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# Avian Malaria-like Disease Found in Young Cranes (*Grus monacha*)

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## Introduction

According to the calculation of the Wild Birds Society of Japan, the population of cranes staying over the coldest season on the Izumi-plain in Kagoshima prefecture, they having been protected as a special natural monument, reached more than 7,000 on January 1, 1983, the highest numbers up to this date. In detail, they were 6,096 Nabezuru (*Grus monacha*), 950 Manazuru (*Grus vipio*), 4 Canadazuru (*Grus canadensis*), and 1 Sodegurozuru (*Grus leucogeranus*). The cranes stay there ordinarily from the last third of October to the first third of March. Starting from the mid-December of 1982, 20 young birds of *Grus monacha* died at a time and 3 of them were brought to the laboratory for pathological examinations.

Microscopy of the paraffin sections of formalin-fixed tissues revealed, in the liver and other organs, a lot of giant cell-like structures which were suspected to be exoerythrocytic forms of avian malaria as plasmodium-like parasites and malaria pigment were found in the erythrocytes. In the present paper we describe a series of study basing on materials restricted to the formalin-fixed organs, so as to provide a foothold to elucidation of avian malaria-like disease in young cranes of Izumi. In so far as we could review, little is known on plasmodial infection of the cranes[5].

## Materials and Methods

*Birds*: Carcasses of 3 young birds of *Grus monacha* weighing 2.0–2.4 kg were conveyed from Izumi to the laboratory of veterinary pathology, having been packed in a box with dry ice.

*Microscopic studies*: The cranes were autopsied and main organs were removed, fixed in 10% formol solution, embedded in paraffin, cut into sections 3–4 $\mu$m thick and were stained with hematoxylin and eosin (HE) for microscopy. Special stains such as periodic acid-Schiff (PAS), Giemsa, Berliner blue staining for iron, Goodpasture's staining for bacteria (Gram) and Ziehl-Neelsen staining for acid-fast bacilli were carried out.

*Electronmicroscopic study*: Formol-fixed liver tissue was washed overnight with running water and diced into 1.5–2.0 $cm^3$ cubes which were refixed in 2.5% glutaraldehyde in the cacodylate buffered solution (pH 7.3) for 2 hours at 4°C. After 1 hour of post-fixation with 2% osmium tetraoxide in the same buffered solution, the samples were dehydrated by passing through serially graded ethanol and were embedded in Epon 812. Topography of the multinucleated giant cell-like structures was

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determined by thick sections stained with toluidine blue and thin sections were made by a diamond knife using an LKB ultratome. After being stained with uranyl acetate and lead citrate, the thin sections were observed by using H–500 electron microscope (Hitachi).

## Results

*Gross findings*: Nutritional states of the birds were slightly undernourished or normal, with little contents held in the gizzard and small intestine. A pseudomembranous colitis and a pseudomembranous tracheitis were found in bird 2 and bird 1, respectively. Pin-point to miliary white spots were observed in the liver of all the 3 birds both on the capsular and cut surfaces. In the lung of bird 3 were recognized diffuse nodules of miliary to soy-bean size with yellowish white tinge, showing slightly firm consistency when made a cut. No remarkable changes were observed in other organs.

*Microscopic findings*: Macroscopic lesions in the lung of bird 3 and in the trachea of bird 1 were diagnosed as aspergillosis with typical conidial heads and hyphae in the pseudomembrane of the latter and in the parabronchi of the former (Figs. 7 and 8). In the lung of bird 1 were found abscesses containing hyphae, strongly eosinophilic foci surrounded with fibroblasts and granulocytes. Hepatitis arising from the portal area was seen diffusely in the liver of all the 3 birds: severe in birds 1 and 3 and moderate in bird 2. Most of the macroscopic white spots seemed to correspond to the larger lesions accompanied with marked fibrosis. The lesions were characterized by remarkable cell infiltration consisting mainly of mononuclear phagocytes and granulocytes with various degrees of fibrosis in the interstitium (Fig. 9). In accordance with the gross greenish color that appeared after formol-fixation, bile thrombi in the hepatic cell cords as well as bile retension in the dilated interlobular bile ducts got numerous (Fig. 10). In the lumen of dilated sinusoids, a number of enlarged macrophages were seen loaded with brownish-yellow pigments and in bird 2 thickened circular lining cells of the sinusoids were appreciably numerous. Necrosis of a very small area, probably of 2–6 hepatocytes, was encountered not infrequently. Interlobular bile ducts showed mild hyperplasia in all the 3 birds. No intranuclear inclusion body was detected.

One peculiar feature of the hepatitis in these birds was that there were many bizarre giant cell-like structures of multinucleated type with various sizes and shapes: 10–40 $\mu$m in length and 10–15 $\mu$m in width (Fig. 11). The numbers of the giant cell-like structures in the liver showed positive correlation with severity of the lesions. Sometimes they were seen in the intact hepatic cell cords replacing a hepatocyte (Fig. 5) or in the bile ducts both in the epithelium and in the lumen (Fig. 12). More often they were found in the vascular spaces in the lesions or confined in the connective tissue. Some of them had eosinophilic cytoplasm-like area at the margin of the multinucleated structure, while others had not. Only in bird 1 was observed a strange focal lesion consisting solely of giant cell-like structures in large packs interspersed with degenerated granulocytes (Fig. 13). Many of the giant cell-like structures in it had a larger and fewer subunits which looked curved fusiform or comma-shaped instead of ordinary round or ovoid ones (Fig. 14).

