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# The immune reaction to heterologous serum causes osteonecrosis in rabbits

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**Abstract** Osteonecrosis (ON) was produced experimentally in rabbits by intravenous injection of horse serum. Eighty adult rabbits were used: 16 were injected twice with isotonic saline (Group A), 24 were injected once with saline and once with horse serum (Group B), and 40 were injected twice with horse serum (Group C). Both femurs of each rabbit were obtained from 2 h to 7 weeks after the final injection an were subjected to histological examination. No pathological changes were seen in Groups A and B. In Group C, 5 of 15 rabbits (33%) showed ON (necrosis of trabecula and bone marrow) in the femoral metaphysis. In Group C, the early major pathological findings in bone marrow are extravasation of erythrocytes in sinusoidal spaces and microthrombi in small arteries and arterioles near the lesion of extravasation. Immune complexes were demonstrated in the kidney within 24 h of the final injection of horse serum. The present study suggests that immunological reaction associated with serum sickness may play an important role in inducible ON and this model will contribute toward clarifying the pathogenesis of ON.

**Key words** Osteonecrosis · Serum sickness
Immune reaction · Animal model

## Introduction

The cause of non-traumatic osteonecrosis (ON) of the femoral head remains unknown, although various hypotheses have been advocated. A number of experiments have been conducted in steroid-treated rabbits [1, 3, 4, 5, 6, 7, 12, 15, 16, 17], but they failed to cause ON reproducibly, despite the presence of fat emboli [1, 3, 4, 6], an increase in the size of fat cells [16], increased intraosseous pressure [17], and fatty degeneration of osteocytes [7]. Thus, none of the theories have been verified and the pathogenesis of ON is still unknown.

Most researchers have used corticosteroids alone in healthy animals, with the exception of Gosling and coworkers [5], who combined corticosteroids with skin heterografts but also failed to cause ON reproducibly. Our previous expeprimental study in rabbits showed that the combined effect of antigen-antibody reaction and high-dose corticosteroids could produce ON [10], but the respective contributions of steroids and hypersensitivity could not be clarified. In this study, we injected rabbits only with horse serum to investigate its role in the causation of ON.

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## Materials and methods

Eighty mature Japanese white rabbits (Kbs-JW) weighing 3.0–3.6 kg were purchased from Kitayama-labes (Nagano, Japan), housed at constant temperature and relative humidity and were allowed free access to food and water.

Animals were divided into three groups. Group A consisted of 16 control animals that received 10 ml/kg of isotonic saline intravenously on two occasions with a 3 week interval between doses. Group B was 24 animals which received 10 ml/kg of whole sterile horse serum (GIBCO Laboratories, Grand Island, New York) intravenously at 3 weeks after a single injection of isotonic saline. Group C comprised 40 animals that received 10 ml/kg of horse serum intravenously on two occasions at a 3 week interval, according to the method of Matsui et al. [10].

Two animals in Group A, 3 animals in Group B and 5 animals in Group C were respectively killed at 2, 4, 8, 24 and 72 h, and 1, 3, 7 weeks after the completion of treatment. The whole femur was fixed in 10% neutral buffered formalin for 5 days and was then decalcifired in 25% formic acid/25% citric acid for 2 weeks. The femur was radiographed with a fine grain film for soft X-ray, cut into transverse 3 mm thick sections, embedded in paraffin wax, cut into 5 μm sections and stained with haematoxylin and eosin.

