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ASPECTS AND PROLIFERATION OF FREE CELLS IN THE COELOMIC FLUID OF NEWTS, *TRITURUS PYRRHOGASTER*

By

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Introduction

In the normal condition, the coelomic fluid of newts contains free cells of several types, which are recognized from the cytological preparations as the monocytes, lymphocytes, granulocytes, histiocytes, and the cell of a peculiar morphology that is characterized by a cytoplasmic inclusion and a bipolar process. As a general rule, these coelomic cells of newts scarecely show their mitotic events. However, as has been reported by Mizutani and Nakahara (1961), when the peritoneal cavity was recieved certain stimuli such as daily abdominal punctures, daily injections of a hypotonic solution, or a saline solution, some mitotic figures were found in the coelomic free cells.

The emergence of large numbers of dividing cells, under the influence of phyto-hemagglutinin (PHA), has been extensively studied on mammalian leukocytes *in vitro* (Nowell 1960, Robbins 1964, Seto 1968a, and others). In contrast to the fact, little knowledge on influence of the administration of PHA to free cells *in vivo* has been presented. Studies of cellualr responses in newt ascites to the PHA-injection are thus interesting in many respects. Also induction of mitosis of an adult amphibian animal *in vivo* is of valuable for cytological and cytogenetical studies not only for contributing toward knowledge on the peritoneal cell of a newt itself. Only a few people have hitherto noticed upon interesting phenomena of free cells in newt peritoneal cavity, especially of characteristic cells with Russell's bodies. It is worthwhile to make further detail observations on the ascites cells of the animal owing to inadequate knowledge on the origin of the peculiar type of cells, which has an important meaning of cellular transformation in an adult animal *in vivo*.

The present study has been undertaken to investigate responses *in vivo* of free cells in the newt peritoneal cavity to three kinds of stimuli, especially to a mitogenic agent. The experiments were also designed to clarify whether the characteristic cells with Russell's bodies have potenciality of proliferation in the adult peritoneal cavity or have to be considered being the end cell in the pathway of differentiation.

Materials and Methods

Adult male and female newts, *Triturus pyrrhogaster*, were collected in the vicinity of Matsue and Kyoto, which had been stocked in a water tank of the laboratory. Animals were regularly fed liver fragments from cow and pig once a week. Free cells occurring in the peritoneal cavity of newts were obtained simply by abdominal puncture and aspiration with a capillary pipette. A drop of peritoneal fluid was placed on a

slide glass and made a smear preparation immediately after aspiration. Methanol-fixed cells were stained with the May-Grünwald Giemsa or Feulgen stain. The observation of living cells with a phase-contrast microscope were carried out by means of the hanging-drop method in combination with liquid paraffin (Makino and Nakahara 1953) or by the Rose culture-chamber method (Seto 1968b).

For inducing mitosis of the coelomic free cell *in vivo*, following procedures were applied : 1) daily injection of 0.1 ml distilled water into a newt peritoneal cavity for 15 days ; 2) daily injection of 0.1 ml amphibian Ringer solution in like manner ; and 3) a single or repeated injection of phytohemagglutinin (PHA-M, Difco Laboratories, Detroit). Each ampulla of PHA-M contains about 50 mg dry substance, which was dissolved in 5 ml of distilled water. This stock solution was diluted further with distilled water to make 10 per cent solution of PHA-M. The animal was injected intraperitoneally 0.1 ml of the working solution.

Results

Observations of Living Free Cells.

As described above, phase-contrast microscopy observation was undertaken for studying dynamic aspects of these free cells. Cells were placed in the Rose chamber together with culture medium for amphibians (Seto 1963) or in a liquid paraffin (Makino and Nakahara 1953). The former preparation could maintain cells in living state for more than 72 hours, and the latter was put to use for the short-term observation which gained higher resolution for a phase-contrast microscope.