Lamina propria and epithelia of villi and crypts in large and small intestines of these 3 cranes were noted to be containing moderate numbers of the giant cell-like structures (Fig. 15). An abscess ranging from the mucous membrane to deep muscular layer was observed in the cecum of bird 3. The pseudomembranous colitis in bird 2 showed marked thickening of the intestinal wall due to necrosis in the mucous membrane and in dilated submucosa with infiltration of granulocytes and monocytes in the distended interstitial tissue among the muscle bundles (Fig. 16).

Extraordinarily dilated blood vessels filled with blood cells were also found in the muscular layer. Extension of inflammatory influence to the cecum and ileum was rather slight.

Parasites that belong to the genus *Tetrameres* were found in the lumina of the profound proper gastric gland in birds 1 and 3 (Fig. 17) accompanied with slight thickening of the glandular stomach.

Damages caused by the stomach worm were minimal. Focal infiltration of mononuclear cells was detected in the muscular layer of the gizzard of bird 2, which seemed not necessarily to be related with the giant cell-like structures.

Many focal lesions of myocarditis were observed in the myocardium of birds 1 and 3 and in some of the lesions were detected the giant cell-like structures both in the vascular space and in the muscle fiber (Fig. 18).

Amorphous necrotic tissue was found distributed throughout the spleen of bird 1 showing splenomegaly (Fig. 19). As the sheathed arteries were well preserved, it might be properly assumed that the necrosis arose from the red pulp where a number of macrophages were seen heavily laden with brown pigments. The giant cell-like structures were numerous in the spleen of bird 1 and few in that of bird 2, in which no necrosis was observed and brown pigments were mostly found in the lumen of splenic sinuses. The spleen of bird 3 was lost and could not be examined.

Nephritis with marked infiltration of granulocytes was diffusely seen in the central vein in bird 1, while cell infiltration in bird 3 was very slight. Birefringent crystals were occasionally found in the tubules of birds 1 and 3 (Fig. 20), and the crystals were in most cases surrounded by foreign body giant cells. The giant cell-like structures were detected in the tubular epithelia (Fig. 21), among inflammatory cells and sometimes in the glomerulus in birds 1 and 3. The kidney of bird 2 showed only granular cytoplasm in the swollen proximal tubules.

In the cortices of the brain and cerebellum in bird 1 were found small foci of glial cells but no obvious giant cell-like structures were detected in these tissues. Lesions found in the 3 cranes were summarized in Table 1.

Table 1. Lesions found in 3 cranes (*Grus monacha*)

| Crane | Aspergillosis Tracheitis | Aspergillosis Pneumonitis | Hepatitis | Focal Myocarditis | Nephritis and nephro-lithiasis | Necrotic Colitis | Tetrameres in glandular stomach |
|---|---|---|---|---|---|---|---|
| No. 1 | ⫲ | + | ⫲ | ++ | ++ | — | ++ |
| No. 2 | — | — | ++ | — | — | ⫲ | — |
| No. 3 | — | ⫲ | ⫲ | ++ | + | — | + |

Key: ⫲ Severe, ++ Moderate, + Slight, and — Negative.

*Further microscopic studies on the giant cell-like structures*:

When stained with HE the giant cell-like structures of multinucleated type were found packed with many round or ovoid nucleus-like subunits having a centrally located chromatin as shown in Fig. 5. Periodic acid-Schiff staining proved that the giant cell-like structures were negative or weakly positive. However, Giemsa staining revealed the subunits of the giant cell-like structures to be stained deep blue in the cytoplasm and pink in the nucleus, the staining property resembling that of merozoites of *Plasmodium* (Fig. 4). Release of the subunits from the giant cell-like structure was detected by careful examinations (Fig. 22). In addition, markedly positive iron reaction was obtained in the liver of all the 3 birds: a fact suggestive of a large amount of hemolysis (Fig. 6). No Gram-positive bacteria nor any acid-fast bacilli were detected.

*Electronmicroscopic study*:

Although small in number the giant cell-like structures were observed. An oval shaped multinucleated giant cell ($16 \times 11$ $\mu$m) having an obvious nucleolus in each nucleus (about 2.5 $\mu$m in width) was found to be containing electron-dense cytoplasm diffusely distributed with membrane-limited circles about 0.7 $\mu$m in diameter (Fig. 23). There was no definite organella in the cytoplasm. In another giant cell ($21 \times 16.5$ $\mu$m) were enclosed several small cells delimited with double membranes ($7.1$–$7.4 \times 3.7$–$5.0$ $\mu$m), each containing a nucleus ($3.7$–$4.6 \times 2.8$–$3.2$ $\mu$m) with a conspicuous nucleolus (Fig. 24). In the electron-dense cytoplasm of both the giant cell and small cells were observed many membrane-limited circles ($1.0$–$1.2 \times 0.6$–$0.9$ $\mu$m) though variation in size and shape was greater than those in the multinucleated giant cell as shown in Fig. 23. The latter giant cell containing small cells seemed to be more mature form than the former multinucleated giant cell.