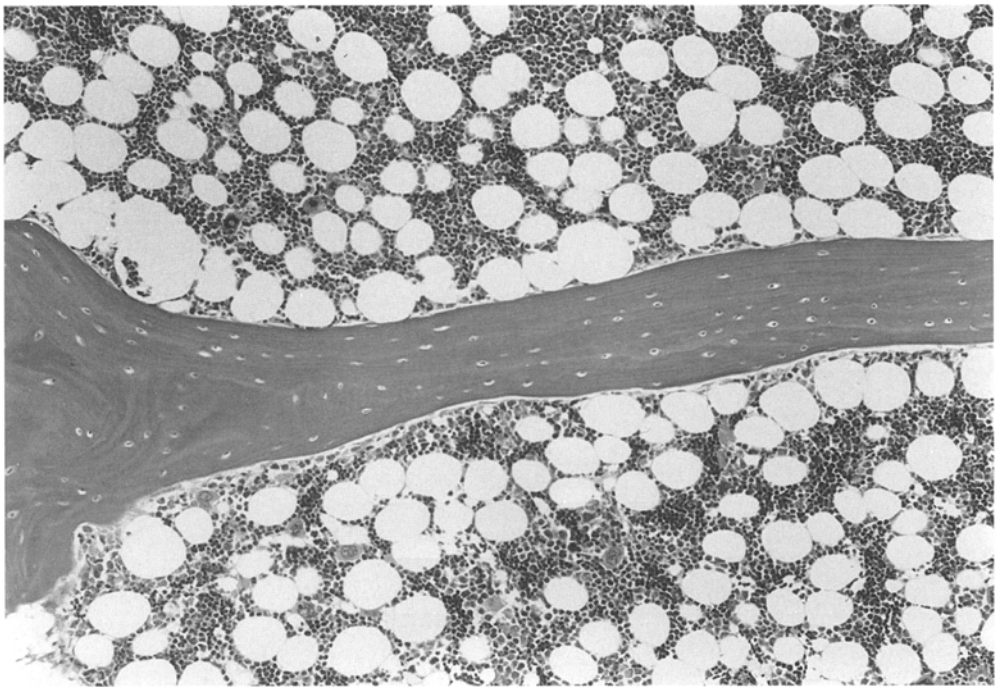

**Fig. 1** Bone trabecula and marrow (Group A). Osteocytes and marrow cells are alive and appositional bone formation are not seen. (H. & E.; original magnification, ×100)

**Table 1** Early pathological findings in Group C

| Time after the second injection | Number of animals | Extravasation of erythrocytes | Microthrombi in arterioles | Immune complex deposition in kidney |
|---|---|---|---|---|
| 2 h | 5 | 2 | 0 | 0 |
| 4 h | 5 | 3 | 2 | 2 |
| 8 h | 5 | 3 | 3 | 3 |
| 24 h | 5 | 3 | 2 | 4 |
| 72 h | 5 | 3 | 3 | – |

**Table 2** Late pathological findings in Group C

| Time after the second Injection | Number of animals | Marrow necrosis | Trabecular necrosis |
|---|---|---|---|
| 1 week | 5 | 2 | 1 |
| 3 weeks | 5 | 2 | 2 |
| 7 weeks | 5 | 2 | 2 |

Empty lacunae of osteocytes with appositional bone formation was defined as trabecular necrosis. Cytolysis or karyolysis of marrow cells and loss of distinct cell borders of adipocytes were the definitive criteria of bone marrow necrosis. Both were determined histologically at 1 week or later after the second administration of horse serum.

Twenty rabbits in Group C, which were serially sacrificed within 24 h of the second injection of the horse serum, were used. Representative sections of excised fresh kidney were embedded unfixed in Tissue-Tek OCT compound (Miles Inc., Tarrytown, New York), frozen by liquid nitrogen, and cut into 7 μm sections using a cryostat (Jung CM 3000) (Leica Instruments GmbH, Nussloch, Germany). An immunofluorescence study was performed to detect immune complexes in glomeruli of kidney. Briefly, sections to detect rabbit IgG were blocked with Block ace, and sections to detect rabbit C3 were blocked with 10% non-immune goat serum (Organon Teknika Co., Durham, North Carolina), and then incubated with antibodies for 2 h in a moist chamber at room temperature. The antibodies were fluorescein isothiocyanate (FITC) conjugated monoclonal rat anti-rabbit IgG (Zymed Laboratories Inc., San Francisco, California) (1:20, diluted with PBS) and FITC-conjugated goat anti-rabbit C3 antibody (Organon Teknika Co., Durham, North Carolina) (1:1000, diluted with PBS). As controls, sections were treated with the identical staining sequence, except that the antibodies were replaced by PBS. The sections were mounted with aqueous mounting medium (Gel mount) (Biomeda Co., Foster City, California), and viewed with a universal microscope spectrophotometer (Carl Zeiss, Germany).

## Results

Neither collapse nor deformity of the proximal femur and the femoral head was seen in any of the groups, radiographically or macroscopically.

Microscopically, there were no pathological findings in Groups A and B (Fig. 1). In Group C, however, various changes were noted such as extensive extravasation of erythrocytes, microthrombi in arterioles, marrow necrosis and trabecular necrosis in the proximal metaphysis and diaphysis of the femur, but rarely in the femoral head (Table 1 and 2).