In the peritoneal fluid, four types of free cells could be easily distinguished in the living state (Figs. 1-6). Monocytes with a oval or bean-shaped nucleus which has a thin but well-defined nuclear membrane was identified from its nucleocytoplasmic ratio, that was relatively low. The cytoplasmic outline was irregular rather than smooth (Fig. 3). When the cell was maintained over 24 hours *in vitro*, the edges of cytoplasm became sharply wavy and number of vacuoles appeared at the inside of cytoplasmic membrane. The phenomenon indicated the occurrence of active pinocytosis or phagocytosis (Figs. 3, 6, and 12). Generally monocytes cannot always be distinguishable from large lymphocytes and are seen to consist of quite different appearance each other.

Lymphocytes of the peritoneal fluid appeared much the same as typical lymphocytes of the blood. They composed from 25 to 30 per cent of the cell population in control newts. Small and large types of these cells could be found. In the present observation, however, large lymphocytes were classified into the monocyte group since these cells were not clearly defined from monocytes. Small lymphocytes have a small amount of translucent cytoplasm which possessed a smooth outline and a round, relatively large sized nucleus. Therefore the nucleo-cytolplasmic ratio was the highest in the free cell population (Figs. 8 & 10). Both monocytes and lymphocytes actively moved around in the culture medium *in vitro*.

Granulocytes, both of neutrophilic and eosinophilic, have a very characteristic polymorphous nucleus such as elongated, lobed, or doughnut-shaped. Nucleolus could hardly be identified in the nucleus. Cytoplasm was usually smoothly wavy in outline, and was packed with minute granules which were identified with acidic or basic stain.

Proportions of granulocytes in the free cell population varied from 6 per cent to over 50 per cent after treatments. This incidental variation in the cell number was observed remarkably after administration of a severe irritant. Frequency of a granulocytic appearance was rapidly affected by the stimuli into a peritoneal cavity (Figs. 7 & 9).

Cells with Russell's bodies were outstandingly large size in the free cell population. They were easily distinguished from other types of free cells on account of having bipolar protoplasmic processes (Fig. 2). The process was clearly observed in the living cells with a phase-contrast microscopy (Fig. 4), while in the stained preparation sometimes it became obscure. As shown in Figures 17–25, these cells were quite polymorphous, indeed, in cytoplasmic processes and in the peculiar cytoplasmic inclusions : Some of bipolar processes formed thick bundles of filaments and some of them consisted of thin multistrands ; cytoplasmic inclusions appeared to be sometimes granular, dot-like, or rod-like crystals .and sometimes be opaque vacuoles containing full of dense substances. In primitive-type cells, cytoplasm contained a vacuole (or vacuoles) instead of polygonal bodies with a high reflaction (Figs. 10, 11, & 15). The nucleus was found lying at the periphery of the cytoplasm or in between the inclusion bodies in a somewhat irregular shape (Figs 23–25). Occasionally binuclear or trinuclear cells with Russell's bodies were found in low frequency (Figs. 21 & 22). It is interesting to note that when coelomic free cells were transferred into *in vitro* conditions these characteristic cells readily tended to degenerate within 24 hours.

Cellular Reactions to Mild Stimuli.

Of three types of stimuli used, the amphibian Ringer solution or distilled water served as a mild irritant. Daily intraperitoneal injections of 0.1 ml of either hypotonic or saline solution to a group of adult newts continued for 15 days. Cytological preparations were made by taking ascites fluid once a couple of days alternatively. These injections caused a slight increase of granulocytes immediately after the treatment. Monocytes and small lymphocytes invariably commanded a majority of the cell population in the peritoneal fluid and these cells respectively took 30 to 40 per cent of total cell number. Characteristic cells with Russell's bodies and bipolar processes consistently appeared in all preparations in low frequency of 1 to 4 per cent. But when the sampling of peritoneal fluid was repeatedly undertaken, these cells gradually decreased in number.

The mitotic event occurred mostly in a group of monocytes. Mitotic figures were found after 7 to 9 days of treatments with a mild irritant *in vivo* (Fig. 9). The frequency was observed by counting the monocytes at mitosis in the total of 2,000 cells of all lymphocyte-type cells in the coelomic fluid. On the seventh day of the daily treatment the mitotic figures appeared most frequently at the rate of 0.3 per cent, though there were individual differences in mitotic activity.

