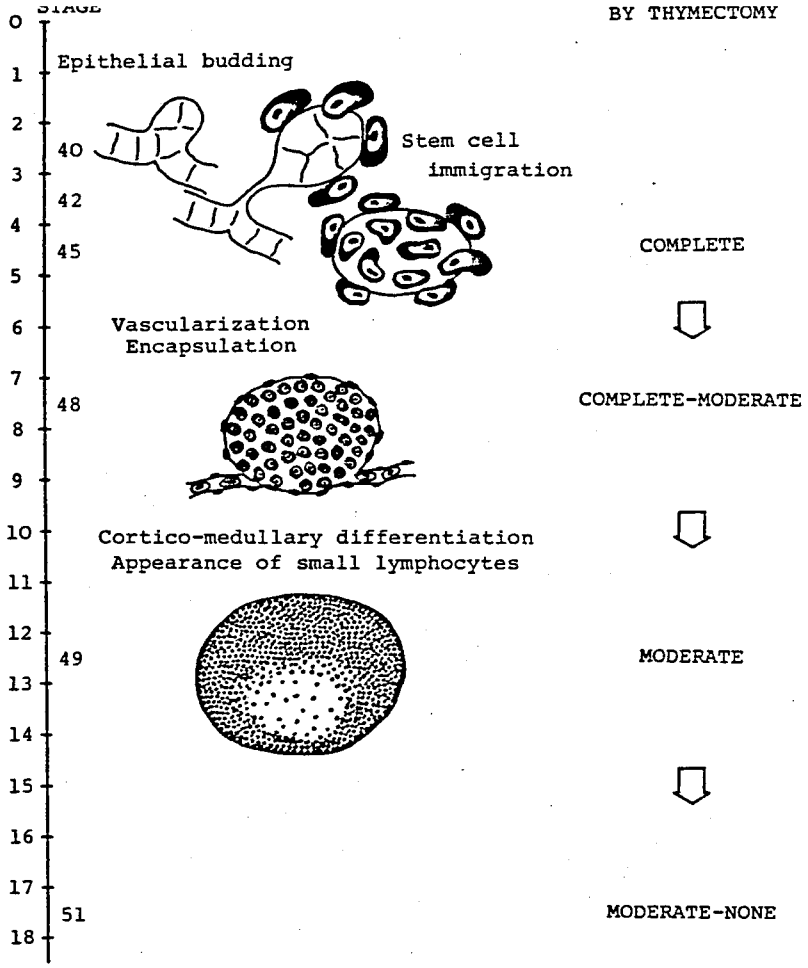
The exact site from which the thymocyte stem cells originated could not be determined in the present study. To date, no substantial evidences have been presented yet to resolve the controversy as to whether the stem cells are originated from blood island or from mesenchyme surrounding the thymus (*cf.* Dieterlen-Lievre, 1975). Investigations on the source of thymocyte stem cells, together with the important questions such as the specific micro-environment for lymphocyte differentiation and the interaction of extrinsic stem cells with particular stages of organ rudiments, should be extremely important for the future researches.

General Conclusions

Several lines of evidences presented in the preceding sections clearly indicate that in *Xenopus*

the thymus functions as the center for the development of both cellular immune response as manifested by skin allograft rejection and the humoral antibody response against the "thymus-dependent antigens" such as RRBC, SRBC, and HGG. Although the abrogation of these responses in later life by thymectomy is similarly seen both in *Xenopus* and mammals (*cf.* Miller and Osoba, 1967), an interesting fact seen in *Xenopus* is that abrogation of the immune response to each specific antigen is correlated well with the particular larval stage at which thymectomy was made. Thus, thymectomy in 4-day larvae completely abrogated the allograft rejection capacity, while thymectomy in 3-week larvae had no effect on it. During these periods, the extent to which allograft rejection capacity is built up is dependent on the stage when thymectomy is made (Text-Fig. 13), that is to say the later the thymectomy is done the fuller allograft rejection capacity develops. The results obtained in other laboratories combined with those in the present study further suggest that the "thymus-dependent" humoral immunity to different antigens is built up only later than the allograft immunity is established (*cf.* Manning and Collie, 1977).

Existence of the immune restoration capacity in suspensions of lymphocytes from thymus, spleen



Text-Fig. 13. Schematic representation of ontogeny of the immunity to allografts in relation to histogenesis of the thymus. The organ commences its histogenesis as a budding of pharyngeal epithelium in 2-day larva (stage 40), and the rudimentary organ receives the precursor stem cells from the mesenchyme at stages 42-45 (3-4 days of age). It is vascularized and encapsulated at 8 days of age (stage 48). Basic histological pattern of the thymus containing a number of small lymphocytes is already established in stage 49 (12-day-old) larva. Thymectomy in the larvae 4-5-day-old or younger completely abraogated their capacity of allograft rejection, whereas the same operation in older larvae resulted in various degrees of suppression of their capacity, depending on their age when the thymectomy was performed. Some of the data used to complete this Text-Fig. are based on experiments by Horton and Manning (1972), Horton and Horton (1975), and Rimmer and Horton (1977).

and blood indicates that the thymus exerts its function by way of lymphocyte "seeding" or peripheralization. Once peripheralized, these thymus-derived (immunocompetent) cells may constitute self-renewing cell lines (clones) in the peripheral lymphoid tissues such as the spleen. In this respect, it can be logically inferred from the present results that the seeding of the lymphocytes pertinent to allograft rejection commences 7-8 days after fertilization and ceases in 2-3 weeks (Text-Fig. 13). It must be recalled that the thymus in 7-8 days of age is at the very beginning of its lymphoidogenesis, *i.e.* it is only 4-5 days after immigration of lymphoid stem cells into epithelial rudiment. Employing the cellular marker as successfully used in the present study, further experiments should be focused in order to substantiate that the seeding of lymphocytes really occurs during these early stages.

It has also been shown that even the thymus rudimentectomy in *Xenopus* larvae cannot abolish their humoral response against the "classical" thymus-independent antigens in mammals, PVP and LPS. This, together with the observation that both serum immunoglobulin levels and a certain lymphoid populaion in peripheral lymphoid tissues are unaffected by thymectomy, strongly indicates that the thymus-independent

immune system exists in *Xenopus*.

It is now concluded that *Xenopus* has the functioanl immune system which is almost comparable to that in mammals and birds. Relatively simple lymphoid system in *Xenopus* as compared with mammals provides an excellent system for understanding the basic mechanism of vertebrate immunity.

SUMMARY

In order to determine the role of the thymus in the establishment of the immune system, early larvae of the South African clawed toad, *Xenopus laevis*, were thymectomized, and the differentiation of lymphoid tissues and immune responses in these animals were compared with that in intact control animals. Observations were also carried out on the thymectomized-and-thymus-implanted animals and thymectomized-and-lymphocyte-injected animals. Also the series of experiments were performed to determine the *in vitro* developmental capacity of the thymus rudiments, the fate of introduced lymphocytes in host animals, and the origin of thymus lymphocytes. The results obtained are summarized as follows:

1) Histologically, the thymus rudiment makes its appearance as an epithelial outpocketing of the second visceral pouch at the Nieuwkoop and Faber (1956) stage 40, and detaches from the epithelium at stage 45. Free basophilic cells are present in and around the thymic bud at stage 43, and in the thymus their number attains an order of a hundred at stages 45-46. Small lymphocytes first appear in the thymus at stage 49. The differentiation of

lymphoid tissues in other organs, such as the spleen, the kidney (mesonephros) and the liver, does not commence before stage 49.

2) A description was made of a number of lympho-epithelial tissues (LETs) which had not been found by previous investigators. These include 4th ventral cavity bodies (VCBs), posterior dorsal cavity bodies (DCBs) in the larvae, and a large number of scattered, gut-associated LET bodies both in the larvae and the adults. In the larvae the lymphoid organs (VCBs and DCBs) in the branchial region are first detectable at stage 49, and the organs along the alimentary tract at stage 48.

3) A technique for thymectomizing the larvae as early as at stage 45 (4 days after fertilization) was devised with a high rate of success, and the larvae thymectomized by it developed at normal rate and metamorphosed to toadlets. But the toadlets were sometimes characterized by the wasting syndrome known in mammals, reflecting their depressed reactivity against microorganisms.

4) The thymectomy of the stage 45 larvae resulted in a complete diminution of lymphocytes from

the anterior DCBs of the larvae, and in a conspicuous lymphocytic diminution in the splenic red pulp of metamorphosed animals. In the thymectomized animals, a small number of lymphocytes were found in the larval gill- and gut-associated LETs, the adult splenic white pulp, and the lymphoid aggregations in the liver and kidney of the larvae and adults. On the other hand, in the thymectomized toadlets lymphocytes were found to accumulate in the gut-associated LETs to an extent as in intact animals.

5) Non-thymectomized toads rejected skin allografts in 16-23 days at 23°C [median survival time (MST), 18.9 ± 1.4 days], as defined by pigment destruction of allografted skins. They rejected the second-set allografts in 6-9 days (MST, 6.9 ± 1.0 days). The toads either thymectomized or sham-operated when they were at stage 51 (18 days old) rejected allografted skin in 16-37 days. In contrast, none of the toads earlier thymectomized at stages 45-47 (4-7 days old) rejected the grafts until the termination of observation (250 days). In these toads, no indication of lymphoid invasion into grafted area was histologically evident.

6) Upon introduction of rabbit red blood cells

(RRBC), both non-thymectomized and sham-operated toads produced antibodies in a titer of 1:64-1:256, accompanied by a conspicuous increase of lymphoid populations in the red and the white pulps of the spleen. The toads thymectomized at stage 51 produced antibodies against RRBC, as did the intact controls. However, those thymectomized at stages 45-47 did not produce antibodies against RRBC, and there was no appreciable alteration of the lymphoid population in the spleen. It was concluded, therefore, that thymus exerts its role in establishment of allograft immunity and anti-RRBC response before stage 47. In spite of the total lack of the above-described immune responses, the thymectomized toads was found to possess a certain level of immunoglobulins, as determined by electrophoresis on cellulose acetate membrane and by immunoelectrophoretic analyses.

7) When injected with polyvinylpyrrolidone (PVP), the toads that had been thymectomized at stage 45 produced the antibodies in titers of 1:256-1:1,024, just as the non-thymectmoized toads did, providing a strong evidence that the thymus-independent immune response is present in this anuran. The antibodies produced in thymectomized toads were of exclusively high molecular weight,

being heat stable and 2-mercaptoethanol sensitive. After immunization with PVP, lymphoid accumulation was prominent in the splenic white pulp of both thymectomized and non-thymectomized toads, but the accumulation in the red pulp was evident only in non-thymectomized toads.

8) The one to one and a half years old toads that had been thymectomized at stage 45 were implanted with histocompatible thymus from 1-year-old toads, and 4 weeks later were given either skin allografts or RRBC to determine their immune responses. In contrast with the perfectly tolerant thymusless controls, the thymus-implanted toads rejected skin allografts after a longer survival time (25-66 days) than the non-thymectomized controls (18-19 days). Rejection of the second-set skin allografts was accelerated in the thymus-implanted individuals (15-27 days). Similarly, the capacity of antibody against RRBC was restored in the thymus-implanted toads to nearly normal extent. Implantation of thymus also restored the histological features of the spleen.

9) Restoration of allograft immunity to approximately normal levels was also achieved after

injecting 1×10^7 thymic or 2×10^6 splenic lymphocytes from histocompatible toads. Lymphocytes from peripheral blood were as effective as thymocytes. The degree of restoration was dependent upon the number of introduced thymocytes. Histoincompatible thymocytes were also effective in restoration, although in a lesser degree. Lymphocytes in a comparable number to those in intact animals was observed in the spleen of all the restored toads.

