

## An Immunocytochemical Study on the Differentiation of Synoviocytes in the Knee Joint of Mouse

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**Abstract**—This study was designed to observe the differentiation of synoviocytes in the knee joint of mouse by immunocytochemical technique.

The feti were collected on the 15th, 16th, 17th, 18th and 19th day of pregnancy, and the 1-day-old and 5-day-old neonates were also collected.

The knee joints were fixed in Bouin's solution and processed and finally embedded in paraplast by routine method. The serial sections were stained in H&E.

In order to visualize the lysozyme and the Ia antigen in the cell, rabbit anti-lysozyme antibody and monoclonal IgG2a anti-mouse I-A<sup>d</sup> were used in the double-bridge peroxidase anti-peroxidase technique.

To observe the ultrastructure of the synoviocytes, the junctional complex, the basal lamina and the type of capillaries in the synovial membrane, some tissues were embedded in Epon 812 by the usual method.

The joint cavities were formed separately in the posterior portion of the patella and between the femur and tibia in the 16-day-old fetus. These were united in the 18-day-old fetus. The synoviocytes, which showed a positive reaction to anti-lysozyme, first appeared in the 18-day-old fetus. These synoviocytes were abundant on the luminal side of the primordial infrapatellar fat pad in the 19-day-old fetus but were absent on the surface of the meniscus and cruciate ligaments.

The Ia antigen-positive synoviocytes first appeared in the 5-day-old neonate. In the types of synovial membrane, the fibrous type first appeared in the 17-day-old fetus, the areolar type in the 18-day-old fetus, and the adipose type in the 5-day-old neonate. The synoviocytes did not have the basal lamina at any age. The desmosomal junctions were observed between the synoviocytes in the 8-week-old adult.

The capillaries supplying blood in the synovial membrane were the fenestrated type with closing diaphragms, but the basal lamina was incomplete.

On the basis of above findings, it is obvious that joint cavities are formed by the fusion of small ones during fetal growth. The lysozyme-positive synoviocytes, which compose the synovial membrane, appear before birth, but the expression of the Ia antigen shows up after birth.

**Key Words:** *Knee joint, Lysozyme, Ia antigen, Synoviocyte, Immunocytochemical*

## INTRODUCTION

The synovial membrane is a tissue which is an inner lining of the synovial joint, except for the articular surface, and it's originated from the embryonic mesenchyme (Williams and Warwick, 1980; Kristic, 1984).

Barland *et al.*(1962) reported that there are two types of synoviocytes in humans. This fact was confirmed by Ghadially and Roy(1966) in the rabbit; Roy and Ghadially(1967) and Graaback(1982) in the rat; and Linck and Porte(1978) in the mouse. The type A synoviocytes have many vacuoles and lysosomes in the cytoplasm, so these cells are regarded as one type of macrophages or M cells (macrophage-like cells). The type B synoviocytes have many rough endoplasmic reticulums in the cytoplasm, and these cells are called S cells (secretory cells) because of the function of protein synthesis and secretion of F cells (fibroblast-like cells). The type A synoviocytes were positive to the lysozyme (=muramidase) which is a major marker of the macrophage (Geiler and Riedel, 1984; Mapp and Revell, 1987), and to Ia antigen, which is one of the markers of the macrophage in the mouse (Klareskog, 1982) using the immunohistochemical technique. In the rheumatoid arthritis of humans, the total number of synoviocytes (Hogg *et al.*, 1985) and the percentage of type A synoviocytes which were positive to the lysozyme(Geiler and Reidel, 1985) increased.

The process of development around the synovial joint has three stages. The first stage is cell condensations and cell necrosis; the second stage is formation of the three-layered mesenchyme; and the third stage is vascular invasion and joint clefting (Whillis, 1940; Mitrovic, 1978). The knee joints of human and rat follow the same process (Haines, 1953; Gardner and O'Rahilly, 1968). But a study on the development of knee joint of the mouse has been lacking.

The present study reports the period of first appearance and distribution of type A synoviocytes during the development of the knee joint using the immunocytochemical technique and electronmicroscopy.

## MATERIALS AND METHODS

### 1. Animals

The feti were collected on the 15th, 16th, 17th, 18th and 19th day of pregnancy, and 1-day-old and 5-day-old neonates were also collected from ICR mice. The day when the vaginal plug was found in the morning, was denoted as first day of pregnancy. The control group was the 8 weeks of age. Each group consisted of 5 animals.