Effects of PHA-M.

In contrast to the mild irritant, phytohemagglutinin (PHA-M) was seemed to be a kind of severe stimulant against the free cells. Newts were recieved 0.1 ml of 10 per cent PHA-M and kept in the same condition thereafter in the water tank. Ascites were taken out at 12, 24, and 48 hours after the treatment. A remarkable change appeared in coelomic cells after the PHA-injection was a considerable increase in granulocytes at

12th hour. This subsequent reaction of intraperitoneal cells was commonly observed after treatment with any kind of chemicals.

Mitotic cells appeared at 24th and 48th hour. On the basis of cytoplasmic shape and size, most dividing cells were regarded as monocytes (Figs. 26–28). Small lymphocytes were also affected by PHA-M and the mitotic figures appeared at the same time as monocytes, though the frequency was lesser than that of the monocyte mitosis. Mitotic rate of 1.0 to 1.2 per cent was obtained when the mitotic figures were most abundant at 24 to 48 hours after treatment in both monocytes and small lymphocytes (Figs. 29–36). Granulocytes have not shown their proliferative figures in the peritoneal cavity even after the PHA-injection. Generally, a mitotic aspect of cells with Russell's bodies was hardly found at any time. However the primitive type cells, suspected by a volume of cytoplasmic inclusions and undeveloped processes of protoplasm, rarely showed mitosis at quite low frequency (Figs. 14–16).

Discussion

The present paper, reported as a preliminary note, has mainly dealt with the cellular responses of coelomic free cells *in vivo* to three kinds of stimuli and aspects of these cells in living and fixed conditions. The ascites fluid of laboratory animals has been widely used for basic studies and experimental purposes of cytology since the treatment *in vivo* and cell preparation from the fluid are easily made, and the response of cells to a stimulus are promptly observed on occasion.

Coelomic free cells of mammalian animals have been extensively studied and the knowledge on these cells has read the conclusion that the peritoneal fluid of laboratory animals contained the same range and similar proportions of cell types as in human, and that the cellular response to stimulation follows a like course (Felix and Dalton 1955). However, only a few cytological studies have been done hitherto in relation to the coelomic free cell from amphibians. Ohuye (1936) has observed the cellular inclusions called Russell's bodies in newt free cells, and by Mizutani and Nakahara (1961) the induction of mitosis with a mild irritant have been examined.

The present study revealed that free cells in the peritoneal cavity were affected by daily injection of a hypotonic solution or a physiological saline solution. And monocytes and small lymphocytes were induced mitosis in low rate by these mild irritants. The result was nearly in accord with findings of Mizutani and Nakahara (1961). A similar fact has been also published by Kano (1953) on rat peritoneal free cells, describing that the increasing mitotic activity of monocytes was occurred *in vivo* by the use of physiological saline or glucose solution.

PHA-M, an agent known as a mitotic initiator, exerted a strong influence upon free cells in the coelomic fluid of newts. The typical effect was observed in an increase of mitotic rate exclusively of lymphocytes and monocytes. Granulocytes also showed gross but temporary increase in number immediately after treatment with the stimulant. However, the increase of granulocytes could be derived from the reaction of a host animal. Responses of cells with Russell's bodies to the stimulus have appeared to be almost insensitive, the cell showed invariable manner in number and morphology before and after treatment. The fact indicated that PHA-M, as well as other mild

irritants, did not directly affected to the cellular transformation of this characteristic cell. However, a primitive type of these cells seemed to be sensitive to stimuli.

Though publications concerning effects of PHA on free cells *in vivo* have been hardly found, some of which were valuable in some respects for the present study. An interesting study of PHA-effect on *Acanthamoeba* by Agrell (1968) has indicated a strong mitogenetic effect upon the free-living soil amoeba. His conclusion has been obtained on the basis of evaluation from the growth rate of the amoeba and it has been considered that PHA acted indirectly through the culture medium. This investigation was noticed in relation to the following point; PHA-effect is indirect to the free-living amoeba which is well-differentiated. Mitotic stimulation by PHA was exclusively occurred in lymphocytes and monocytes *in vivo*, as well as *in vitro* cultivation. Kano (1953) has also indicated the similar fact. Agreeable results were acquired by Gamble (1966) on the effects of PHA on mouse spleen cells *in vivo*.