Extravasation of erythrocytes, which was spotty and multiple at first, appeared from 2 h after treatment (Fig. 2) and was the initial pathological finding. It was likely to oc-

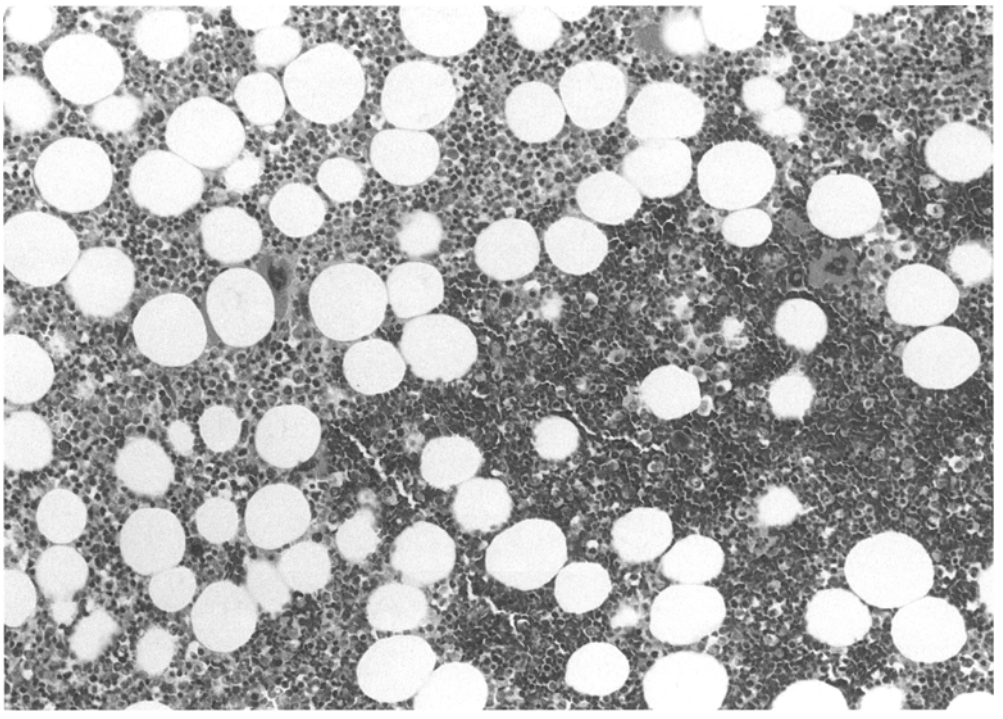

**Fig. 2** Extravasation of erythrocytes (Group C). Increase in number of erythrocytes are seen in marrow space. (H. & E.; original magnification, ×100)