10) The thymus rudiments together with their surrounding tissues were removed from the larvae at 3-13 days of age (stages 42-49), and were organ-cultured for 10-14 days. The differentiation of epithelial cysts was observed in the rudiments taken from the 5-day-old or older larvae, and the differentiation of myoid cells was evident in those taken from the 7-day-old or older larvae. However, the lymphocytes differentiated only in the rudiments taken from 8-9-day-old or older larvae. When grafted to the tail of histocompatible tadpoles, all the rudiments taken from the larvae ranging 3-11 days of age come to contain lymphocytes as well as other cell types.

11) The thymus rudiments taken from the

3-11-day-old diploid larvae (stages 42-49) were grafted into histocompatible, triploid 40-day-old tadpoles (stages 56-58). Seven to sixteen days later, the ploidies of lymphocytes in differentiated thymuses were determined microspectrophotometrically for the amount of their nuclear DNA. It was found that the rate of graft-type (diploid) lymphocytes increased clearly as the donors got older (43 % on an average from 3-day-old rudiment *versus* 63 % on an average from 4-day-old rudiment). Thus, in the grafts derived from 5-11-day-old larvae most lymphocytes (82 % on an average) were of graft-origin. These results provide a strong support for the view that lymphoid precursor cells of the thymus are of extrinsic in origin, immigrating from somewhere into the rudiments 3-4 days after fertilization.

12) The thymus rudiments together with surrounding tissues were extirpated from the 4-day-old larvae, and were subjected to a brief culture for microscopic observation. Under *in vitro* condition, a number of large mesenchymal cells showed amoeboid movement with prominent pseudopodia. These cells migrated actively toward the rudiment, at the speed of 10-35 μm/10 min, and were finally engulfed by the rudiment. In none of the preparations from 5-day-old

larvae were found such migrating cells. Histological examination of these cells indicated that they correspond to the lymphoid stem cells of the thymus, referred to in the previous studies.

13) These results indicate that the thymus in *Xenopus laevis* functions at the very early stage of its histogenesis, by way of lymphocyte seeding, for establishing immune reactions such as skin allograft rejection and humoral antibody production against "thymus-dependent" antigens. It was also stressed that the thymus-independent immune system is present in this species.

14) Usefulness of this animal as an excellent model for further immunobiological investigations was discussed, in particular from the ontogenic and phylogenetic viewpoint.

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EXPLANATION OF PLATE I

Unless otherwise stated, all the sections were stained with Delafield's hematoxylin and eosine.

Fig. 1. Section of a thymus from a 2-year-old toad, showing deeply stained cortex and paler medulla. × 25.

Fig. 2. Higher magnifacation of the thymus shown in Fig. 1. The trabucular invasion from the capsule and associated melanophores are seen. × 125.

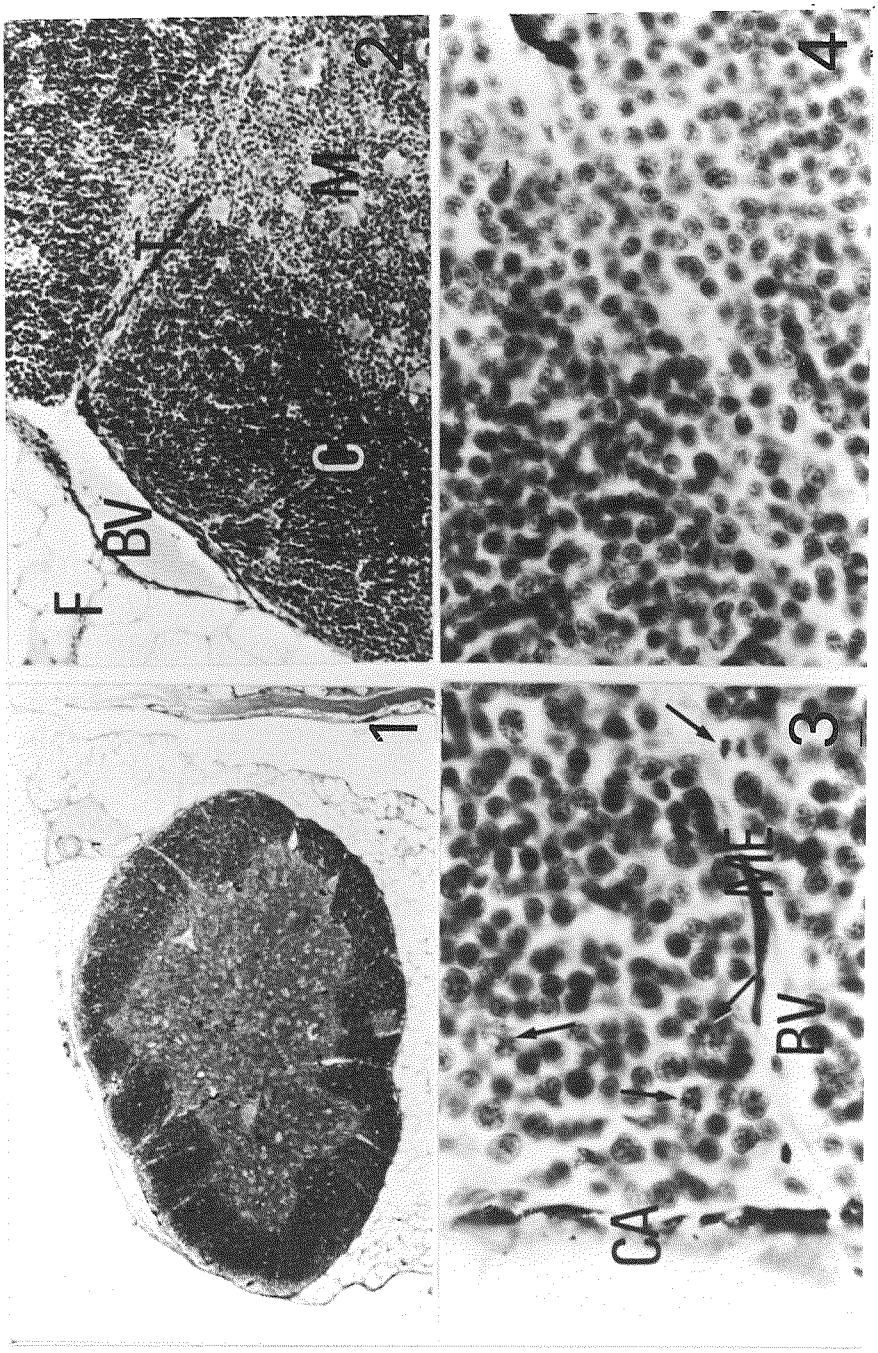
Fig. 3. Outer part of the thymus shown in Fig. 1, indicating predominant medium-sized lymphocytes and a high rate of mitosis (arrows). × 630.

Fig. 4. Small lymphocytes tightly packed in inner cortex of the same thymus as shown in Fig. 3. × 630.

ABBREVIATIONS

BV, blood vessel
C, cortex
CA, capsule
F, fatty tissue
M, medulla
ME, melanophore
T, trabecular invasion

PLATE I



EXPLANATION OF PLATE II

Figs. 5-8. Various epithelial derivatives found in the medulla of the thymus. × 630.

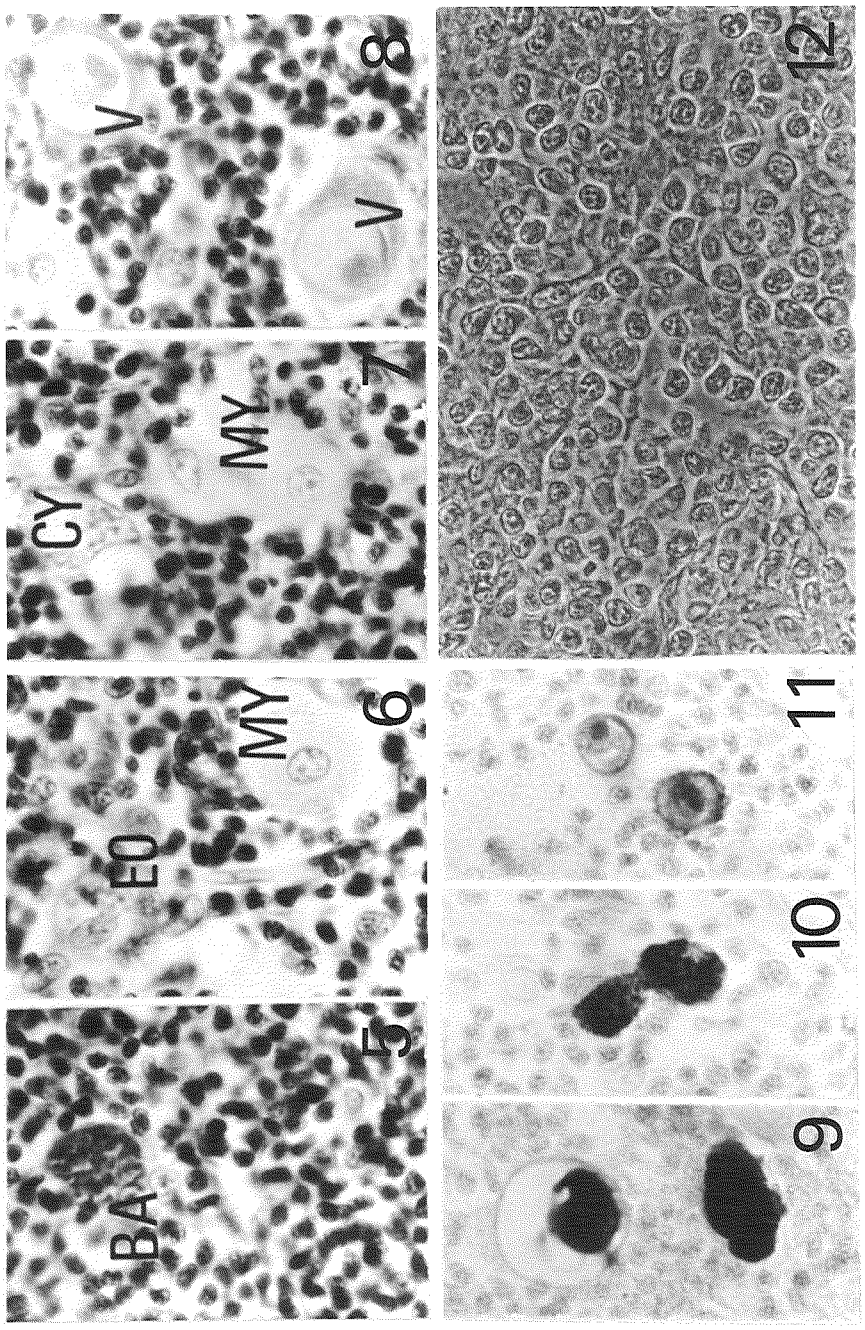
Figs. 9-11. Demonstration of the PAS-positive substances in the medullary cells of the thymus in adult toad. PAS-positive substances are present in a variety of forms. Stained with PAS method. × 630.

Fig. 12. A section of Carnoy-fixed thymus of a toadlet as seen by a phase contrast microscope, showing dendritic reticular fiber of epithelial nature running among lymphoid cells. Unstained. × 630.

ABBREVIATIONS

BA, cell containing basophilic granules
CY, cell containing cystic space
EO, cell containing eosinophilic granules
MY, myoid cell
V, intercellular vacuole (cyst)

PLATE II



EXPLANATION OF PLATE III

Fig. 13. Left thymic rudiment (arrow) of a stage 45 (4-day-old) larva, as seen through transmission light. Fresh material. × 62.5.

Fig. 14. Thymus of a 4-day-old (stage 45) larva. Deeply stained free cells are scattered among paler epithelial cells. Glycol methacrylate-embedded thin section stained with toluidine blue. × 1,000.