Table 2. Grade of infestation with the tissue-form schizonts (exoerythrocytic forms)

| Crane | Liver | Spleen | Intestines | Myocardium | Lung | Kidney |
|---|---|---|---|---|---|---|
| No. 1 | ## | ## | ## | ## | + | ## |
| No. 2 | + | + | + | − | − | − |
| No. 3 | ## | N. D. | ## | + | + | + |

N. D.: Not detectable as the organ was lost.

*Parasitology*:

From these findings described above, we suspected the infection with a kind of hemolytic protozoon such as *Plasmodium* and sought for the parasites in the cytoplasm of red blood cells on a liver section stained with Giemsa's solution, and succeeded in detecting those (about $2$–$3 \times 3$–$5$ $\mu$m) with malaria pigment (Figs. 1, 2 and 3). Therefore, we inferred that the multinucleated giant cell-like structures found in the cranes might be the exoerythrocytic forms of avian malaria. Grading of organs infested by the exoerythrocytic forms or tissue-form schizonts is shown in Table 2.

## Discussion

Many giant cell-like structures were detected in the liver and other organs of the 3 young cranes found dead in the field of Izumi, their wintering place. In common to the 3 birds hepatitis and hemosiderosis were marked in the liver, and the numbers of the giant cell-like structures were proportionate to the severity of the lesions. As observed in the epithelial cells, in the interstitial tissue and in the vascular space, the giant cell-like structures were supposed to be intracellular parasites causing destruction of parenchymal cells and inflammation as seen in the liver and myocardium. The giant cell-like structures were PAS-negative or weakly positive, which denied the possibility of protozoae such as coccidium, histomonas, etc.. However, Giemsa stain showed the staining property of round or ovoid nucleus-like subunits in the multinucleated type of giant cell-like structures resembling that of merozoites of *Plasmodium*. Moreover, release of subunits from the giant cell-like structure was detected by careful examinations. Electronmicroscopy disclosed the fact that a giant cell-like structure was really a multinucleated giant cell which had distinct nuclei and electron-dense cytoplasm though it contained no ordinary organella. Another picture of the multinucleated giant cell showed many small cells contained in it. Putting all these findings together, we suspected an infection with such a hemolytic parasite as *Plasmodium*. The eryth-

rocytic parasites were also found accompanied with malaria pigment-like bodies. We now deduce that the giant cell-like structures might be the exoerythrocytic forms of avian malaria.

The assumption that the giant cell-like structure might be a syncytium due to infection with paramyxovirus[3] was rejected as no inclusion body was detected. The virus-induced cell fusion usually produced multinucleated giant cells which were far larger and freer in shape and size than those in the present case.

The present erythrocytic forms bear some resemblance to those of *P. juxtanucleare*[1,2,4] but what we infer to be exoerythrocytic forms in the present case contain far larger merozoites than those of *P. juxtanucleare*.

The parasitic hepatites in these cranes were severe in cases associated with aspergillosis, and even in a moderate case there was association with severe pseudomembranous colitis. These evidences indicated that some debilitating factors might have contributed to the development of the disease in young cranes. Even participation of unidentified parasites other than *Plasmodium* in causing the hepatitis might be probable.

As far as we could review, avian malaria in the cranes seemed to be rare[5]. Further studies will be required for accumulation of more cases, and for isolation and identification of *Plasmodium* before elucidation of the true role of avian malaria in diseases of cranes.

## Summary

Three young cranes (*Grus monacha*) found dead in Izumi, the wintering place of the bird, were examined pathologically in mid-December in 1982. Severe to moderate hepatites with marked infiltration of mononuclear phagocytes and granulocytes were found in all the cranes, in which bizarre pleomorphic giant cell-like structures were numerous. The giant cell-like structures were recognized in the liver, spleen, intestines, heart, lung and kidney, in diminishing order. They existed mostly in the vascular space or in the parenchymal cells. Electronmicroscopy of the once formol-fixed liver tissue disclosed a multinucleated giant cell with electrondense cytoplasm and unusual organella. Another giant cell contained several small cells. These subunits of the multinucleated giant cell-like structres were proved negative or weakly positive to PAS, while Giemsa stain revealed their staining property resembling that of merozoites of *Plasmodium*. Livers of all the 3 birds showed marked hemosiderosis. From these findings we suspected that these giant cells were the exoerythrocytic forms of a hemolytic protozoon, *Plasmodium* the causative agent, and we sought for the erythrocytic forms in sections stained with Giemsa, and succeeded in detecting the erythrocytic form-like parasites with malaria pigment-like bodies.