Although characteristic cells in newt ascites having cytoplasmic inclusions have not shown any direct and immediate responses to stimulants described above, in three or four weeks later a remarkable increase of this type of cells have been observed in the coelomic fluid of the treated newts. The phenomenon was prominently noted in animals recieved the PHA-injection. Therefore, a suspection is made from this interesting fact that the characteristic cell may be transformed from lymphocytes or monocytes gradually in the peritoneal cavity. Further extensive study has to be done to prove a possible cellualr transformation.

Summary

Aspects of free cells in the coelomic fluid of newts have been studied before and after treatments of such irritants *in vivo* as ; 1) daily injection of a hypotonic solution, 2) daily injection of a isotonic saline solution, and 3) a single injection of 10% PHA-M solution.

When the peritoneal cavity recieved any of these stimuli, the relative proportions of most free cells in the cavity varied. Granulocytes rapidly and remarkably increased due possibly to the reflection of physiological changes of the host animal after the treatment.

Cellular responses to PHA-M were positively occurred in lymphocytes and monocytes, which indicated the mitotic initiating agent was proved to has the same effect to free cells *in vivo* as cultured lymphocytes *in vitro*.

Mitotic activities of monocytes and lymphocytes *in vivo* have been raised by the PHA-injection up to about 1 per cent within 24 hours of treatment. Characteristic cells with Russell's bodies have not been directly affected by a mild irritant or a mitogenetic agent.

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PLATE I

(Figures 1-6)