### 2. Processing of tissue

The pregnant mice were killed by cervical dislocation, and the uteri were opened. The knee joints of the feti and neonates were dissected under a dissecting microscope and fixed in Bouin's solution for 12 hours. After fixation, dehydration and clearing of the tissues were carried out by the usual method. Finally, the tissues were embedded in paraplast and serially cut at 6 micrometers. Each section was stained with hematoxylin and eosin.

For electronmicroscopic observation, the trimmed tissues were fixed in 0.2% glutaraldehyde and 2% paraformaldehyde solution for 2 hours, and postfixed in 1% osmium tetroxide for 1 hour. After the routine procedure, the tissues were embedded in Epon 812.

### 3. Using antibodies

#### a) Labelling lysozyme

The primary antibody was rabbit IgG anti-lysozyme (=muramidase) (Dakopatts a/s, Denmark), the secondary antibody was goat IgG anti-rabbit IgG (Sigma Chemical Co., USA), and the tertiary antibody was rabbit peroxidase anti-peroxidase soluble complex (PAP) (Sigma Chemical Co., USA).

#### b) Labelling Ia antigen

The primary antibody was monoclonal IgG2a anti-mouse I-Ad (Becton Dickinson Immunocytometry System, USA), the secondary antibody was goat IgG anti-mouse IgG (Sigma Chemical Co. USA), and the tertiary antibody was mouse peroxidase anti-peroxidase soluble complex (PAP) (Sigma Chemical Co., USA).

#### 4. Immunocytochemical reaction

To remove the endogenous peroxidase, deparaffinized sections were treated with fresh 0.3% hydrogen peroxide in methanol for 30 minutes, and sections were rinsed in three changes of pH 7.2, 0.02 M phosphate buffered saline (PBS) for 10 minutes. Sections were incubated with 0.5% normal goat serum for 10 minutes in the moist chamber and drained. Then sections were covered with the primary antibodies, which were titrated previously in optimal dilution, at room temperature for 30 minutes. After reaction, the sections were washed in 3 changes of PBS for 10 minutes. Next the washed sections were incubated with the secondary antibody, and then the tertiary antibody in order. To increase sensitivity, the secondary antibody and tertiary antibody were applied again in the double bridge PAP method (Vacca et al, 1975). To visualize horseradish peroxidase, a substrate solution was used for 1 minute. The substrate solution was composed of 10 mg 3,3'-diaminobenzidine tetrahydrochloride and 5  $\mu$ l 30% hydrogen peroxide in 40 ml PBS.

The nuclei were counterstained by hematoxylin. The tissues were dehydrated, cleared and coverslipped by using a synthetic mounting medium.

## RESULTS

### 1. The 8 weeks of age

The synoviocytes, which were located around the patella, were all positive to lysozyme and Ia antigen as 1 or 2 layers (Fig. 2, 3), and there was no difference in regional distribution. But none of the synoviocytes were positive to lysozyme or Ia antigen on the surface of the meniscus and cruciate ligaments (Fig. 4, 5).

### 2. The 15-day-old fetus

The joint cavity was not formed. The cartilage of the femur, tibia, and patella were seen. The cells, which would be a cruciate ligament, were arranged in a parallel direction, and the primordium of synovial fold, which had many blood vessels, were also seen (Fig. 6). At the ends of cartilage, cell condensation and necrosis of a few cells were seen.

### 3. The 16-day-old fetus

This period was the first appearance of a joint cavity behind the patella (Fig.7). In the posterior region of the infrapatellar tendon, many capillaries were located between the mesenchymal cells, and another cavity was newly formed between the cartilage of the femur and tibia (Fig. 8), but none of synoviocytes were positive to lysozyme and Ia antigen.

### 4. The 17-day-old fetus

The joint cavity located above and below the meniscus, was connected, but the joint cavity located behind the patella was formed independently. The cruciate ligament was exposed partially to the joint cavity (Fig. 10, 11).

The large, saccular vessels, which were lined by endothelial cells with many red blood cells, were located in a parallel direction along the cruciate ligaments (Fig. 9, 10, 11). A large triangular vessel was located at the triangular zone, which was composed of cartilage of the femur, anterior cruciate ligament, and posterior cruciate ligament (Fig. 10). This vessel was connected with another large vessel which was located posterior to the cruciate ligament and finally drained into the popliteal vein. Some cells were observed in the joint cavity with eosinophilic cytoplasm and vacuoles. These were positive to lysozyme, but negative to Ia antigen (Fig. 11). The fibrous type synovial membrane first appeared in the suprapatellar region (Table 1), but none of synoviocytes were positive to lysozyme.