Figs. 15-18. Phase contrast micrographs of squashed thymuses of the 4-day-old (Fig. 15), 6-day-old (Fig. 16), 8-day-old (Fig. 17), and 12-day-old larvae (Fig. 18), respectively. The lymphoid cells apparently decrease in size as the animals grow. Arrows (white) indicate the reticulo-epithelial cells dispersed among predominant free lymphoid cells. × 1,000.

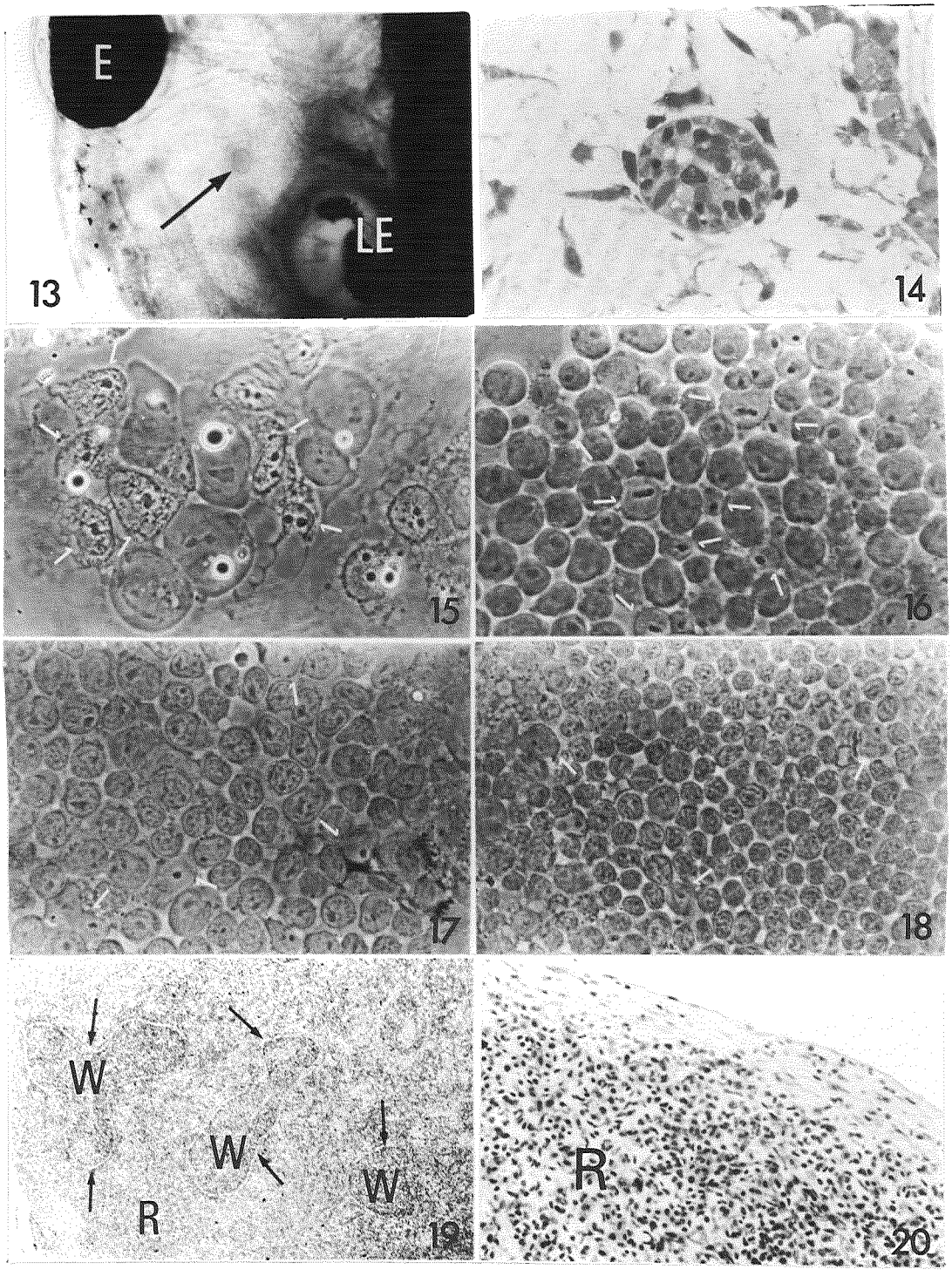
Fig. 19. Part of an adult spleen showing the white and the red pulps. Arrows indicate the reticular boundary zone between the two pulps. × 100.

Fig. 20. Part of an adult spleen showing a thick connective tissue encapsulating the organ. × 200.

ABBREVIATIONS

E, eye
LE, larval ear
R, red pulp
W, white pulp

PLATE III



EXPLANATION OF PLATE IV

Fig. 21. Higher magnification of a part of an adult spleen showing the boundary layer between the red and the white pulps. The white pulp comprises of a large number of small and medium-sized lymphocytes, and paler staining cells (arrows) having lobulated nuclei and prominent nucleoli. Lymphocytes are also seen in the red pulp near the boundary layer. × 500.

Fig. 22. Lymphocyte aggregation in the red pulp, showing its lack of association with the white pulp follicles. × 200.

Fig. 23. Spleen rudiment at an initial stage of histogenesis, comprising an accumulation of a small number of mesenchymal cells, in a larva at stage 45 (4-day-old). × 200.

Fig. 24. Spleen from a larva at stage 51, showing the red and the white pulps with a boundary layer (arrows). × 200.

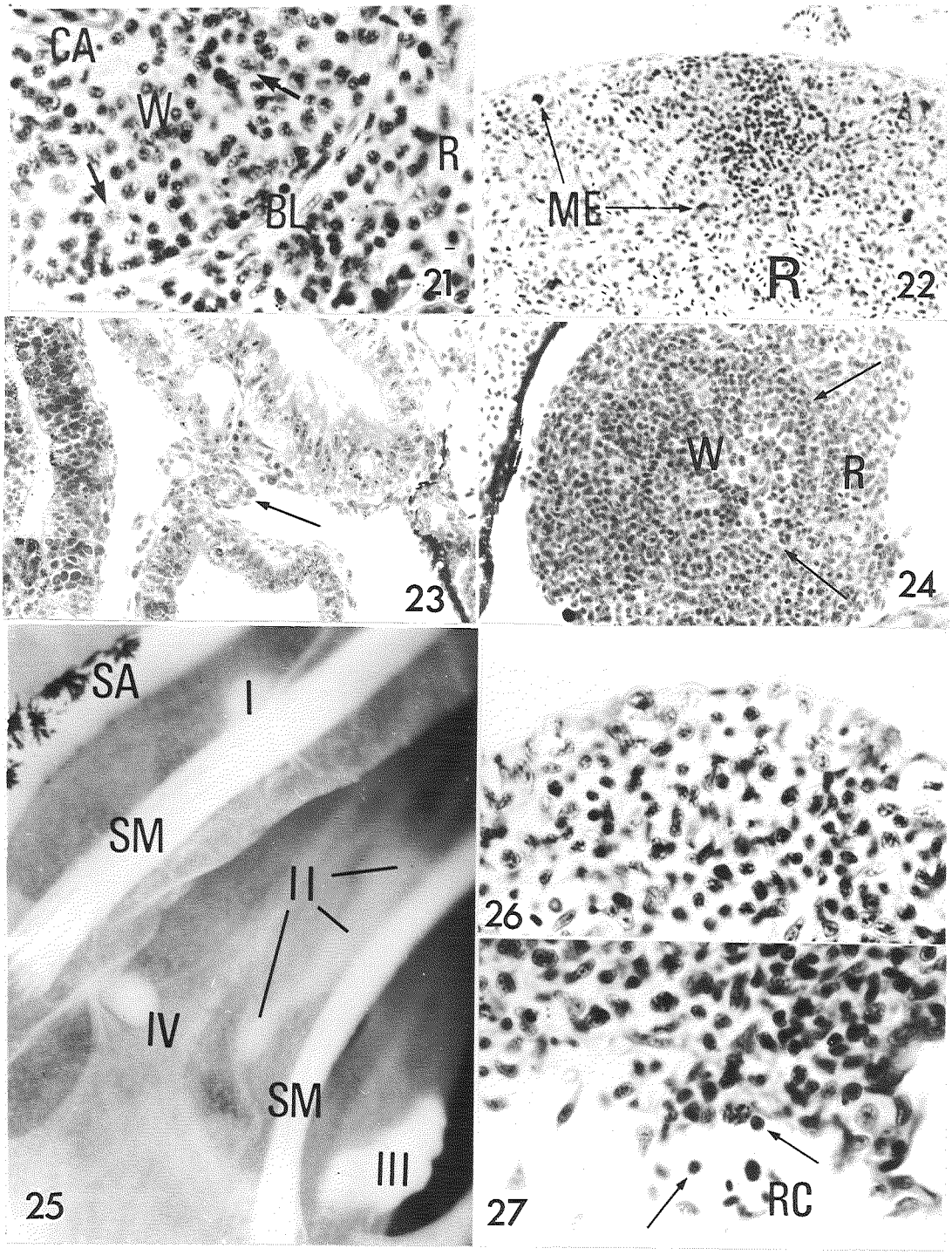
Fig. 25. Ventral view of the right branchial region of a Bouin-fixed larva at stage 56, showing 4 groups of ventral cavity bodies (VCBs). The first and the second VCBs are seen through the floor of branchial chamber. Operculum has been removed. Anterior to the upper. Stained with borax-carmine. × 80.

Fig. 26. Apical portion of the first VCB, showing accumulation of small and medium-sized lymphocytes together with a loose distribution of connective tissue cells. Note a heavy infiltration of lymphocytes into the "dome epithelium". × 500.

Fig. 27. Basal portion of the first VCB, in association with the blood vessel containing erythrocytes and small lymphocytes (arrows). Note that there is no definite demarcation between lymphoid aggregation and surrounding connective tissue. × 500.

ABBREVIATIONS

I, first VCB
II, second VCB
III, third VCB
IV, fourth VCB
BL, boundary layer
CA, central arteriole
ME, melanophore
R, red pulp
RC, erythrocyte
SA, systemic artery
SM, subarcual muscle
W, white pulp


CA
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EXPLANATION OF PLATE V

Fig. 28. A sagittal section of a stage 56 larva through its branchial region, showing the four VCBs there. Anterior to the right. × 100.

Fig. 29. Apical portion of the third VCB. Note the "giant inclusion" containing basophilic particles of various sizes. A mitotic figure (arrow) is seen at right to the inclusion. × 500.

Fig. 30. A sagittal section of the anterior DCB, forming a "dome epithelium" of a stage 56 larva. Note the presence of a "giant inclusion" (arrow) similar to that shown in Fig. 29. × 200.

Fig. 31. A section of the pharyngo-esophagus of a larva at stage 56, showing heavy lymphoid invasion into the epithelium. × 200.

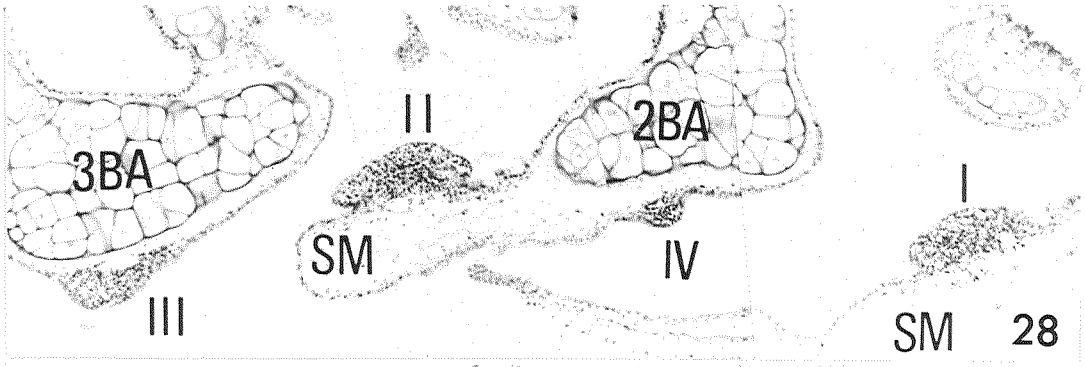
Fig. 32. Lymphoid accumulation in connective tissue among gastric glands of a larva at stage 56. Arrows indicate small lymphocytes within gland cells. × 200.