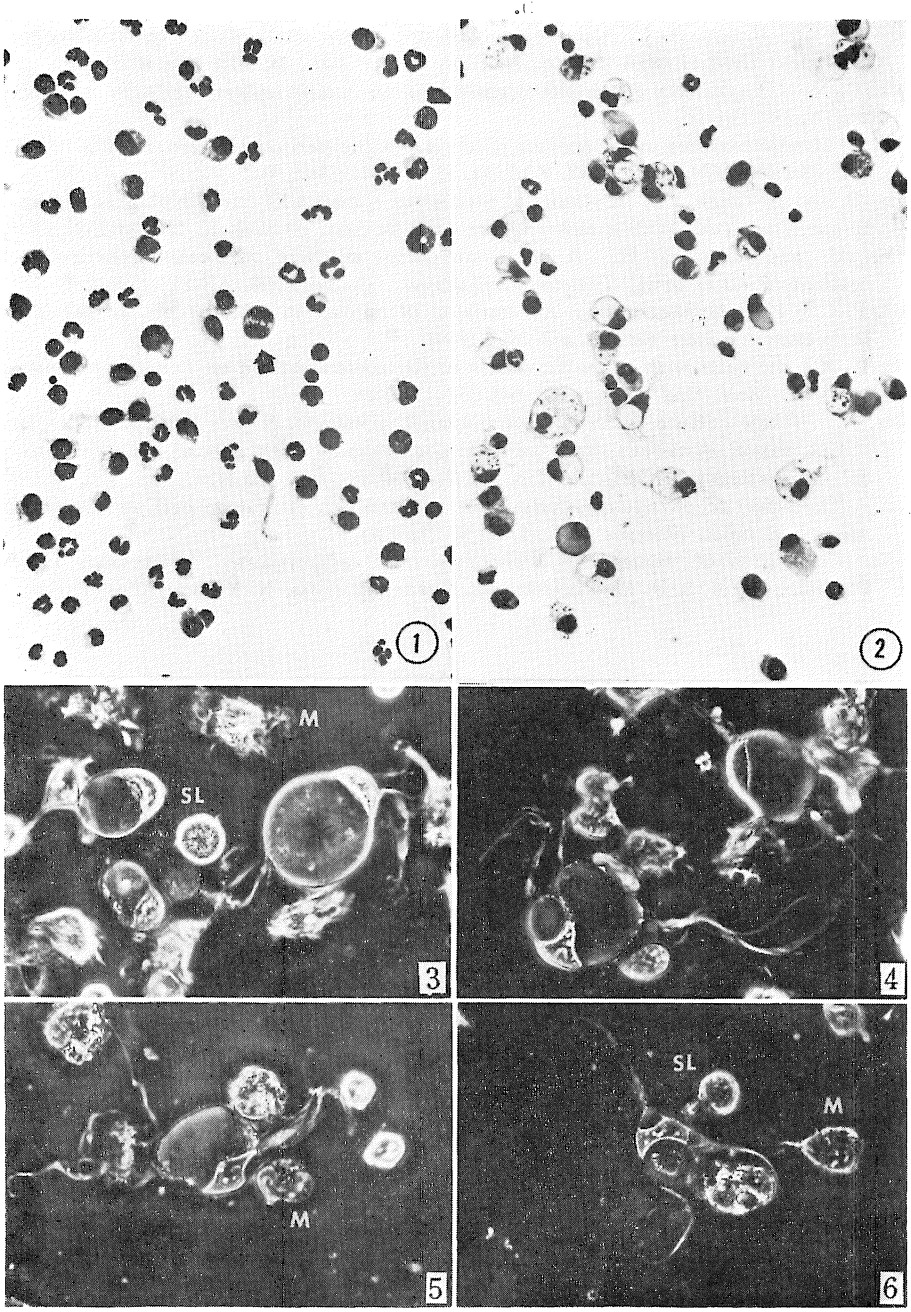

PLATE I

FIGURES 1–2. A general view of coelomic free cells of newts. Smear preparations, methanol-fixed, and May-Grünwald Giemsa stain. 1, cells from the treated newt with 0.1 ml of 10 per cent PHA-M solution intraperitoneally ; granulocytes unusually appear in abundance after the treatment and a mitotic cell is observed at 24 hours after PHA-injection (arrow). 2, cells from the newt that has been maintained for 4 weeks after the treatment. × 150.

FIGURES 3–6. Living free cells in peritoneal fluid which maintained in the Rose culture chamber, taken with bright-medium objectives of phase-contrast microscope (Nikon). Various types of cells with Russell's bodies are distinguished. Bipolar protoplasmic processes are clearly seen in the living preparation rather than the fixed. Small lymphocytes (SL) and monocytes (M) are movable, and pinocytotic activities of monocytes are commonly observed. × 400.

PLATE II

(Figures 7-16)