**Table 1.** Types of synovial membrane

|         | Age\ Type | Fibrous | Areolar | Adipose |
|---------|-----------|---------|---------|---------|
| Adult   | 8w        | +       | +       | +       |
| Fetus   | 15d       | -       | -       | -       |
|         | 16d       | -       | -       | -       |
|         | 17d       | +       | -       | -       |
|         | 18d       | +       | +       | -       |
|         | 19d       | +       | +       | -       |
| Neonate | 1d        | +       | +       | -       |
|         | 5d        | +       | +       | +       |

### 5. The 18-day-old fetus

The joint cavities, which were formed separately, were joined as one. Many vessels were located in the primordium of the infrapatellar fat pad, and some vessels crossed the joint cavity through a bridge of connective tissue (Fig. 13, 14).

The small-sized vessels supplied the posterior cruciate ligament at the anterior and superior portion of it. The large triangular vessel, which appeared in the 17-day-old fetus, showed a decrease in diameter. The anterior and posterior cruciate ligaments became elongated and were exposed to the joint cavity.

Some synoviocytes were positive to lysozyme, but their frequency and stainability were low, and they were negative to Ia antigen (Table 2). The areolar type synovial membrane first appeared in the infrapatellar region.

**Table 2.** An immunohistochemical reaction for synoviocytes

|         | Age | Lysozyme | Ia Antigen |
|---------|-----|----------|------------|
| Adult   | 8w  | +        | +          |
| Fetus   | 15d | -        | -          |
|         | 16d | -        | -          |
|         | 17d | -        | -          |
|         | 18d | ±        | -          |
|         | 19d | +        | -          |
| Neonate | 1d  | +        | -          |
|         | 5d  | +        | +          |

### 6. The 19-day-old fetus

The joint cavities were well-developed and connected to each other. The infrapatellar fat pad was not developed, and there were many mesenchymal cells (Fig. 15, 16).

The synoviocytes, which were positive to lysozyme (Fig. 17), were located in one layer at the primordial infrapatellar fat pad, and these were more abundant at the lower portion. However, these synoviocytes were negative to Ia antigen, and no cells were positive to lysozyme and Ia antigen on the surface of the meniscus and cruciate ligaments.

### 7. The 1-day-old neonate

Many synoviocytes were positive to lysozyme but negative to Ia antigen (Fig. 18, 19).

### 8. The 5-day-old neonate

Many synoviocytes were positive to lysozyme and also positive to Ia antigen (Fig. 20, 21). The localization of Ia antigen was the cellular surface, and the adipose type synovial membrane first appeared.

### 9. Findings in electronmicroscopy

From the 17-day-old fetus, the two types of synoviocytes could be identified (Fig. 22, 23) without basal lamina.

The desmosome was observed in the 8 weeks of age mouse (Fig. 24), but there was no desmosome at this stage of development because the synoviocytes were arranged loosely.

The endothelial cells of capillaries were fenestrated with closing diaphragms, which were located in synovial membrane; the basal lamina was incomplete (Fig. 25).

## DISCUSSION

There are two types of synoviocytes, but they cannot be distinguished by routine staining methods in light microscopy. Geller and Riedel (1985) reported on the type A synoviocytes which contain lysozyme by PAP technique. Mapp and Revell (1987) also reported on the type A synoviocytes which contain lysozyme by immunogold labelling technique, and Palmer *et al.* (1985) reported that the type A synoviocytes could be labelled by the monoclonal antibody specific for macrophage and HLA-DR, and the type B synoviocytes could be labelled by the monoclonal antibody anti-Thy-1.

The lysozyme is a mucopeptide located in various cells, such as the Paneth cells of the small intestine, proximal tubular cells of the kidney, polymorphonuclear leukocytes, eosinophils and macrophages, with a molecular weight of about 12,000 (Speece, 1965; Mason and Taylor, 1975; Kloclars and Reitamo, 1975; Reitamo, 1978; Franken *et al.*, 1989). The type A synoviocytes could be almost

all labelled all by peroxidase which was injected into the joint cavity. However, only a small amount of type B synoviocytes could be labelled by pinocytosis (Shannon and Graham, 1971).