## References

1) Akiba, K.: Studies on the Avian Malaria. II. On the Species of *Plasmodium* from Chicken in Japan. (*P. japonicum* Ishiguro 1957, a Synonym for *P. juxtanucleare* Versiana, Gomes 1941.) *Proc. 47th Meeting of Jap. J. vet. Sci.*, **21**, 18 (1959) (in Japanese)
2) Akiba, K.: Avian Malaria in the Chicken. in Horiuchi, T. (ed), *Diagnosis of Poultry Diseases.* p. 450–458, Ie-no-Hikari Kyokai., Tokyo (1982) (in Japanese)
3) Docherty, D. E. and Henning, D. J.: The Isolation of a Herpesvirus from Captive Cranes with an Inclusion Body Disease. *Avian Dis.*, **24**, 278–283 (1980)
4) Itagaki, K. and Tsubokura, M.: Studies on Avian Malaria in Japan. I. Life Cycle, especially Exoerythrocytic Growth of the Parasite. *Jap. J. vet. Sci.* **30**, 1–6 (1968) (in Japanese with English Summary)

5) Miyata, A.: *Kisei-Genseidobutsu (Parasitic Protozoae)* I & II. p. 1293–1304. Kiseidobutsu Kanko-Kai (Association for Publication on Parasitic Animals) in Institute of Tropical Medicine of Nagasaki University, Nagasaki. (1979) (in Japanese)

## Explanation of figures

Fig. 1. An erythrocytic form-like parasite accompanied with malaria pigment found in the liver section of bird 3. Giemsa staining. ×975

Fig. 2. The same as Fig. 1. ×650

Fig. 3. The same as Fig. 1. ×650

Fig. 4. A multinucleated giant cell-like structure shown by Giemsa stain, which was inferred to be an exoerythrocytic form of avian malaria. Note deep blue cytoplasm and pinkish nuclei of the merozoites. ×650

Fig. 5. A presumptive exoerythrocytic form parasitized in the hepatocyte. Hematoxylin and eosin (HE). ×390

Fig. 6. Marked hemosiderosis in the liver of bird 3. Berliner blue staining for iron. ×293

Fig. 7. Pseudomembranous tracheitis in bird 1. HE. ×26

Fig. 8. Aspergillosis in the lung of bird 3 showing necrotic tissue in the nodule (center to right) and the surrounding normal tissue (left). Conidial heads are seen in the parabronchi. HE. ×19.5

Fig. 9. Hepatitic lesion in bird 3, showing marked granulocyte infiltration and increase in fibrous tissue. Note many exoerythrocytic form-like bodies (giant cell-like structures). HE. ×130

Fig. 10. Hepatic lesion in the portal area in bird 3. Note dilated bile ducts and retention of bile. HE. ×97.5

Fig. 11. Hepatitic lesion in bird 3, showing marked pleomorphism of the giant cell-like structures. HE. ×130

Fig. 12. The giant cell-like structures parasitized in the epithelium of a bile duct (upper left) and in the hepatocyte (lower right). HE. ×130

Fig. 13. A focal lesion consisting of many giant cell-like structures and degenerated granulocytes in the liver of bird 1. HE. ×65

Fig. 14. Higher magnification of the Fig. 13, showing extraordinarily larger and fewer subunits with strange, curved fusiform or comma shapes in the giant cell-like structures. Giemsa. ×390

Fig. 15. The giant cell-like structures found in the epithelium and in the lamina propria of duodenum in bird 2. HE. ×260

Fig. 16. Pseudomembranous colitis in bird 2, showing necrosis of the mucous membrane (right) and marked dilatation of submucosa in filtrated with granulocytes and mononuclears (center). HE. ×19.5

Fig. 17. Tetrameres in the profound proper gastric gland in bird 1. HE. ×26

Fig. 18. Myocarditis found in bird 1, showing the giant cell-like structures in the muscle fiber and vascular lumen. HE. ×195

Fig. 19. Spleen of bird 1 showing necrosis (lower right) and intact sheathed arteries (center to left). HE. ×130

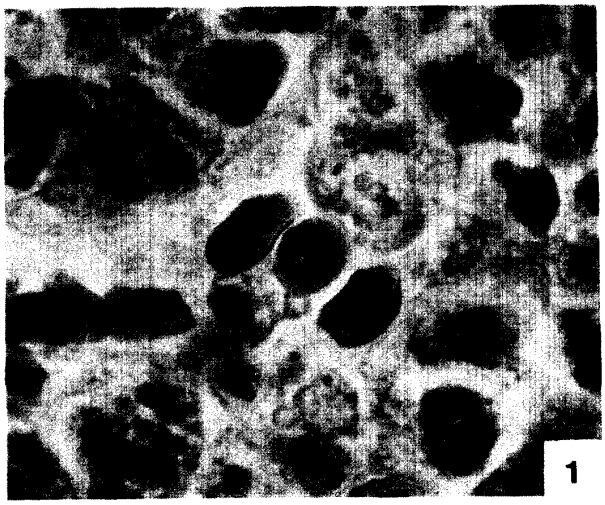
Fig. 20. Intratubular deposition of birefringent crystals in the kidney of bird 1. HE. ×260

Fig. 21. The giant cell-like structure in the tubular epithelium in the kidney of bird 1. HE. ×260

Fig. 22. Release of subunits (merozoites) from the giant cell-like structure (exoerythrocytic form) found in the liver (upper right) of bird 3. Giemsa. ×390

Fig. 23. Electronmicrograph of the giant cell-like structure (presumptive exoerythrocytic form) having obvious nuclei and electron-dense cytoplasm without any distinct organella excepting many membrane-limited circles. ×6624