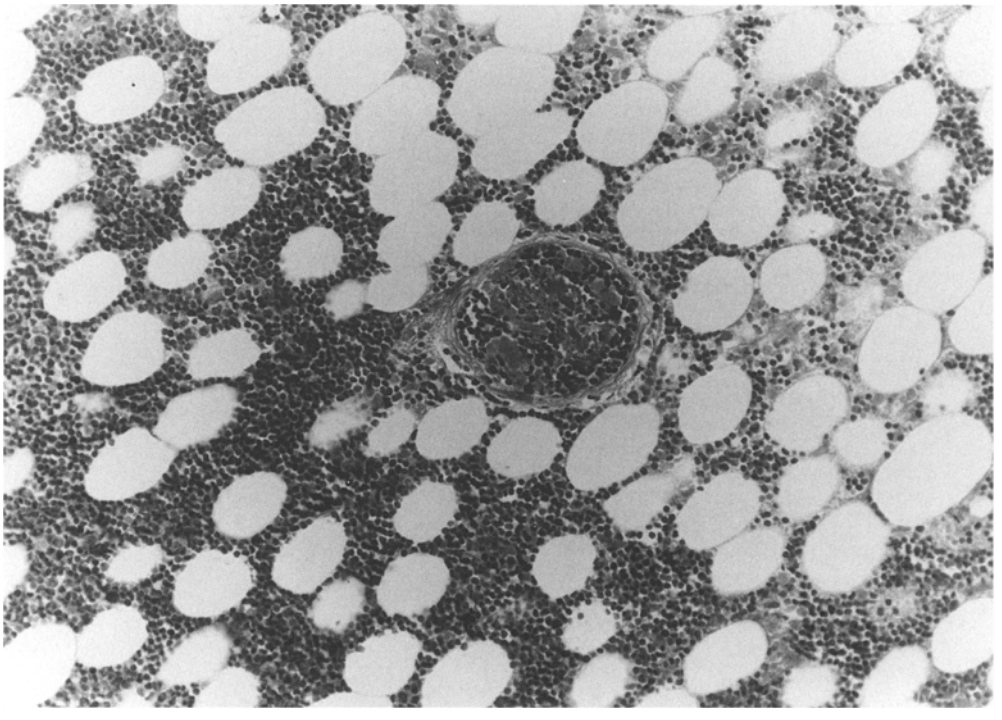

**Fig. 3** Microthrombi formation (Group C). A small artery is filled with thrombi near haematoma. (H. & E.; original magnification, ×100)

cur through sinusoids since there were no ruptured larger blood vessels observed around extravasated erythrocytes.

Microthrombus formation was observed mainly from 4 h or later (Figs. 3 and 4). Small arteries and arterioles filled with microthrombi were usually seen near the lesion of massive extravasation.

ON (necrosis of trabecula with bone marrow necrosis) was histologically evident after 1 week (Fig. 5). The incidence of ON was 33% (5 of 15 rabbits). The dead trabecula with marrow necrosis had empty lacunae and were surrounded by living osteoblasts (Fig. 6). Fibrovascular proliferation which is extensive in the reparative process, was widey observed 3 weeks after treatment (Fig. 7).

Immune complexes could be detected as a granular pattern in glomeruli of the kidney using immunofluores-

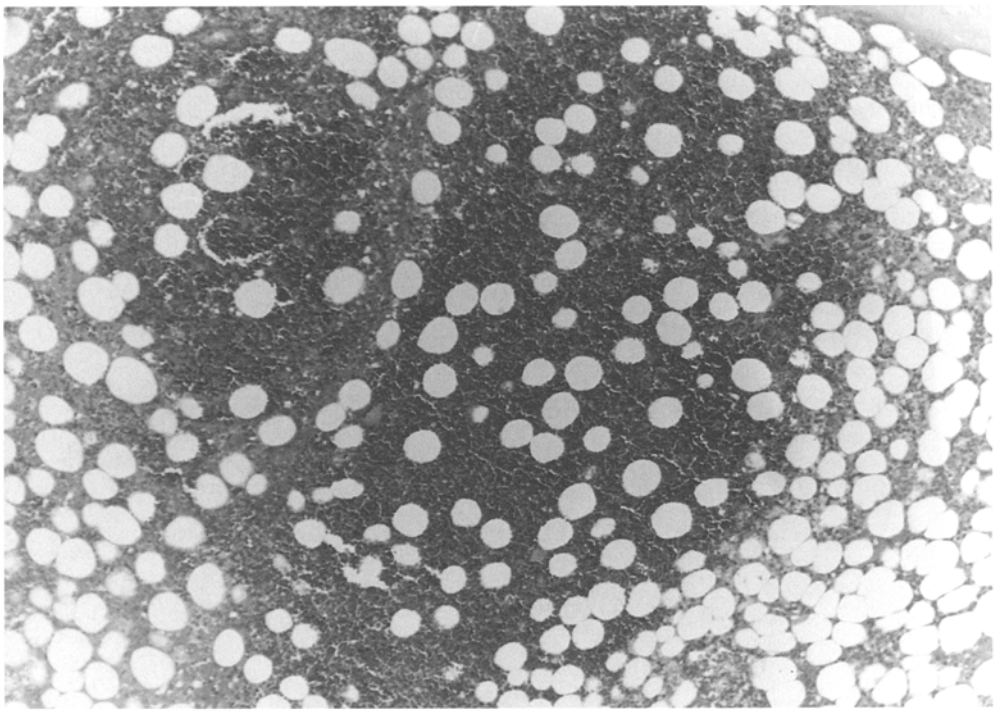

**Fig. 4** Massive extravasation of erythrocytes (Group C). Mass of erythrocytes is observed in the sinusoidal space of bone marrow. (H. & E.; original magnification, ×40)