The type A synoviocytes were positive to the murine Ia antigen, which is a marker of macrophage (Klareskog *et al.*, 1982; Burmester *et al.*, 1983; Fournier *et al.*, 1986), and also positive to the HLA-D antigen and macrophage specific antigen(Hogg *et al.*, 1985; Palmer *et al.*, 1985). So the type A synoviocytes have characteristics of macrophage, morphologically and functionally.

The reason for using anti-lysozyme antibody in this study was its usefulness in labelling any stage of macrophage, that is, immature macrophage, primed macrophage and activated macrophage(Male *et al.*, 1987). It is very common to use proteolytic enzymes for exposing antigen in fixed tissues, but we used the double bridge PAP technique without enzymatic digestion, because the sensitivity of this technique is 20-50 times that of the single bridge PAP technique.

Ia antigen is one of the major murine histocom-

patibility antigens, and is located on the surfaces of lymphocytes and macrophages(Male *et al.*, 1987). Ia antigen is equivalent to human HLA-DP, HLA-DQ and HLA-DR(Stites *et al.*, 1987), and Ia antigen is a kind of glycoprotein with murine I-A region and I-E region(Weir, 1986). Primed macrophages and activated macrophages participate in the activation of helper T lymphocytes by expression of Ia antigen(Male, 1987), so Ia antigen is a functional marker of macrophage. The first appearance of Ia antigen was in the 5-day-old neonate mice in this experiment(Table 1).

The locations of Ia antigen were both in the intracytoplasm and on the cell surface. This finding was consistent with Klareskog *et al.*(1982) who reported the intracytoplasmic location of Ia antigen.

The origin of the type A synoviocyte has been controversial. One theory is that it is caused by the differentiation of mesenchymal cells. The other theory is the migration of blood monocytes to the synovial membranes(Kristic, 1984; Hogg *et al.*, 1985). In this experiment, the first appearance of the type A synoviocytes was at the joint cavity

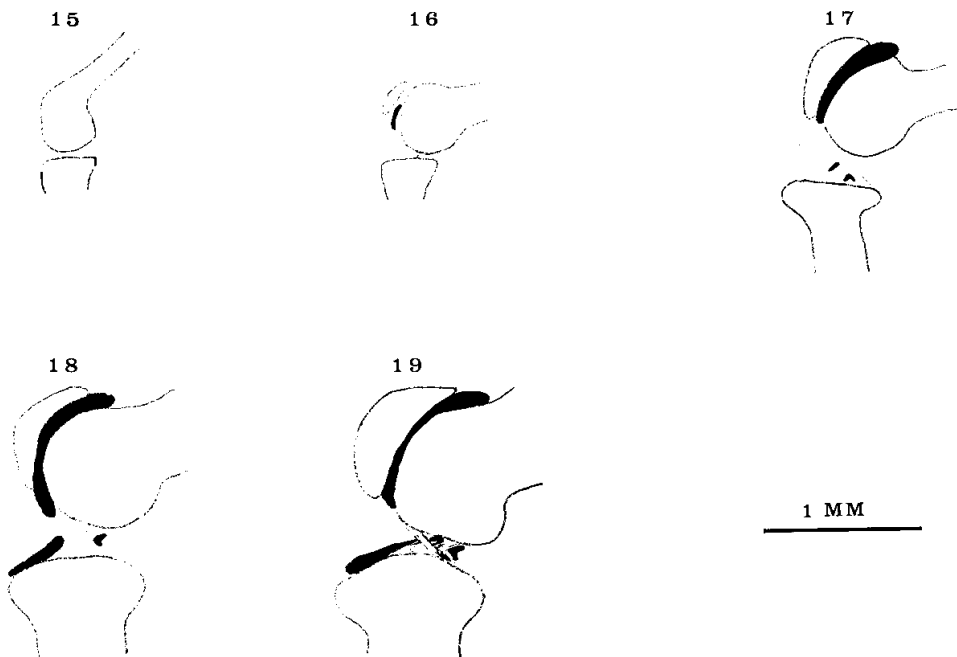


Fig. 1. A schematic figure of the development of joint cavity. The joint cavities are filled with black color. The bar is 1 mm.

in the 17-day-old fetus. Later, many type A synoviocytes were located at the primordium of the synovial fold which had many blood vessels. So it is necessary to follow up the migration of cells in the blood vessels.