Fig. 33. The lympho-epithelial tissue (LET) body in the duodenum of a larva at stage 56, showing a lymphoid aggregation between its epithelium and peritoneum. × 200.

Fig. 34. The LET body in the larval ileum, showing a few lymphocytes invading the gut epithelium. × 200.

ABBREVIATIONS

I, first VCB
II, second VCB
III, third VCB
IV, fourth VCB
2BA, second branchial arch
3BA, third branchial arch
SM, subarcual muscle


3BA
II
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I
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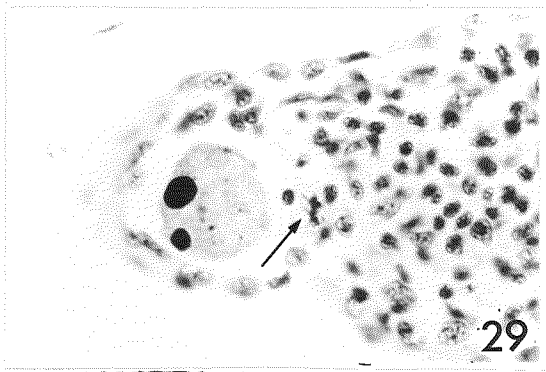
SM
28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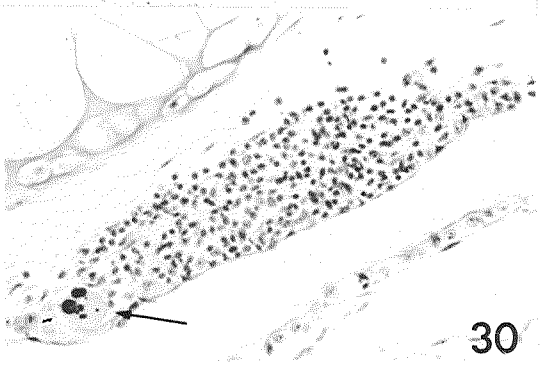
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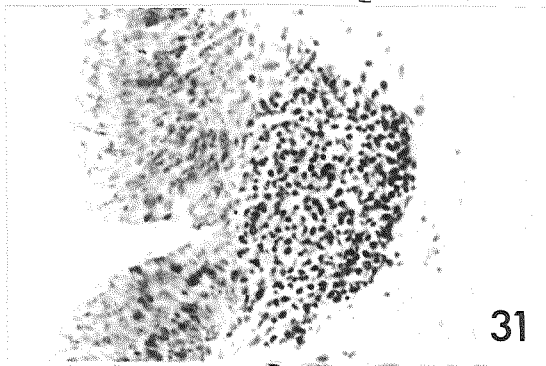
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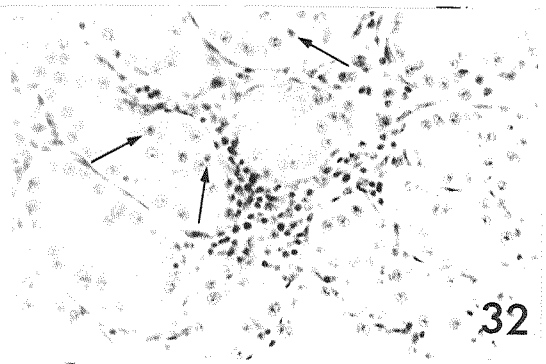
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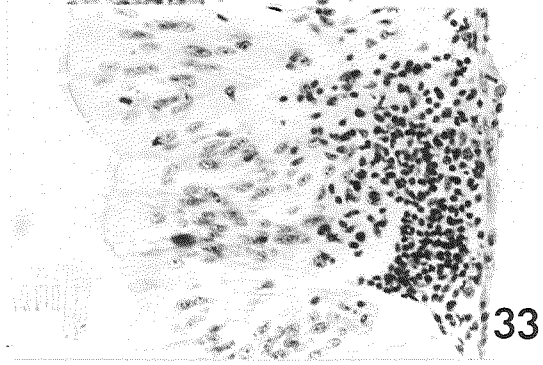
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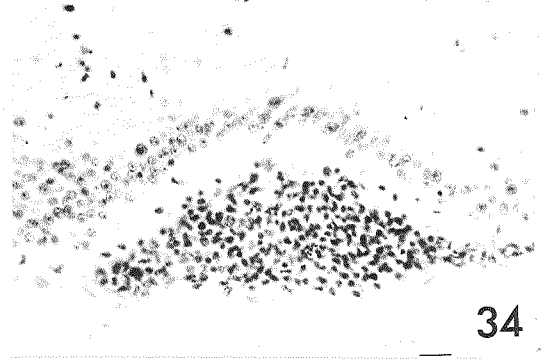
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EXPLANATION OF PLATE VI

Fig. 35. The LET body in the rectum of a larva at stage 56. The space in the lymphoid accumulation (arrow) is an artifact made during the preparation. × 200.

Fig. 36. Lymphoid accumulation (arrow) in the gut of a larva at stage 49. Note the close relationship of accumulated lymphocytes with the gut epithelium. × 200.

Fig. 37. A cross section of the mesonephros of a larva at stage 56. Myeloid tissue under the ventromedial wall of the kidney (arrows) is prominent. × 200.

Fig. 38. A higher magnification of the myeloid tissue in larval kidney shown in Fig. 37, showing the predominance of neutrophilic granulocytes in the tissue. × 500.

Fig. 39. Lymphoid aggregation between the renal tubules of the kidney in a toad. × 500.

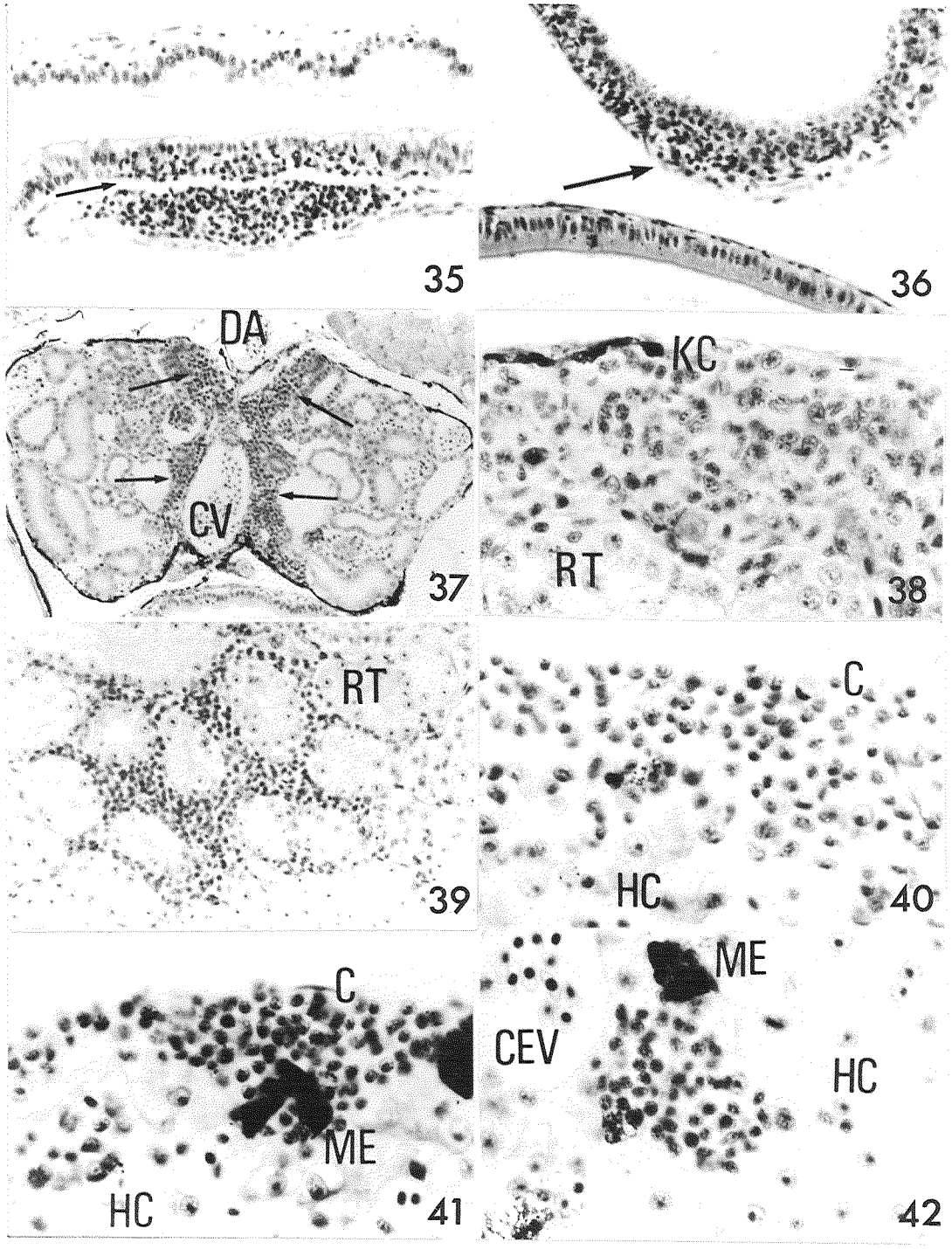
Fig. 40. Subcapsular region of the liver in a toad, showing an accumulation of free cells beneath the thin liver capsule. Among free cells, many neutrophils and other types of granulocytes are seen. × 500.

Fig. 41. Aggregation of small lymphocytes beneath the liver capsule. × 500.

Fig. 42. Lymphoid aggregation around the central vein of the liver in a toad. × 500.

ABBREVIATIONS

C, capsule
CEV, central vein
CV, caval vein
DA, dorsal aorta
HC, hepatic cell
KC, kidney capsule
ME, melanophore
RT, renal tubule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41
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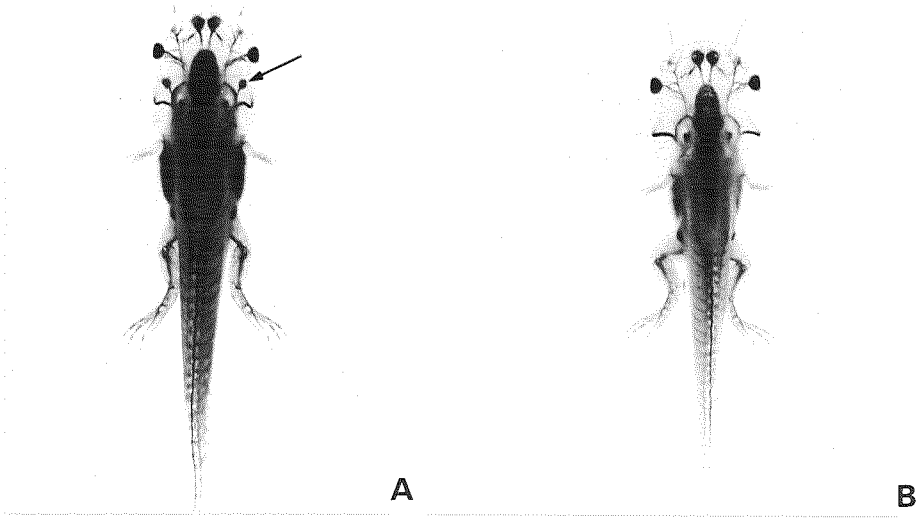
EXPLANATION OF PLATE VII

Fig. 43. *Xenopus* larvae at stage 59, either intact (A) or previously thymectomized at stage 45 (B). The arrow indicates the right thymus. Fresh materials. × 1.5.