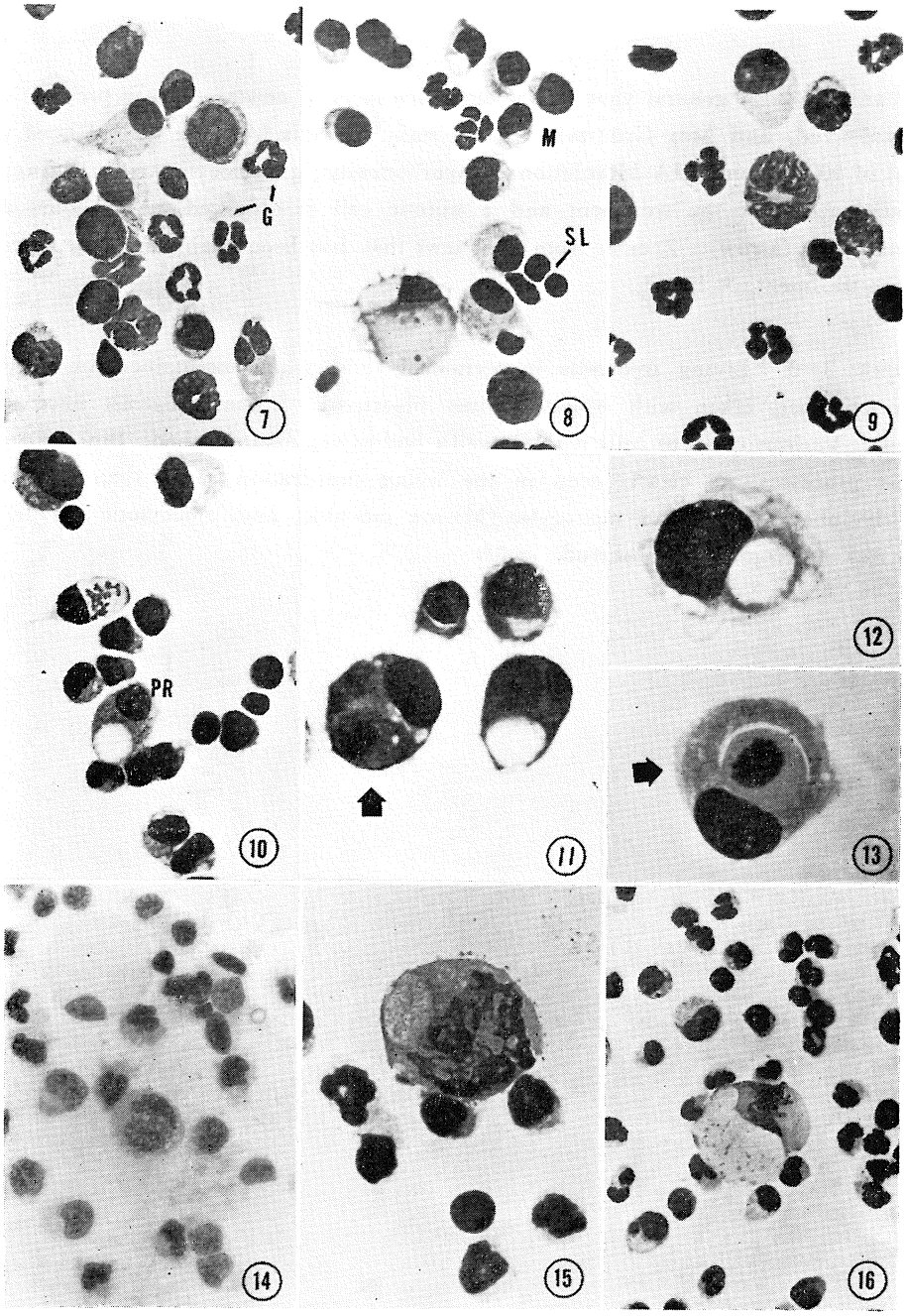

PLATE II

FIGURES 7–10. A group of cells from ascites of non-treated and treated newts, consisting of monocytes (M), small lymphocytes (SL), granulocytes (G), and cells with Russell's body. 7 & 8, from a normal newt. 9, from the treated newt by injection of a hypotonic solution for 7 days. 10, from the treated newt with a saline solution; a primitive type of the Russell's cell is suppositively pointed out (PR). × 400.

FIGURES 11–13. Pinocytotic and phagocytotic activities are seen in monocytes. 11 & 13, cells taking a erythrocyte in their cytoplasm (arrow). 12, a pinocytotic cell. × 600.

FIGURES 14–16. Vacuolized cells and a cell having bipolar cytoplasmic processes rarely show mitosis after 24 hours of PHA-M treatment. These cells possibly differentiate into characteristic cells with Russell's body. 14 & 16, × 400. 15, ×600.

PLATE III

(Figures 17-25)