The development of joint cavities falls into three stages. The first is the formation of three layers at the interzone. The second is the appearance of blood vessels in the loose connective tissue, and the third stage is the formation of the joint cavity (Whillis, 1940; Gray and Gardner, 1950; Gardner and O'Rahilly, 1968; O'Rahilly and Gardner, 1975; Mitrovic, 1975).

The formation of joint cavity in the knee begins in the 16-day-old fetus in the rat and at the Streeter stage 23 (end of 8 weeks after ovulation) in humans (Gardner and O'Rahilly, 1968). In this experiment, that began at the 16-day-old fetus in mouse. Blood vessels in the 15-day-old fetus appeared between the femur and tibia, and large vessels located between the two cruciate ligaments and femur were very interesting finding.

The size of these vessels decreased as development progressed. On the surface cruciate ligaments, which were exposed to the joint cavity, the type A synoviocytes did not exist, whereas these cells exist in humans (Williams and Warwick, 1980).

The cell junction in the synovial membrane has been reported in rats (Roy and Ghadially, 1967; Muranane and Feagans, 1970), in mice (Linck and Porte, 1978). The existence of the basal lamina in the synovial membrane has been controversial, but we could not find the basal lamina. While the endothelial cells of the capillaries were fenestrated with closing diaphragms.

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≡ 국문초록 ≡

## 생쥐 활액세포의 분화에 관한 면역세포화학적 연구

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생쥐 배자에서 태령 증가에 따른 슬관절의 발생과정을 밝히기 위하여 태령 제15일, 제16일, 제17일, 제18일, 제19일, 생후 1일, 생후 5일에 슬관절 부위를 적출하여 일부는 parplast에 포매하여 조직을 연속 절편을 만들어 H&E 염색, 리조짐 및 Ia 항원에 대한 항체로 이중 연결 PAP(double bridge peroxidase-anti-peroxidase) 기법을 시행하여 관절강의 형성 과정과 활액막에서 리조짐(lysozyme) 양성 A형 활액세포 및 Ia항원 양성 A형 활액세포의 출현 시기와 분포를 관찰하였다. 또한 일부의 조직은 Epon 812에 포매하여 전자현미경을 이용하여 활액세포의 유형과 이들 세포 사이의 복합 연결, 기저막 그리고 활액막 주위에 분포하는 모세혈관의 유형 등을 관찰하였다.

관절강은 슬개골 후방에서, 대퇴골과 경골 사이에서 태령 제16일부터 독립적으로 형성되었다가 태령 제18일에 하나로 연결되었다.

리조짐 양성 활액세포는 태령 제18일부터 처음 출현하기 시작하여 태생말일인 태령 제19일에 슬개골 아래 지방체 원기 표면에서 가장 많이 분포하였으나 관절반월이나 십자인대 표면에서는 관찰되지 않았다.

한편 Ia 항원 양성 활액세포는 출생 5일에 처음 관찰되었다. 활액막의 유형은 태령 제17일에서 섬유형, 태령 제18일에서 소극형, 출생 5일에서 지방형이 처음 출현하기 시작하였다. 기저막은 전 연령에서 관찰되지 않았으며 부착반은 생후 8주인 대조군에서만 관찰되었다. 모세혈관의 내피세포는 그 세포질에 격막으로 단혀진 구멍을 갖고 있는 유창내피세포로 이루어져 있으며 불완전한 기저막을 갖고 있었다.

이상의 실험결과로 보아 관절강은 발생과정을 거침에 따라 각각의 독립된 관절강이 융합되어 출생 전에 완성되고 활액막을 구성하는 리조짐양성 A형 활액세포는 태생말기인 태령 18일에 관찰되나 Ia 양성 A형 활액세포는 출생 5일이나 관찰되는 것을 알 수 있었다.