Fig. 44. Cross sections of the spleens of non-thymectomized (A) and thymectomized (B) toads that were sacrificed 28 days after RRBC administration. Note the difference in size of the lymphoid populations in and around the white pulps (deeply stained areas) between the two animals. × 20.

Fig. 45. The white pulps of the spleens of non-thymectomized (A) and thymectomized (B) toads. Note the difference in the volume of white pulps (arrows). Both animals had received RRBC administration. × 100.

43



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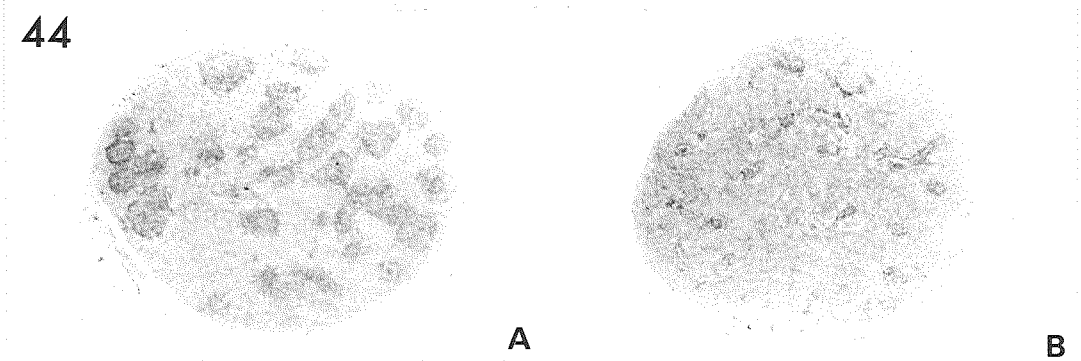

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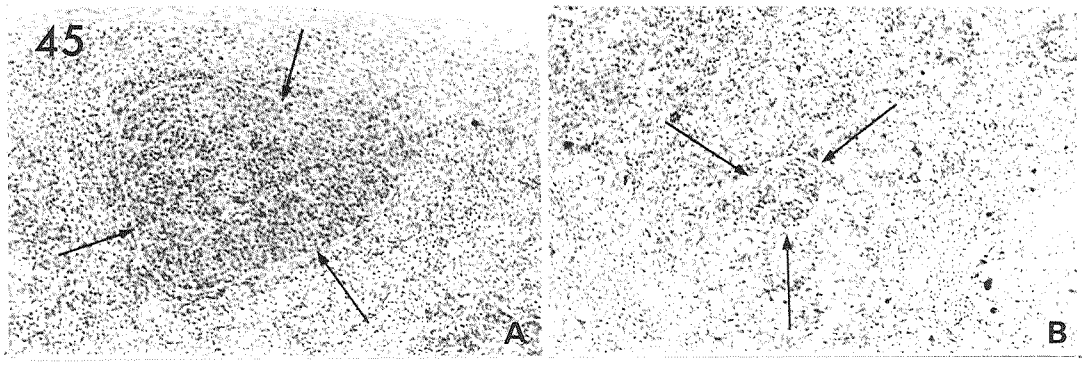


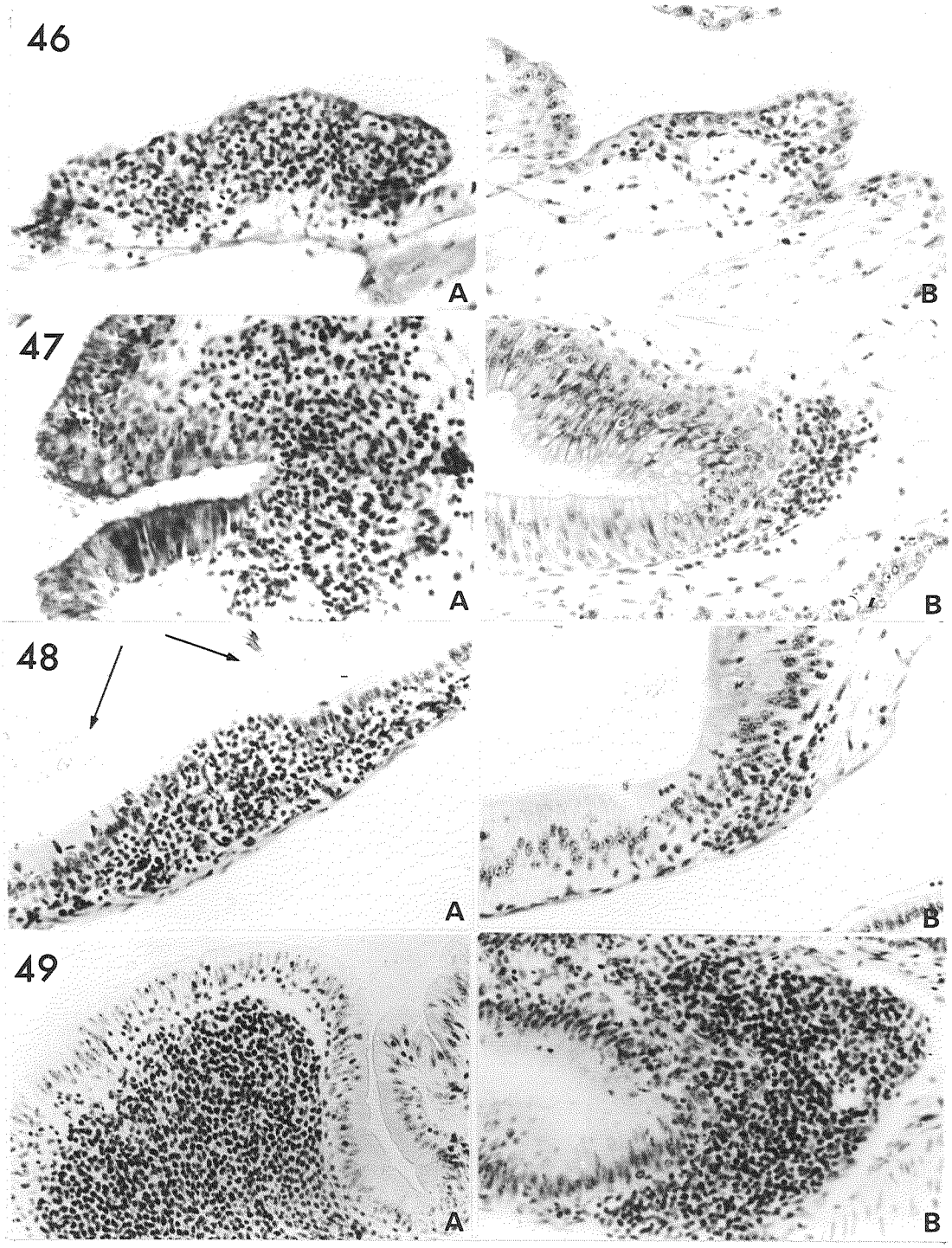
EXPLANATION OF PLATE VIII

Fig. 46. The second VCBs of the control (A) and the thymectomized (B) larvae at stage 56, showing the distinct difference in size of lymphoid population between the two. × 200.

Fig. 47. The posterior DCBs of control (A) and the thymectomized (B) larvae at stage 56, showing the difference in the degree of lymphoid accumulation and invasion into epithelium. × 200.

Fig. 48. The LET bodies in the ileum of the control (A) and the thymectomized (B) larvae at stage 56. Difference in size of the lymphoid population is evident. Sometimes the animal is parasitized by *Opalina* (arrows). × 200.

Fig. 49. The LET bodies in the small intestine of the control (A) and the thymectomized (B) toads. Note that the bodies in both animals are occupied by lymphoid populations of the similar size. × 200.


46
A
B
47
A
B
48
A
B
49
A
B

EXPLANATION OF PLATE IX

Fig. 50. Liver of an early-thymectomized toad which died of some specific infectious disease. Necrotic foci are evident among the normal hepatic cells. × 100.

Fig. 51. A higher magnification of the liver shown in Fig. 50, showing necrotic focus with a large number of melanin granules and cellular debris. × 200.

Fig. 52. A large blood vessel of the liver in the toad shown in Fig. 50, containing a large number of monocytic cells. × 200.

Fig. 53. The lung of a normal toad. Lumens are divided by septae with melanophores. × 100.

Fig. 54. Lung of an early-thymectomized-and-infected toad, showing lumens filled with blood. Compare with Fig. 53. × 100.

Fig. 55. Same lung as shown in Fig. 54. A lymphoid aggregation is seen in the septum. × 100.

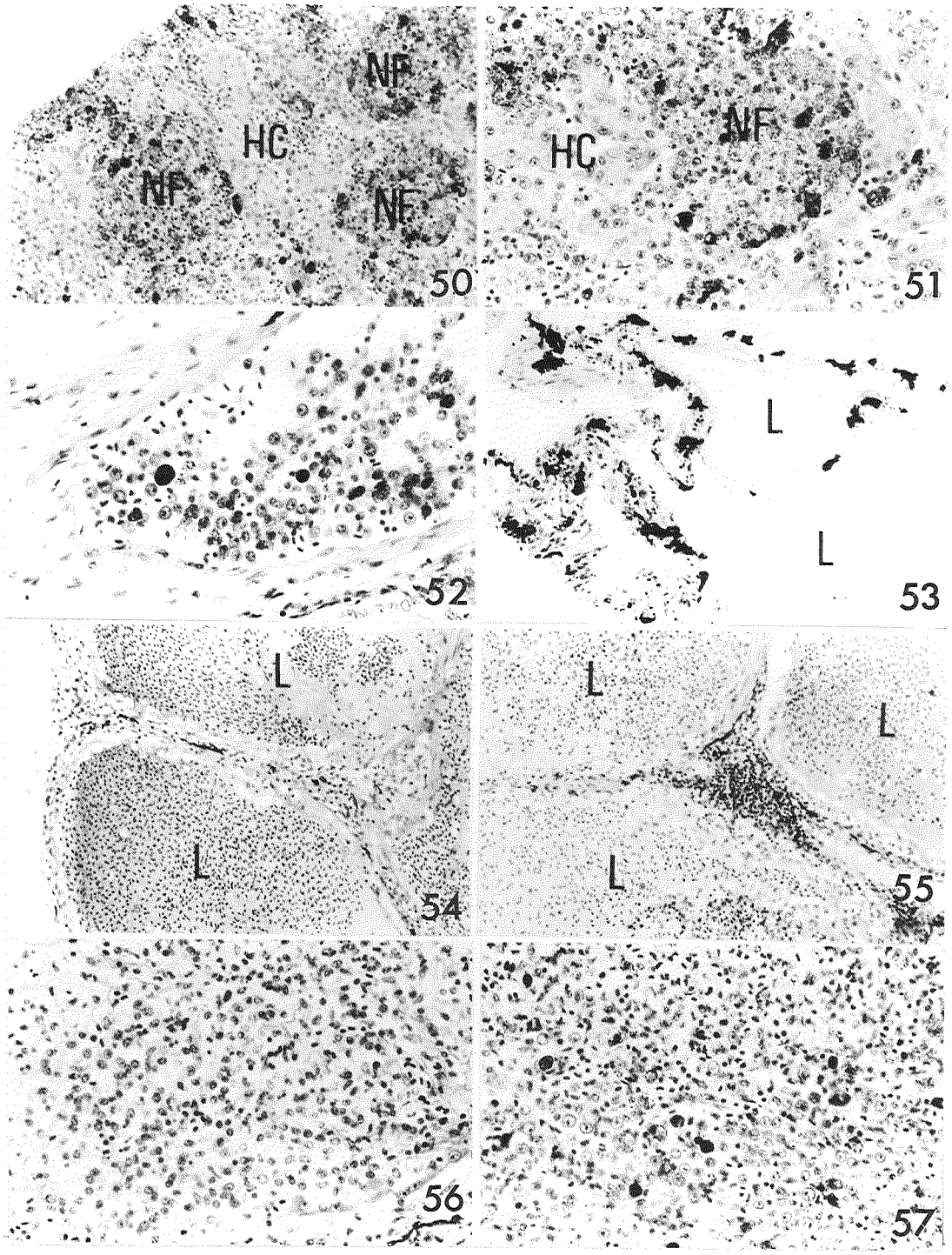
Fig. 56. A part of the lumen of infected lung showing a large population of monocytic cells. × 200.