Fig. 24. Electronmicrograph of another exoerythrocytic form, containing distinct small cells (presumptive merozoites). ×4968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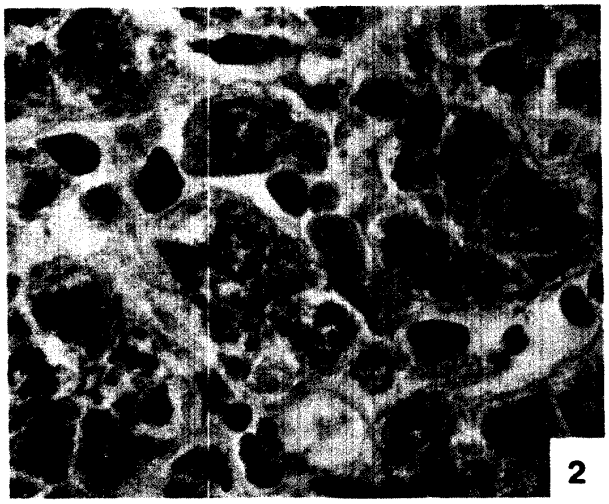
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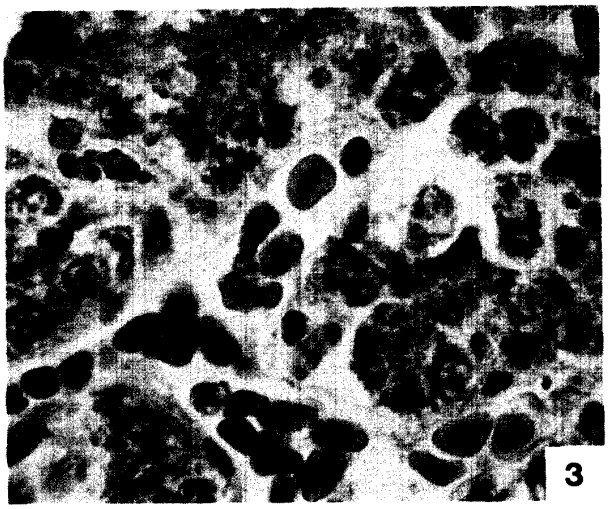
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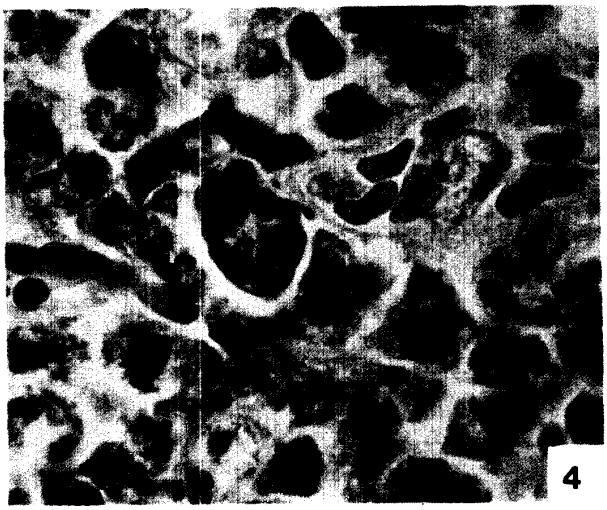
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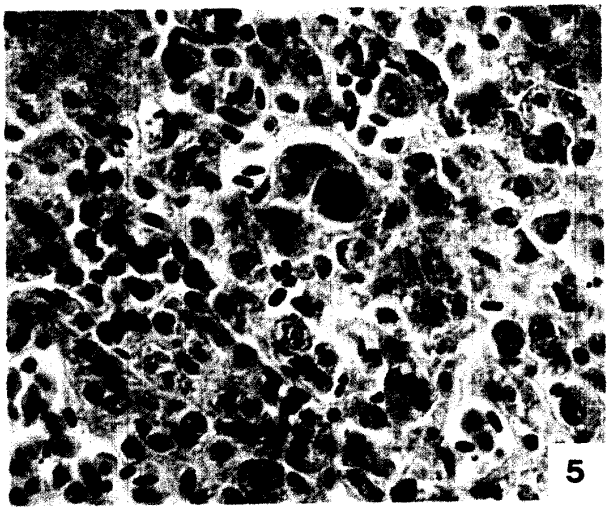