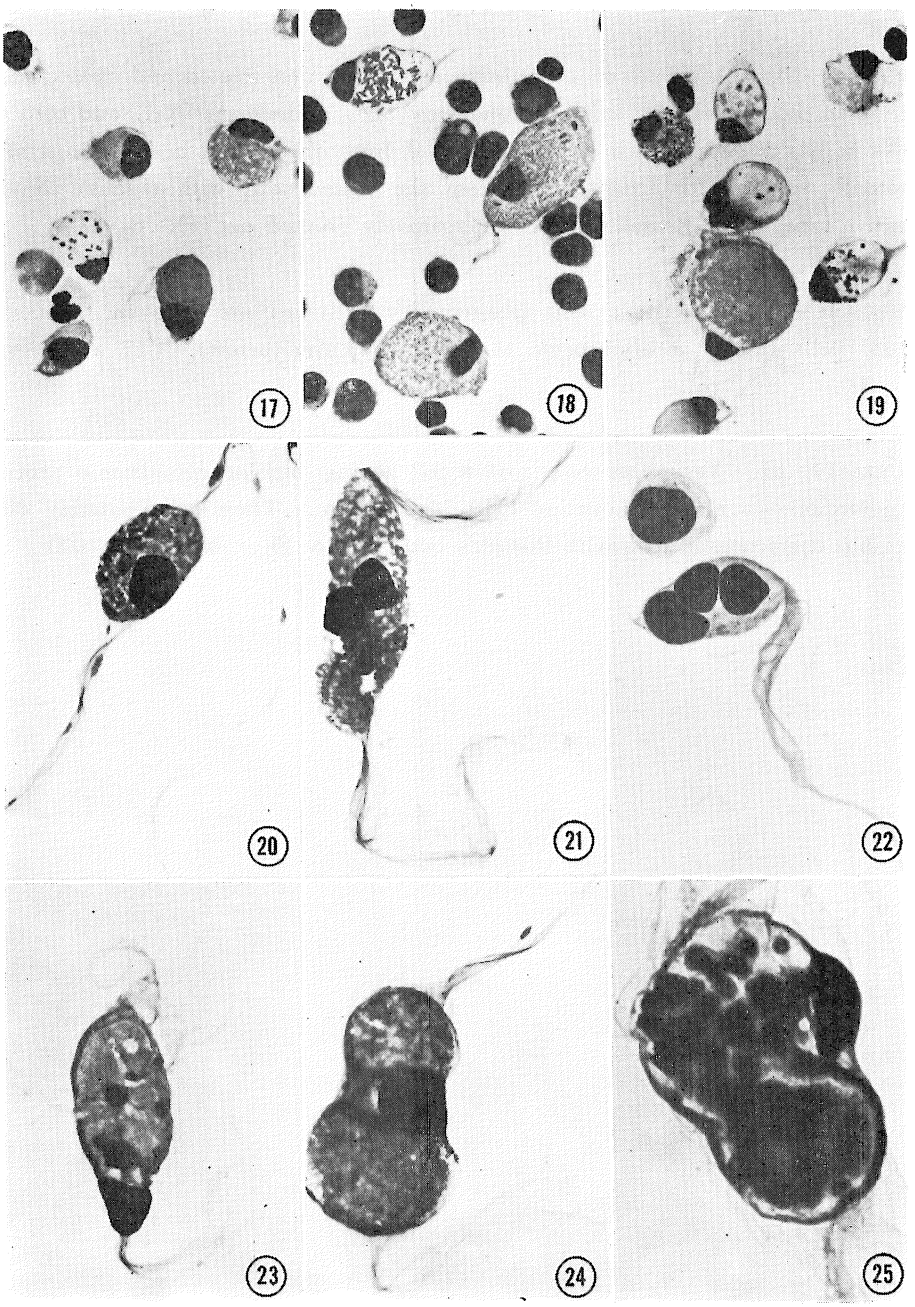

PLATE III

FIGURES 17–19. Various types of characteristic cells with Russell's bodies. Cytoplasmic inclusions appear to be sometimes granular, dot-like, or rod-like crystals and sometimes be opaque vacuoles containing full of dense substances. ×400.

FIGURES 20–22. Occasionally, binuclear and trinuclear cells are found in contrast with a regular type of mononuclear cells. × 600.

FIGURES 23–25. The nuclei lie on the periphery of the cytoplasm or in between vacuoles. Cytoplasmic processes are also quite polymorphous, some of them consist of thin multistrands and some are thick bundles of filaments. 23 & 24, ×400. 25, ×600.