Fig. 57. Red pulp in the spleen of an infected toad. Besides the erythrocytic series cells, the monocytic cells similar to those observed in liver and lung are abundant. × 200.

ABBREVIATIONS

HC, hepatic cell
L, lumen
NF, necrotic focus

PLATE IX



EXPLANATION OF PLATE X

Fig. 58. Section through the graft and the host skin 15 hr after the grafting, showing complete fusion of the two. Arrows indicate the boundary between the two. × 100.

Fig. 59. Higher magnification of the base of the cut edge of the skin shown in Fig. 58. A number of polymorphonuclear leukocytes (arrows) are seen to accumulate in this region. × 500.

Fig. 60. Allografted skin in the rejection phase I (2 days after the grafting), showing that the graft still retains a normal structure. × 100.

Fig. 61. The edge of allograft (arrows) at the end of the rejection phase I (3 days after the grafting). Several cells with phagocytic inclusions are conspicuous. × 500.

Fig. 62. Basal portion of an allograft at the end of the rejection phase II (8 days after the grafting), showing lymphocytic aggregation around dilated blood capillary in connective tissue. × 500.

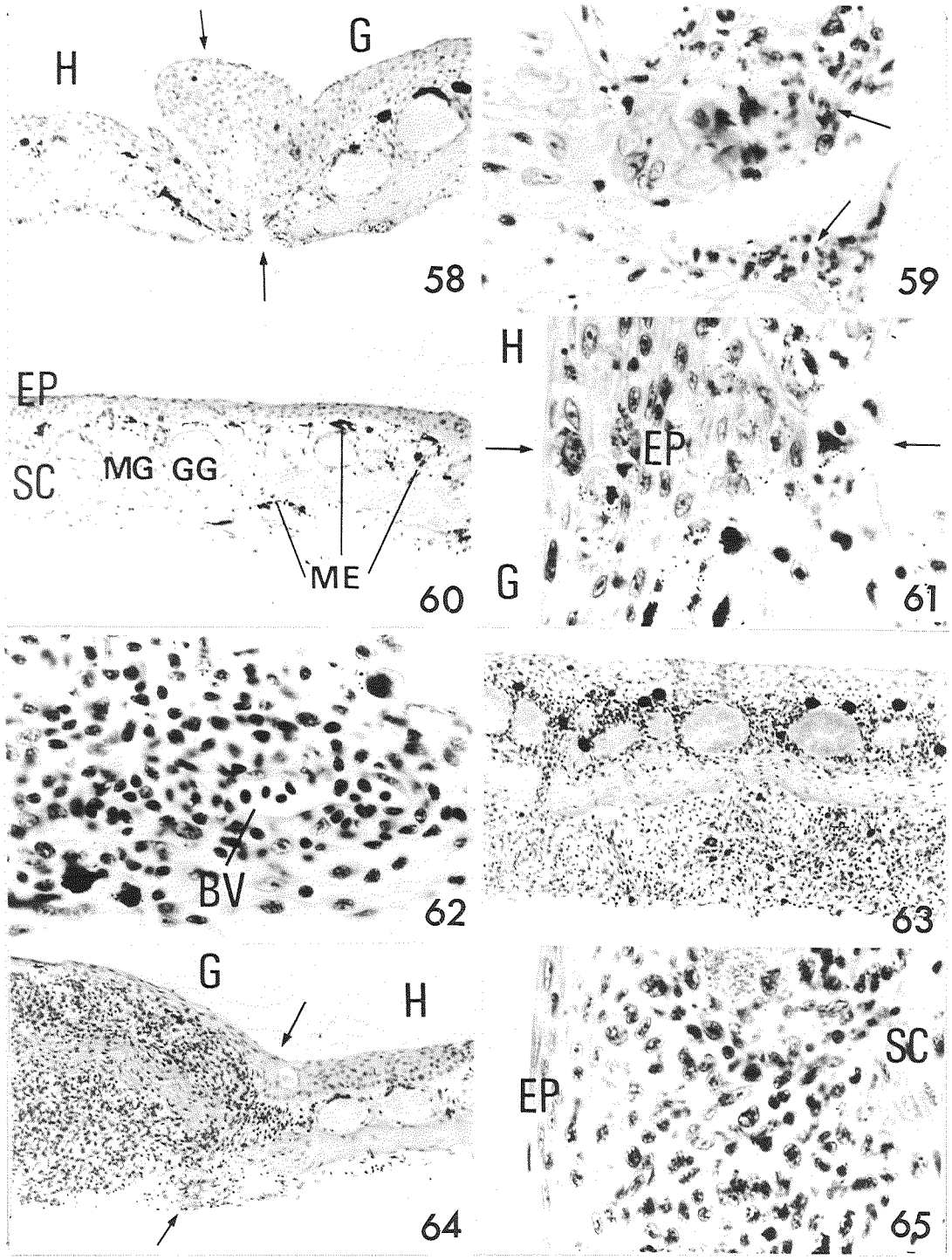
Fig. 63. Allografted skin in the rejection phase III (14 days after the grafting). Although the graft is heavily invaded by lymphocytes, melanophores and gland cells still look intact. × 100.

Fig. 64. Allografted skin in the rejection phase IV (16 days after the grafting), showing the climax of its destruction. The lymphcoytic invasion is so heavy that the whole grafted area is occupied by lymphocytes. Arrows indicate the boundary of the graft. × 100.

Fig. 65. Part of the graft shown in Fig. 64, showing aggregation of small and medium-sized lymphocytes. × 500.

ABBREVIATIONS

BV, blood vessel
EP, epidermis
G, graft
GG, granular gland
H, host skin
MG, mucous gland
SC, stratum compactum


H
G
58
59
EP
SC
MG GG
ME
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G
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BV
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G
H
64
SC
EP
65

EXPLANATION OF PLATE XI

Fig. 66. Allografted skin near the end of the rejection phase IV (18 days after the grafting), showing a decrease in number of accumulated lymphocytes as compared with the graft shown in Fig. 64. × 100.

Fig. 67. Higher magnification of the lymphoid aggregation as shown in Fig. 66. A large number of phagocytes are evident with their engulfed melanin granules. Compare with Fig. 65. × 500.

Fig. 68. Allografted skin in the final stage of rejection (30 days after the grafting). Note the stratum compactum is under destruction. Most lymphocytes have disappeared. Arrows indicate the boudary. × 100.

Fig. 69. Higher magnification of the grafted area after the "end" of its rejection. A small number of phagocytes (arrows) still remain in the area. × 500.

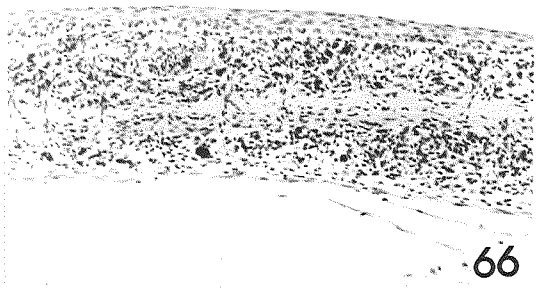
Fig. 70. The skin in allografted area 35 days after the grafting, showing the complete displacement of the graft by unorganized tissues of host origin. Inflammatory cells are no longer observed. × 100.

Fig. 71. Allografted skin in an early-thymectomized toad, 122 days after the grafting. Demarcation of the graft and the host skin (arrows) is less conspicuous. Note that the graft looks completely normal without any signs of lymphocytic invasion. × 100.

Fig. 72. Surface view of allografted skins in non-thymectomized (A) and early-thymectomized (B) toads, 135 days after the grafting, respectively. Arrows indicate the original outline of the graft. The grafted area in intact toad has been replaced by host tissues together with chromatophores. Fresh materials. × 30.