## LEGENDS FOR FIGURES

- Fig. 2.** Photomicrography of the 8-week-old mouse. Suprapatellar region. Many lysozyme-positive synoviocytes are seen. Immunohistochemical reaction. x400.
- Fig. 3.** Photomicrography of the 8-week-old mouse. Suprapatellar region. Many Ia antigen-positive synoviocytes are seen, but their cytoplasm are also seen as positive reaction. Immunohistochemical reaction. x400.
- Fig. 4.** Photomicrography of the 8-week-old mouse. Junction of the synovial membrane and the meniscus. S=synovium, M=meniscus. Immunohistochemical reaction. x125.
- Fig. 5.** Photomicrography of the 8-week-old mouse. Anterior cruciate ligament. No synoviocyte is located on the surface of ligament. HE. x400.
- Fig. 6.** Photomicrography of the 15-day-old fetus. The cartilage of femur and tibia, the primordium of synovial fold, which has many blood vessels, are seen. AC=anterior cruciate ligament, F=cartilage of femur, IT=infrapatellar tendon, PS=primordium of synovial fold, T=cartilage of tibia. HE. x125.
- Fig. 7.** Photomicrography of the 16-day-old fetus. The joint cavity is formed between the patella and the cartilage of femur. P=patella, IT=infrapatellar tendon. HE. x125.
- Fig. 8.** Photomicrography of the 16-day-old fetus. The joint cavity is formed between the cartilage of femur and the cartilage of tibia. Many necrotic cells are seen. HE. x250.
- Fig. 9.** Photomicrography of the 17-day-old fetus. Large vessels are connected in the lower portion of the anterior cruciate ligament. AC=anterior cruciate ligament. HE. x125.
- Fig.10.** Photomicrography of the 17-day-old fetus. Adjacent section of Figure 5. A large triangular vessel is seen. A slit-like joint cavity is formed just anterior to the lower portion posterior cruciate ligament(arrowheads), but lysozyme-positive cells are absent. Immunohistochemical reaction. x125.
- Fig.11.** Photomicrography of the 17-day-old fetus. Many cells are seen in the joint cavity, their cytoplasm eosinophilic and foamy (arrowheads). AC=anterior cruciate ligament. HE. x250.
- Fig.12.** Photomicrography of the 17-day-old fetus. One lysozyme-positive cell is in the joint cavity(arrowhead). The morphology of cell is similar to that of Figure 7. Immunohistochemical reaction. x400.
- Fig.13.** Photomicrography of the 18-day-old fetus. A bridge of connective tissue, which contains blood vessels, is laid in the joint cavity(arrows). HE. x125.
- Fig.14.** Photomicrography of the 18-day-old fetus. Joint cavity widens at the anterior and posterior area of cruciate ligaments. AC=anterior cruciate ligament. HE. x125.
- Fig.15.** Photomicrography of the 19-day-old fetus. Both cruciate ligaments are fully exposed to the joint cavity. AC=anterior cruciate ligament. HE. x125.
- Fig.16.** Photomicrography of the 19-day-old fetus. Adjacent section of Figure 11. The synovial fold has many lysozyme positive cells. C=joint cavity. Immunohistochemical reaction. x125.
- Fig.17.** Photomicrography of the 19-day-old fetus. Infrapatellar synovial fold region. One layer of lysozyme-positive synoviocytes are seen(arrowheads). Immunohistochemical reaction. x400.
- Fig.18.** Photomicrography of the 1-day-old neonate. Posterior wall of the synovial cavity. A few lysozyme-positive synoviocytes are seen(arrowheads). Immunohistochemical reaction. x250.
- Fig.19.** Photomicrography of the 1-day-old neonate. Adjacent section of Figure 14. No synoviocyte is positive for Ia antigen. Immunohistochemical reaction. x250.
- Fig.20.** Photomicrography of the 5-day-old neonate. Infrapatellar region. A single layer of lysozyme-positive synoviocytes is seen. Immunohistochemical reaction. x400.
- Fig.21.** Photomicrography of the 5-day-old neonate. Infrapatellar region. A single layer of Ia positive synoviocytes is seen. Immunohistochemical reaction. x400.
- Fig.22.** Electronmicrography of the 18-day-old fetus. A typical type A synoviocyte is seen. Its cytoplasm has many vacuoles, but no basal lamina is seen. x6,700.
- Fig.23.** Electronmicrography of the 17-day-old fetus. A typical type B synoviocyte is seen. The rough endoplasmic reticulum is prominent, and the Golgi apparatus is also seen, but no basal lamina is visible. x10,000.
- Fig.24.** Electronmicrography of the 8-week-old mouse. A desmosome (arrowheads) is seen between type B synoviocytes. x20,000.
- Fig.25.** Electronmicrography of the 18-day-old fetus. The endothelial cell of capillary is fenestrated with closing diaphragms (arrowheads). The basal lamina is incomplete. R=red blood cell in capillary. x50,000