PLATE IV

(Figures 26-36)

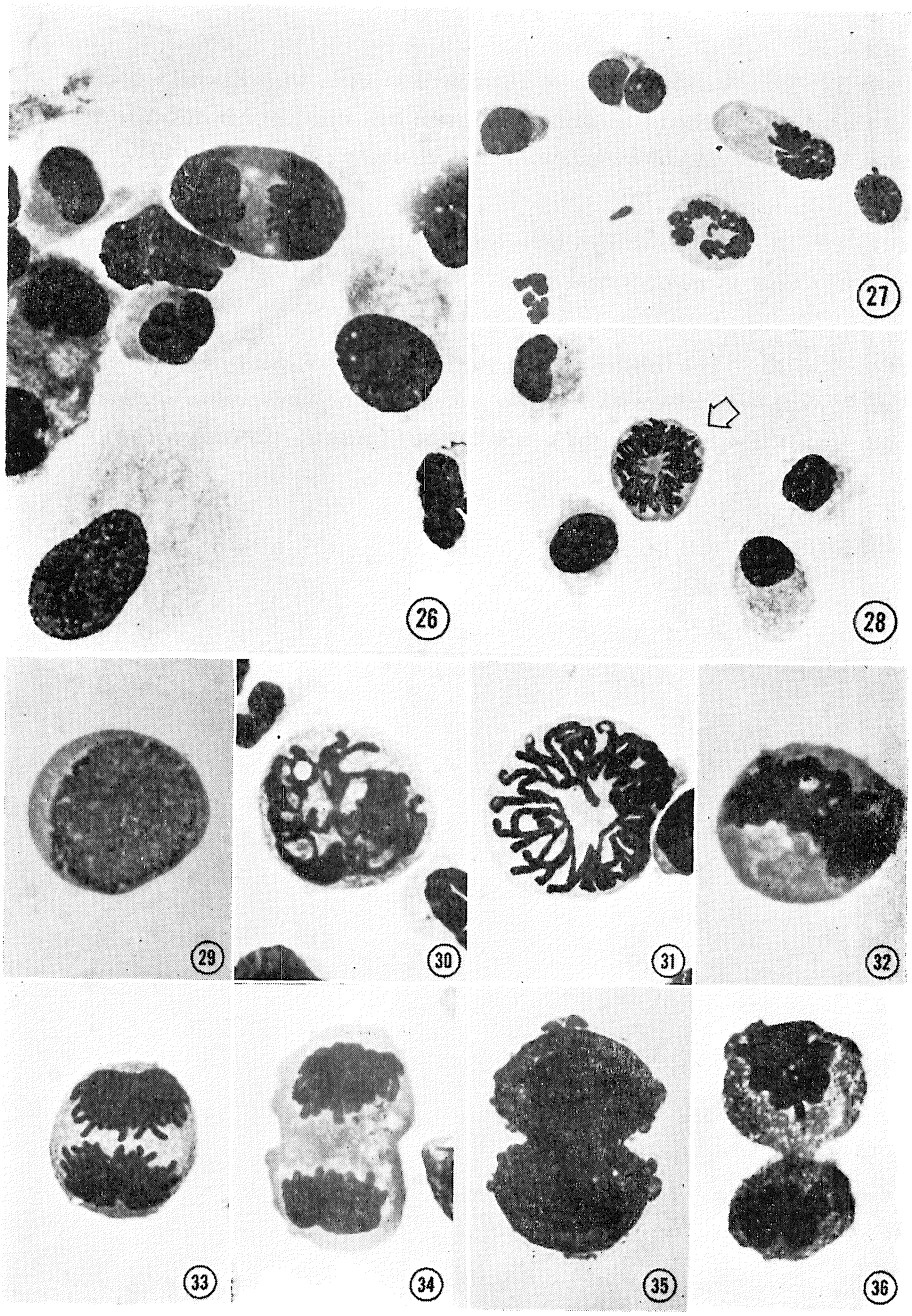

PLATE IV

FIGURES 26–36. Mitotic figures of monocytes and lymphocytes, which are obtained from peritoneal cavity of the newt treated with PHA-M *in vivo*. 26, a cell in the anaphase stage of mitosis. 27 & 28, metaphasic cells seen in the preparation of 24 hours after treatment. A polyploid cell infrequently appears (arrow). × 400.

29–36, cells in various mitotic stages from prophase to telophase. Smear preparations, May-Grünwald Giemsa stain. × 800.