ABBREVIATIONS

EP, epidermis
G, graft
H, host skin
SC, stratum compactum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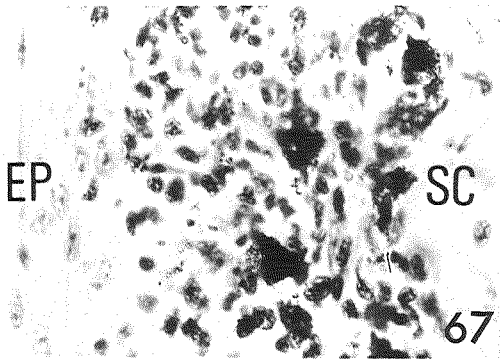
EP
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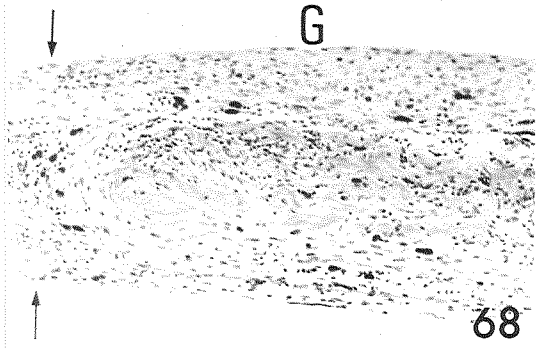
G
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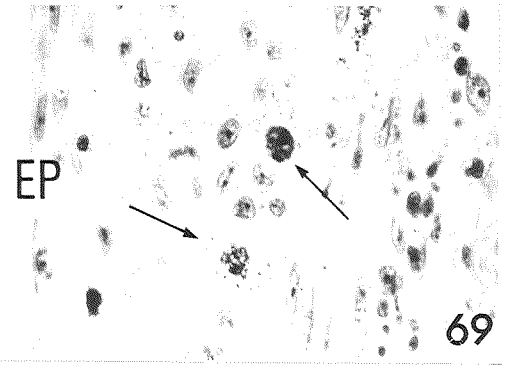
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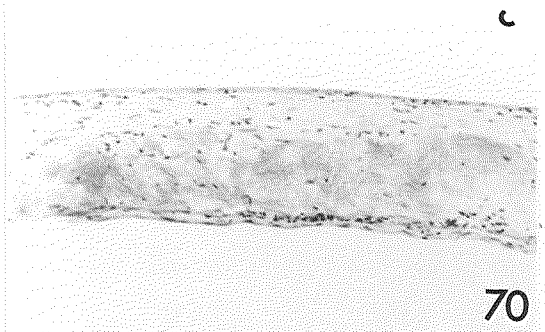
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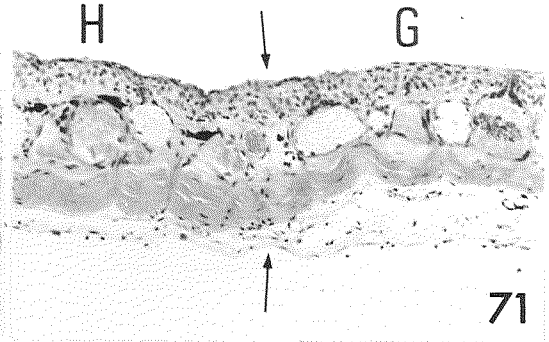
H
G
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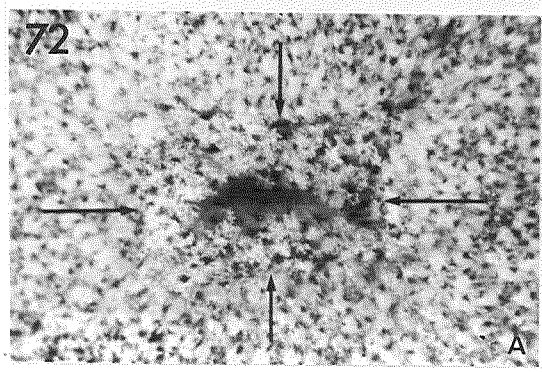
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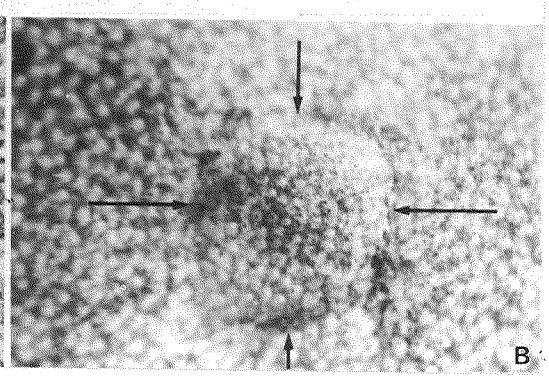
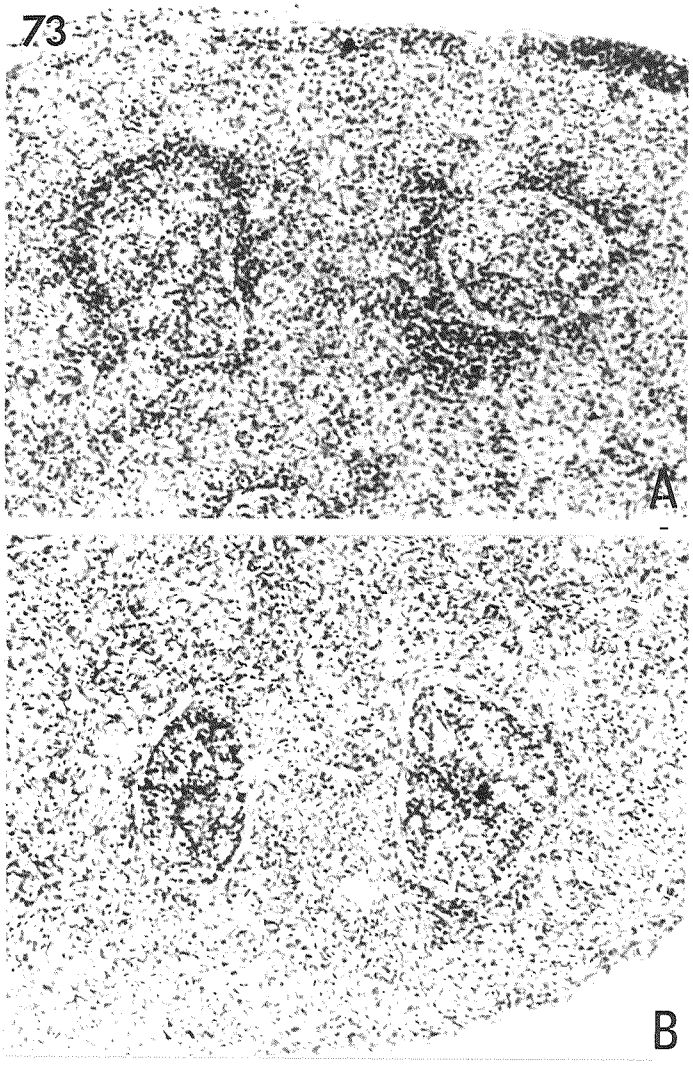
A


B

EXPLANATION OF PLATE XII

Fig. 73. Sections through a part of the spleens from a non-thymectomized (A) and an early-thymectomized (B) toads. Animals received three injections of polyvinylpyrrolidone, and fixed four weeks after the last injection. Note a remarkable difference in lymphoid accumulation in the red pulps surrounding the white pulps, but not in the white pulps, between the two specimens. × 150.

PLATE XII



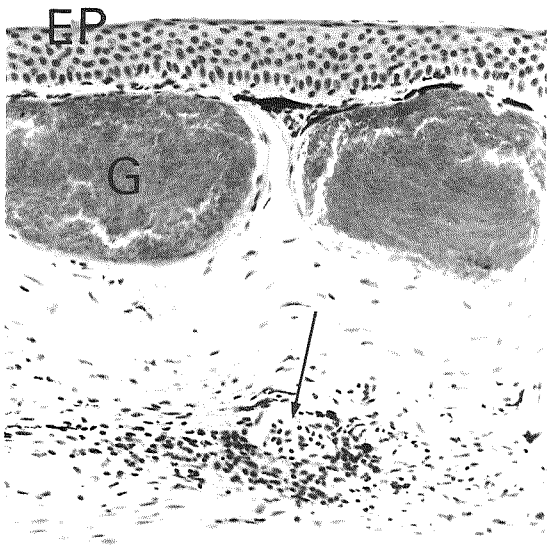
EXPLANATION OF PLATE XIII

Fig. 74. Section through a second-set skin allograft in a thymectomized-and-thymus-implanted toad which was still carrying a healthy first-set graft (fixed 35 days after second-set grafting). Only a small number of lymphocytes are aggregated around a dilated blood vessel (arrow). × 150.

Fig. 75. A pair of implanted thymuses 9 weeks after their implantation, showing perfect integrity of the organs. × 50.

ABBREVIATIONS

EP, epidermis
G, skin gland (granular gland)
VS, ventral skin of host animal



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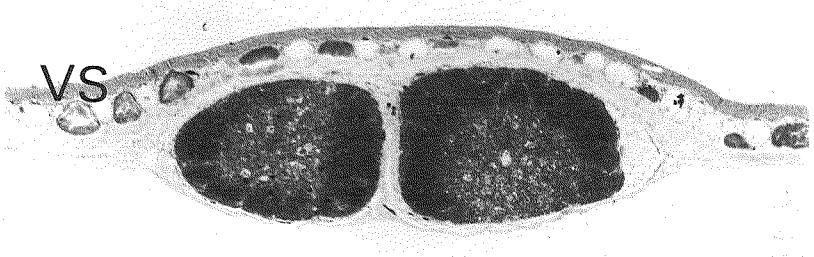


75

EXPLANATION OF PLATE XIV

Figs. 76-83. Glycol methacrylate-embedded, thin sections stained with toluidine blue.

Fig. 76. Thymus rudiment from a 5-day-old larva. Deeply stained lymphocytic precursor cells and paler-staining epithelial cells are readily distinguished. The arrow indicate a cell in mitosis. × 750.

Fig. 77. Thymus taken from a 5-day-old larva and cultured *in vitro* for 10 days. Note the absence of lymphoid cells. × 750.

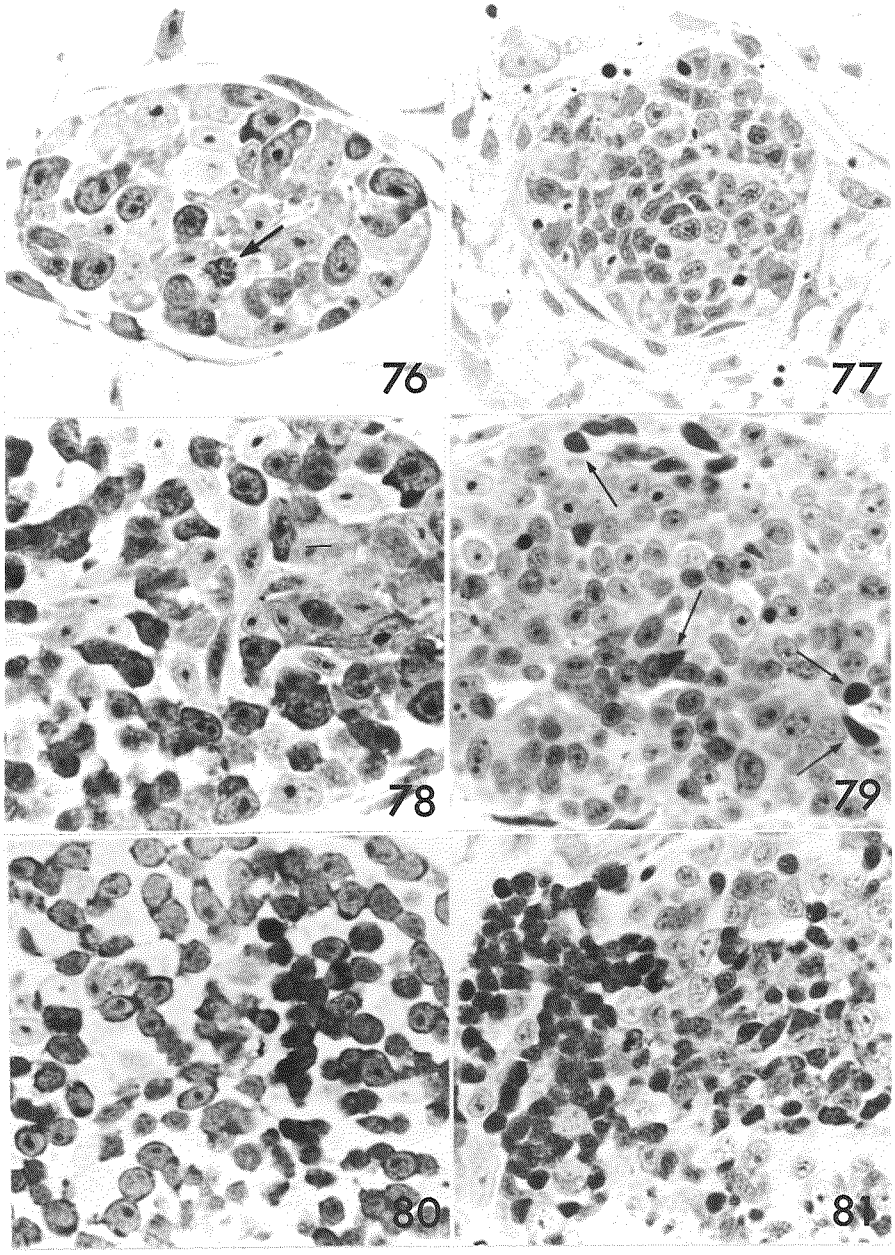
Fig. 78. Thymus from a 7-day-old larva, having a number of large lymphocytes with deeply stained cytoplasm. × 750.

Fig. 79. Thymus taken from a 7-day-old larva and cultured *in vitro* for 10 days. Note that the the thymus consists of more epithelial cells and fewer lymphoid cells (arrows), as compared with the one shown in Fig. 78. × 750.

Fig. 80. Thymus from a 9-day-old larva, showing that the size of lymphocytes is smaller than in the previous stage (Fig. 78). × 750.

Fig. 81. Thymus taken from a 9-day-old larva and cultured *in vitro* for 10 days. Typical small lymphocytes are differentiated. To left, a densely packed lymphoid aggregation is seen. × 750.