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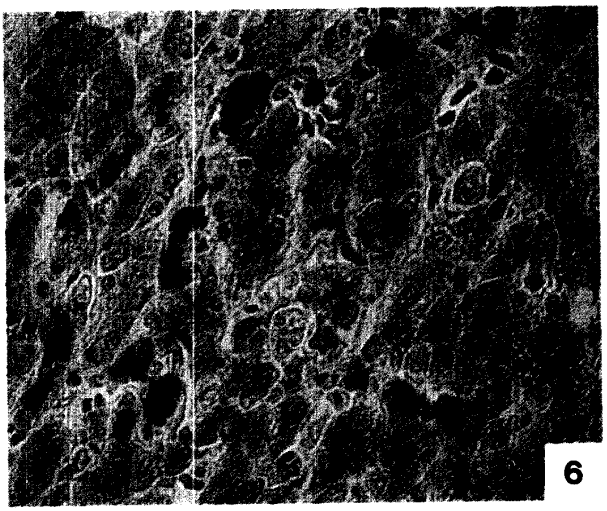
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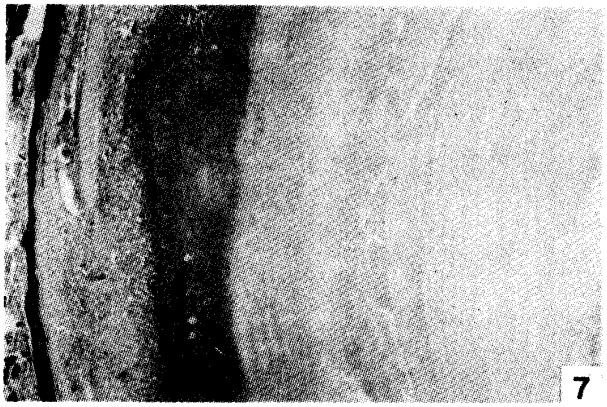
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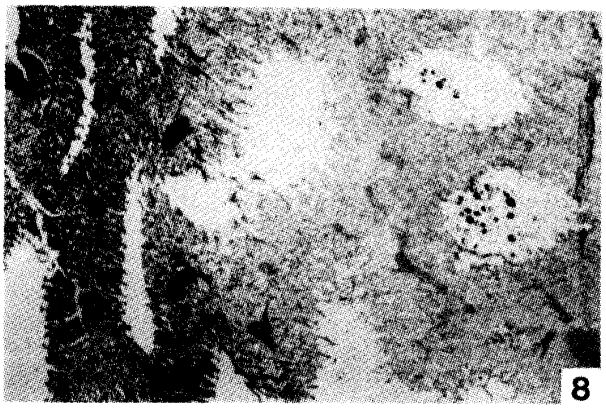
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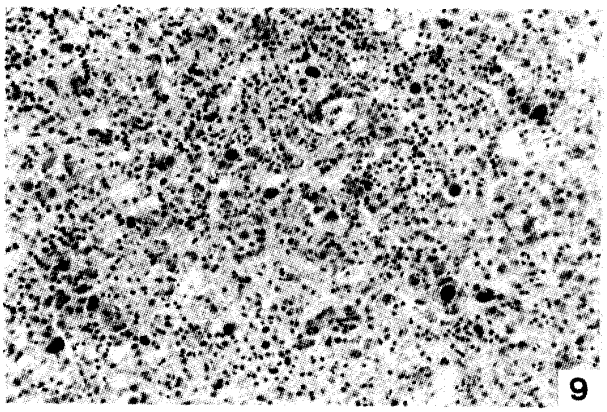
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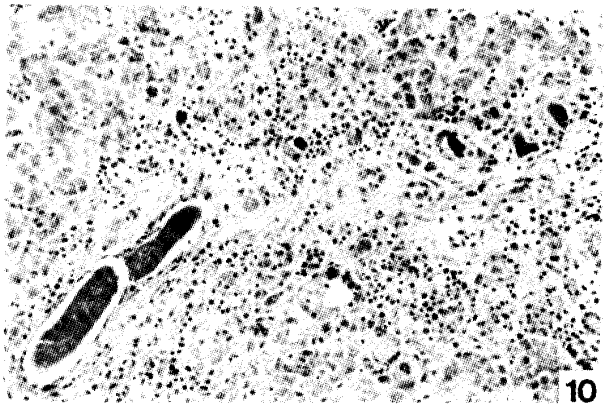
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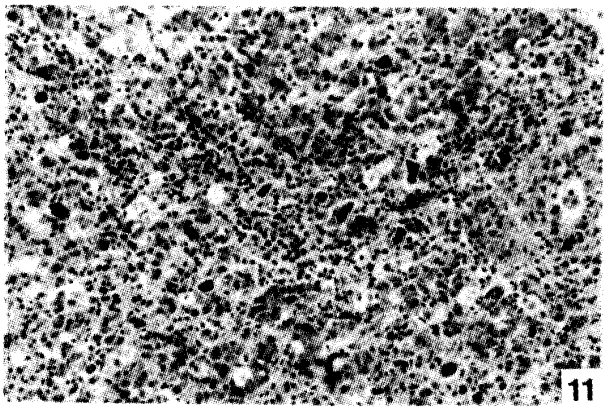