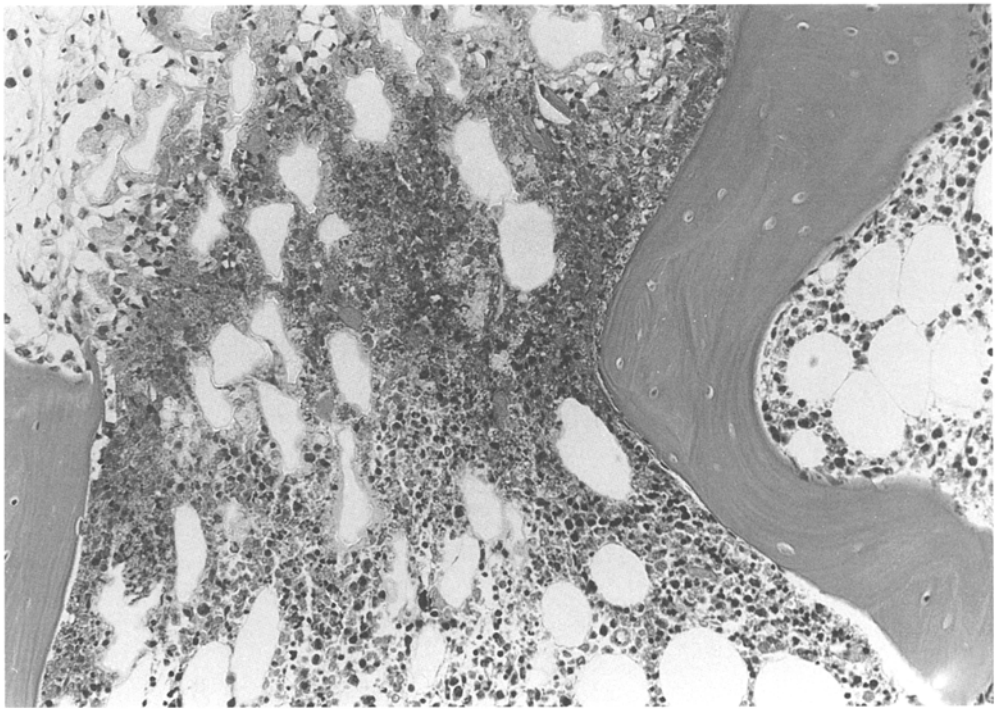

**Fig. 5** Marrow necrosis (Group C). It features death of marrow cells and adipocytes. (H. & E.; original magnification, ×100)

cence (Fig. 8). Immune complex deposition was observed in the kidney mainly from 4 h or later (Table 1).

## Discussion

It is well known that repeated injection of heterologous protein can induce an antigen-antibody reaction in many organs of the rabbit [2, 13], but pathological findings in the bone marrow have rarely been reported. The present study showed that two injections of heterologous serum produced extravasation of erythrocytes, microthrombosis and ON in the femur of mature rabbits at a high incidence.

Patients with steroid-associated ON have various underlying pathologies including collagen diseases and re-