PLATE XIV

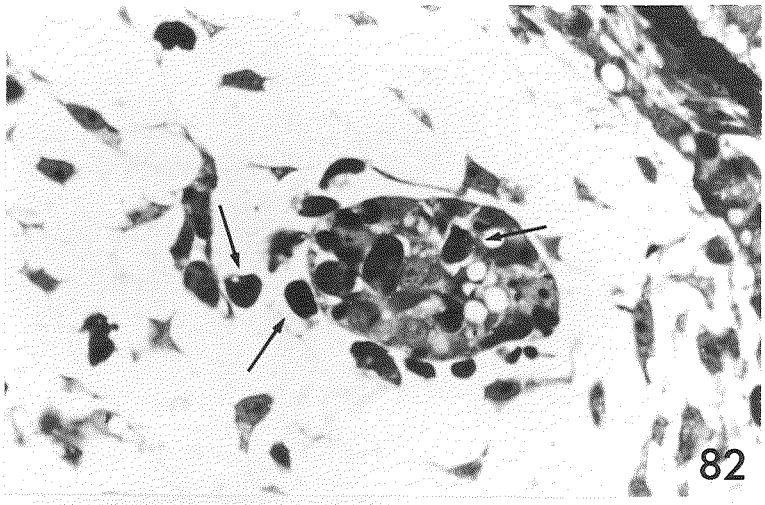


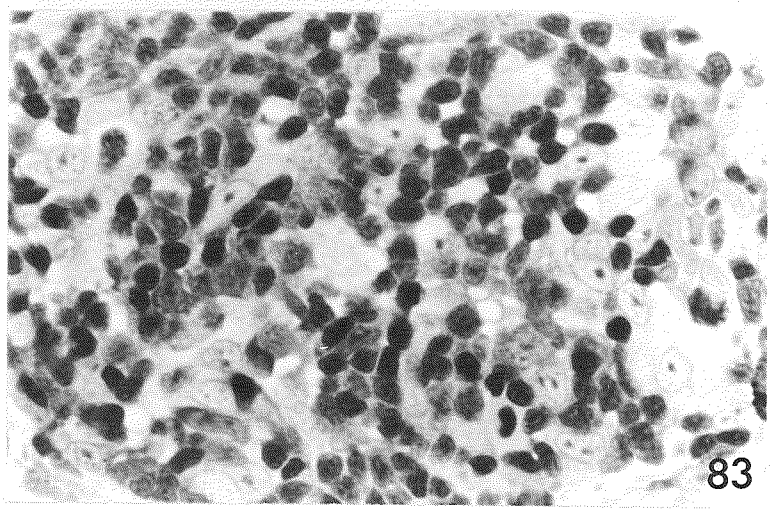
EXPLANATION OF PLATE XV

Fig. 82. Thymus rudiment from a 3-day-old larva. The rudiment comprises yolk-containing epithelial cells and deeply stained lymphoid precursor cells (arrows). Note that similar lymphoid precursor cells are also seen in the mesenchyme around the rudiment. × 750.

Fig. 83. Thymus taken from a 3-day-old larva as a rudiment, grafted into 40-day-old tadpole, and fixed 10 days later. Differentiation of typical small lymphocytes proceeded excellently. × 750.

PLATE XV





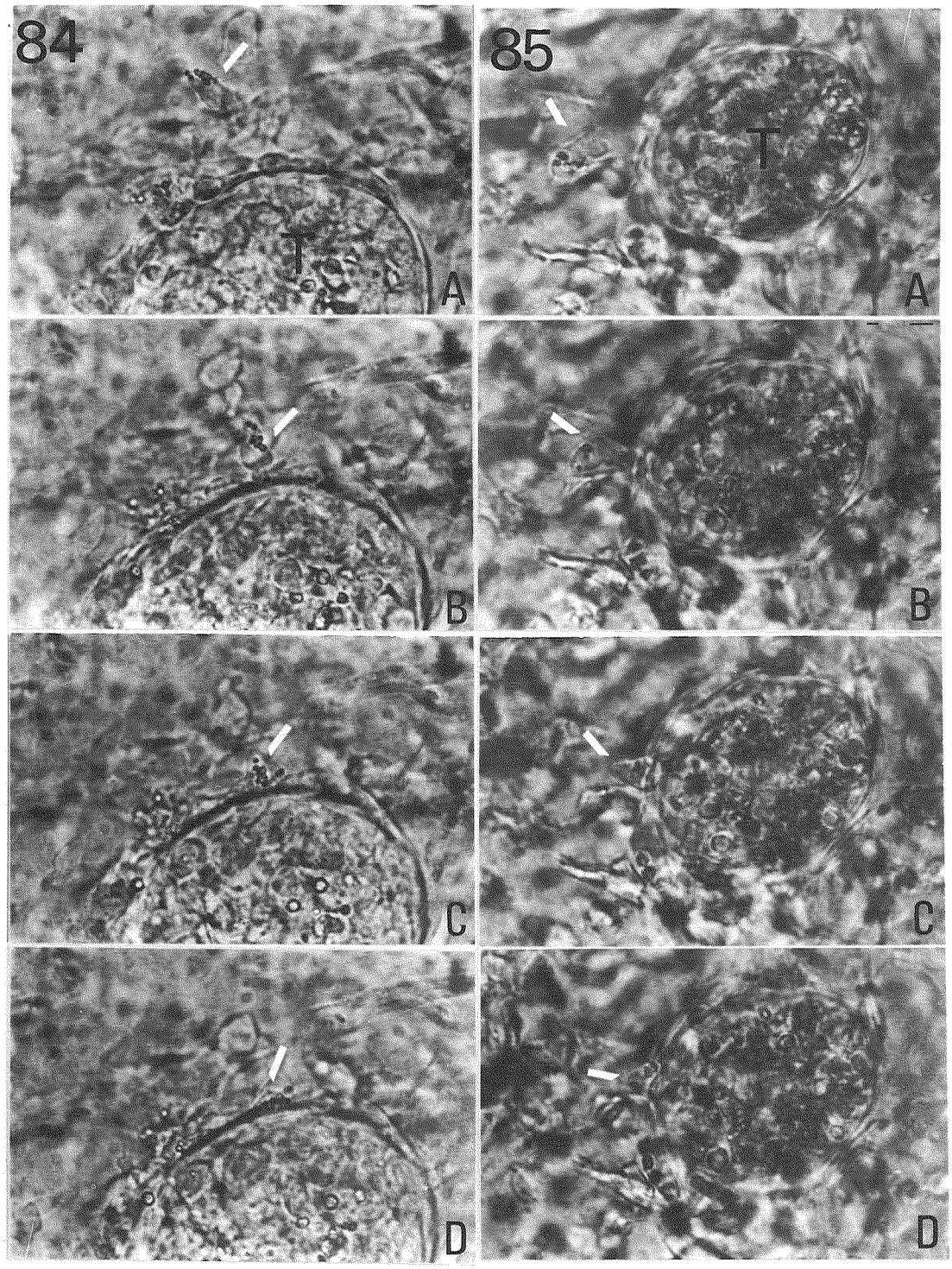
EXPLANATION OF PLATE XVI

Figs. 84 and 85. Two series of microphotographs showing 4 different stages of migration of amoeboid cells (arrows) in mesenchyme toward thymus rudiment (T) in 4-day-old larvae. The same cell is indicated by an arrow in each photograph. Photograped in the live specimen. × 1,000.

Fig. 84. Photographed at 0 (A), 3 (B), 5 (C), and 8 min (D), respectively.

Fig. 85. Photographed at 0 (A), 8 (B), 10 (C), and 13 min (D), respectively.

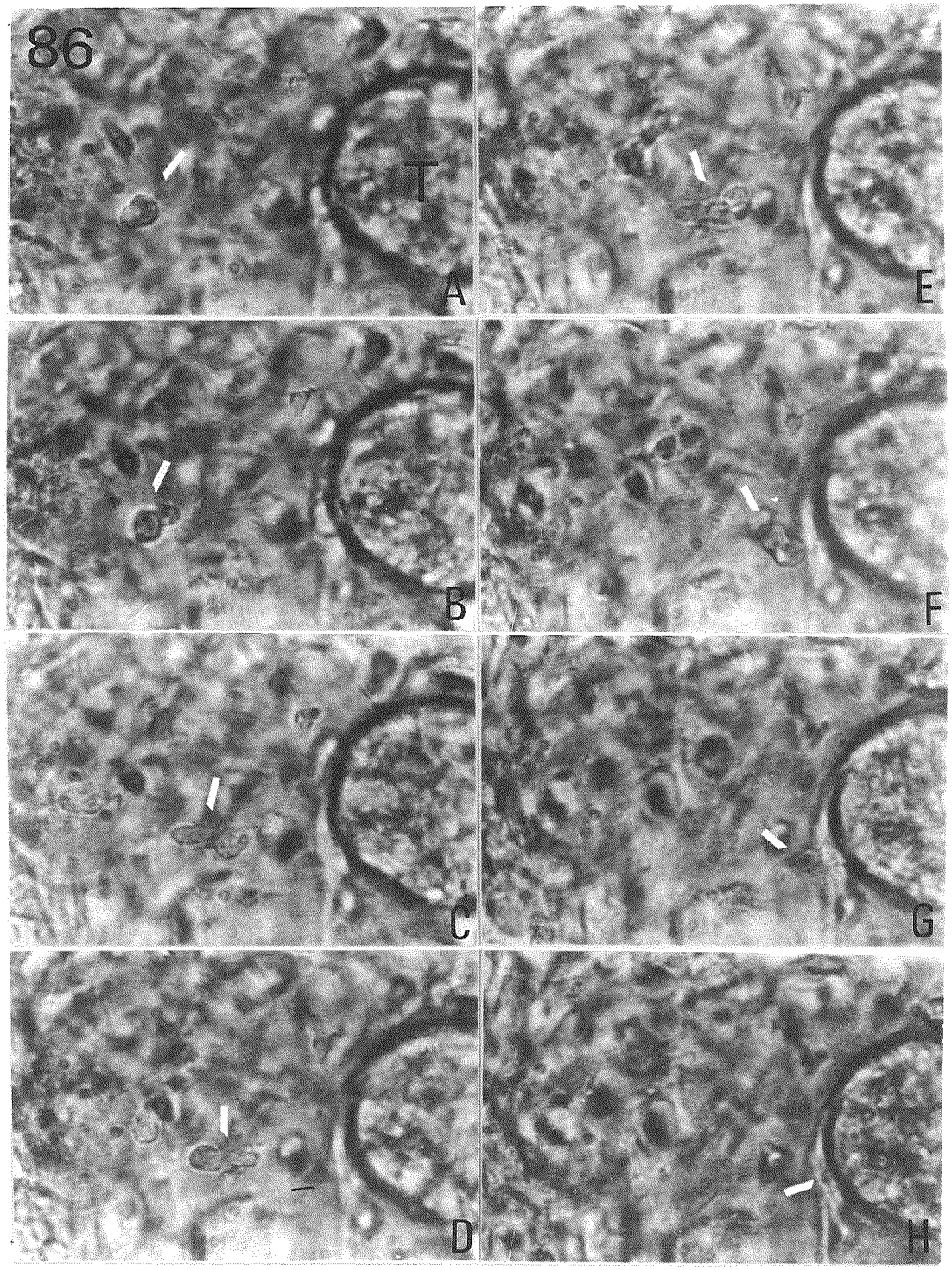
PLATE XVI



EXPLANATION OF PLATE XVII

Fig. 86. A series of microphotographs showing 8 different stages of migration of an amoeboid cell (an arrow in each photograph) toward the thymus rudiment (T) of a 4-day-old larva. Photographed at 0 (A), 7 (B), 17 (C), 22 (D), 46 (E), 60 (F), 66 (G), and 73 min (H), respectively. The cell in the course of migration, exhibited variable shapes by protruding lobopodia in several directions, drew toward the thymus rudiment, and was ultimately engulfed by the latter. × 1,000.

PLATE XVII



EXPLANATION OF PLATE XVIII

Fig. 87. Higher magnification of amoeboid cells (arrows) in mesenchyme from 4-day-old larvae. Squashed preparations of fresh materials, observed by a phase contrast microscope. Yolk granules and mitochondria in the cytoplasm look granular. × 2,000.

PLATE XVIII