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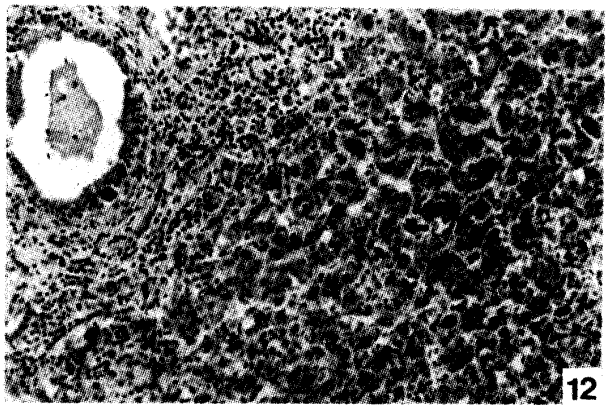
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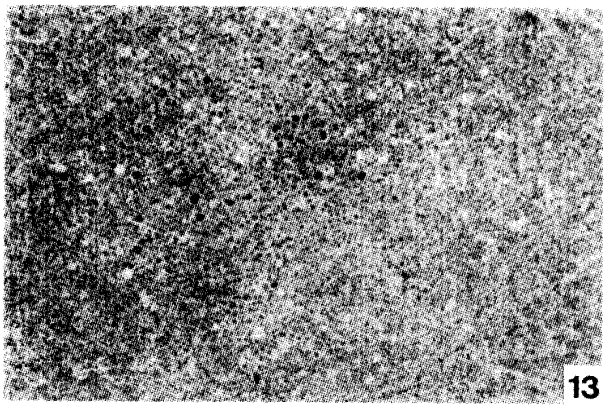
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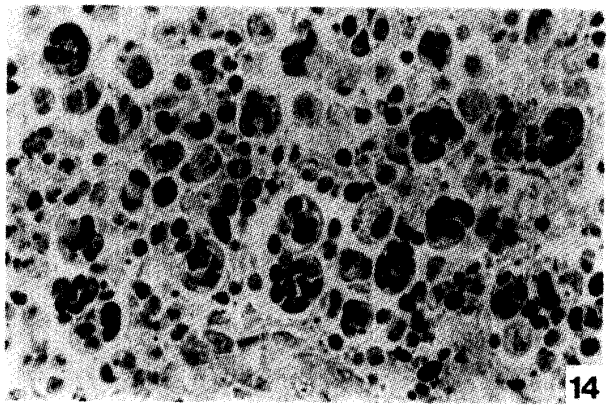
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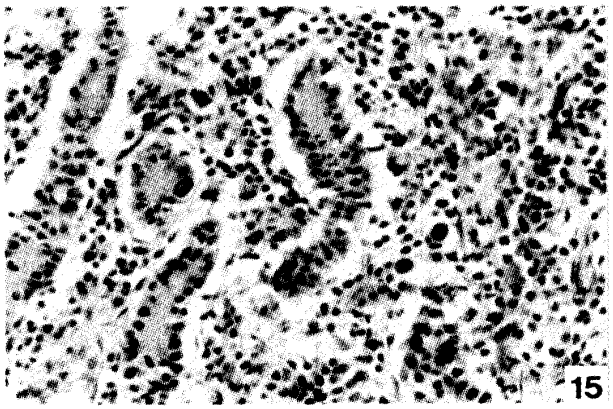
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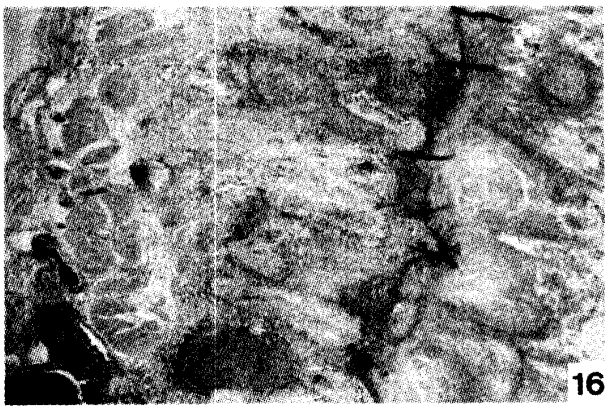
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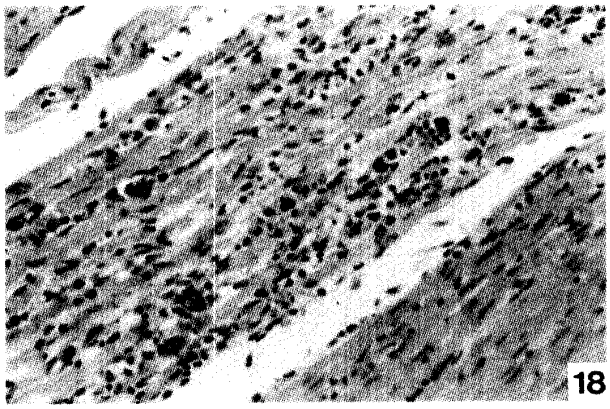
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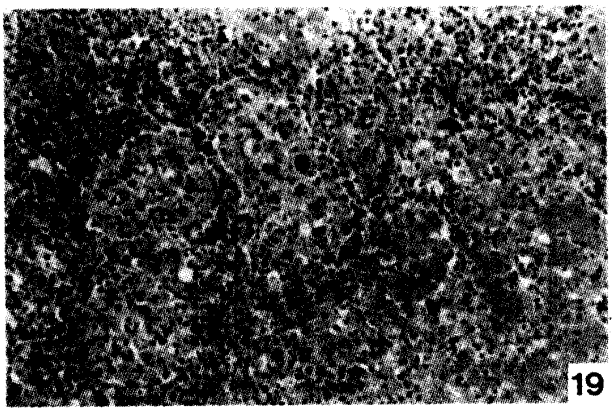
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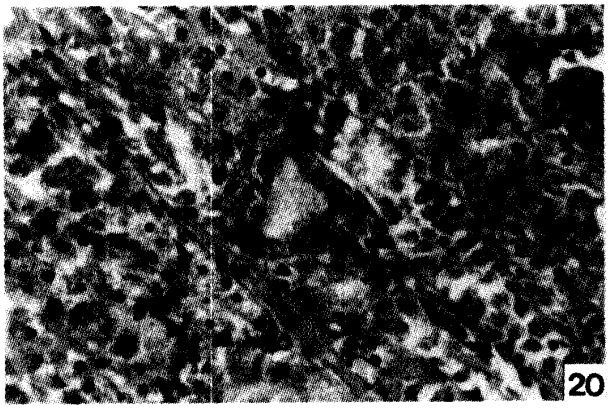
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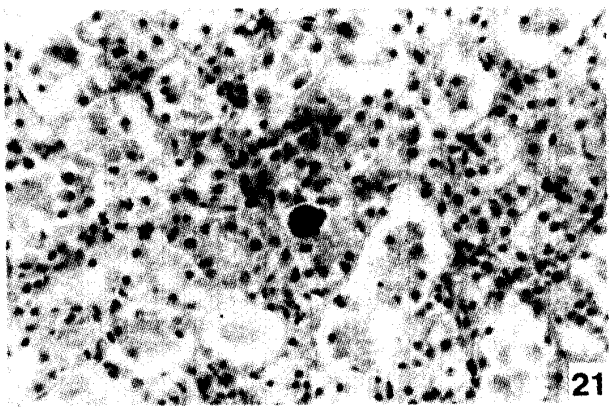
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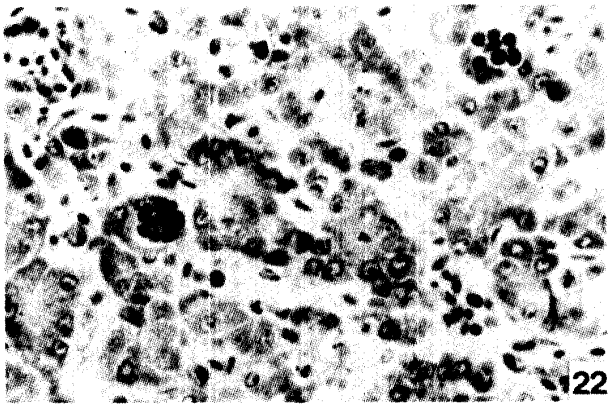
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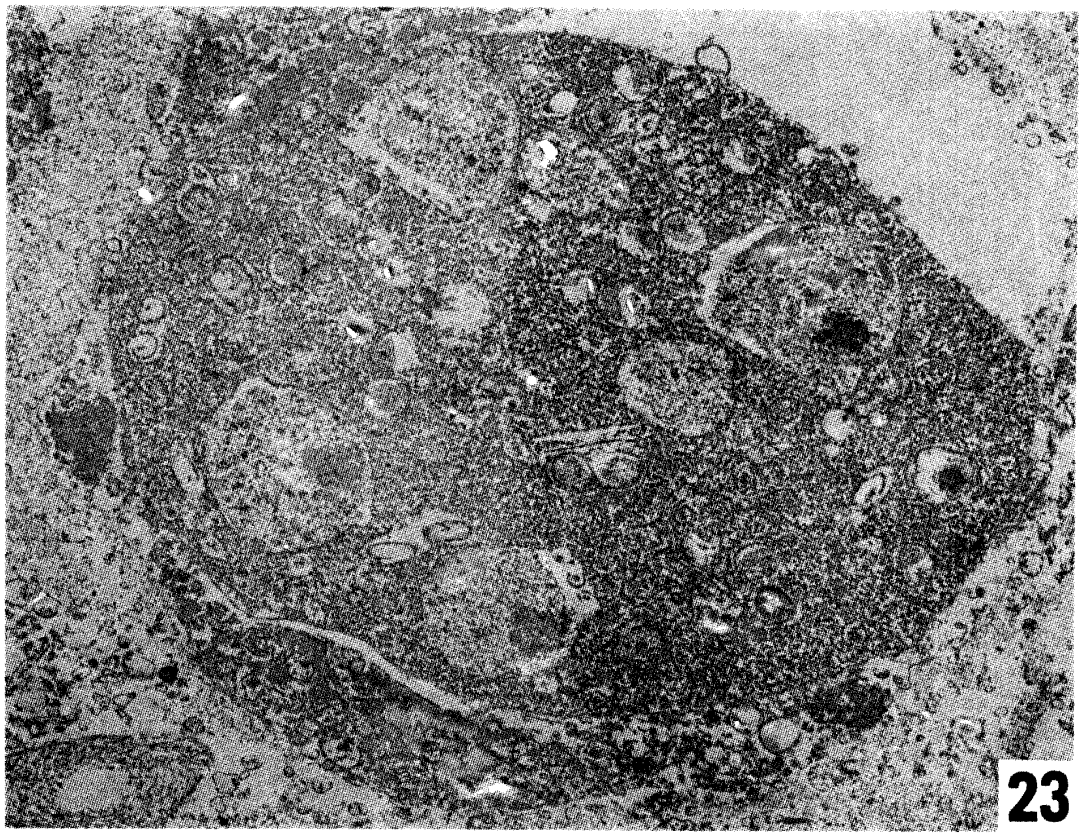

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