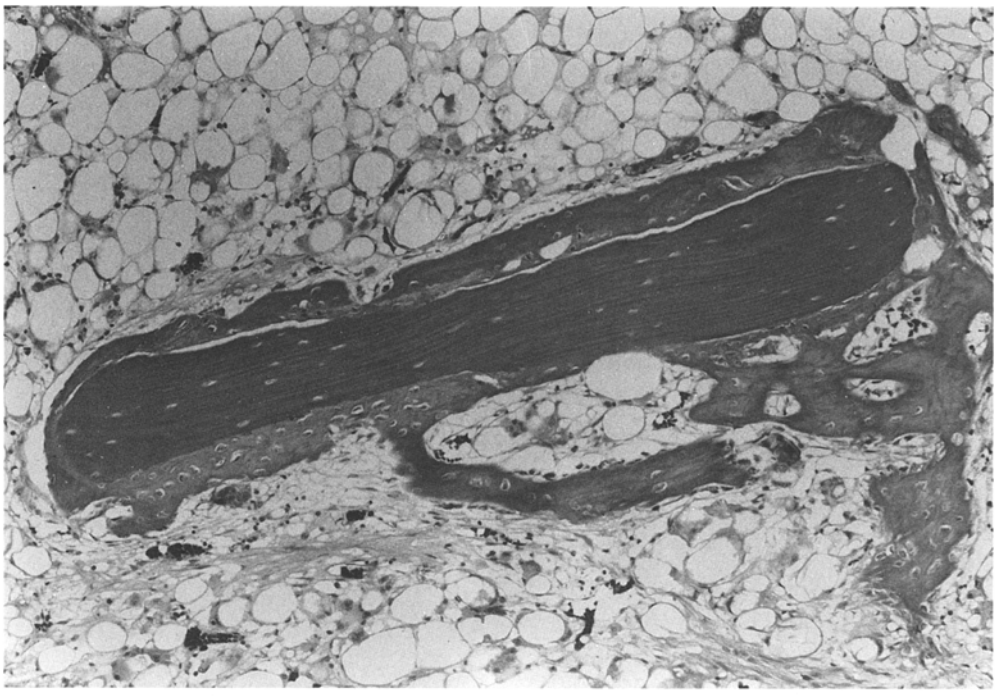

**Fig. 6** Osteonecrosis (Group C). Empty lacunae of trabecula surrounded by osteoblasts and new bone are seen in addition to bone marrow death. (H. & E.; original magnification, ×40)

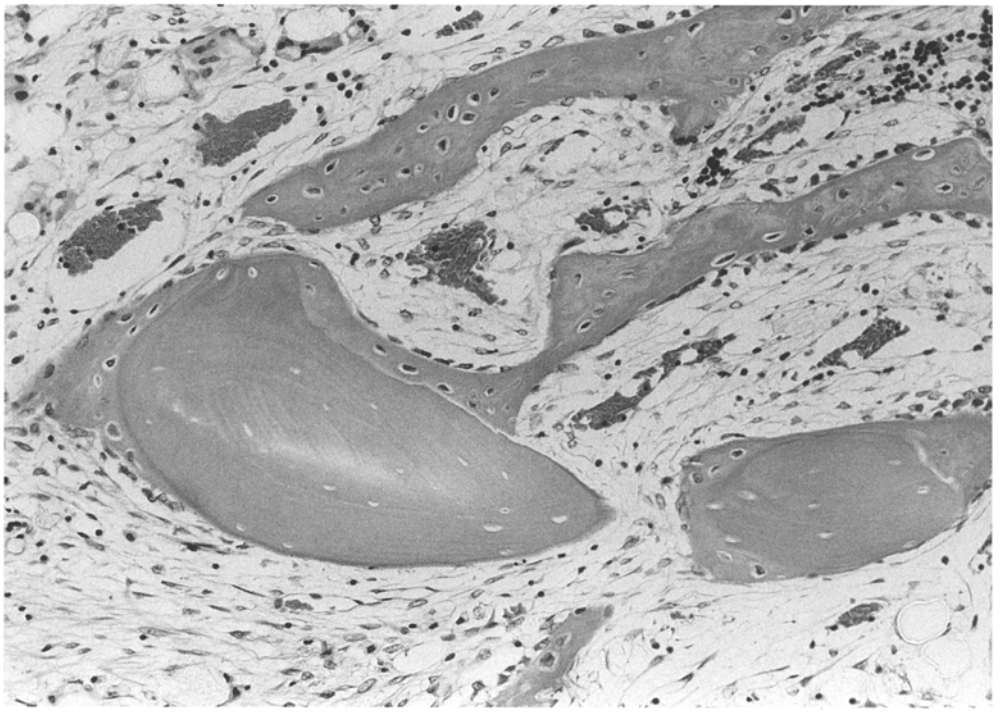

**Fig. 7** Osteonecrosis (Group C). Fibrous tissue infiltrates necrotic area and appositional bone is formed on dead trabecula. (H. & E.; original magnification, ×100)

nal transplantation [8, 9] usually characterized by antigen-antibody reactions. Immune complex deposition could cause the release of vasoactive amines from mast cells and basophils through complement activation leading to increased vascular permeability, which has been found in classical experimental serum sickness. In this rabbit serum sickness model, immune complexes could be demonstrated in the kidney where immune complex deposition is often observed in patients with systemic lupus erythematosus [8]. These findings suggest that some kind of immunological reaction, presumably immune complex deposition, may produce extravasation of erythrocytes and microthrombi in arterioles followed by ON. In the present serum sickness model, immune complexes may be formed in bone as well as in kidney. Further investigation is clearly needed to examine whether immune complex deposition exists in bone and could induce the early major pathological findings in bone marrow (extravasation of erythrocytes and microthrombi in arterioles).

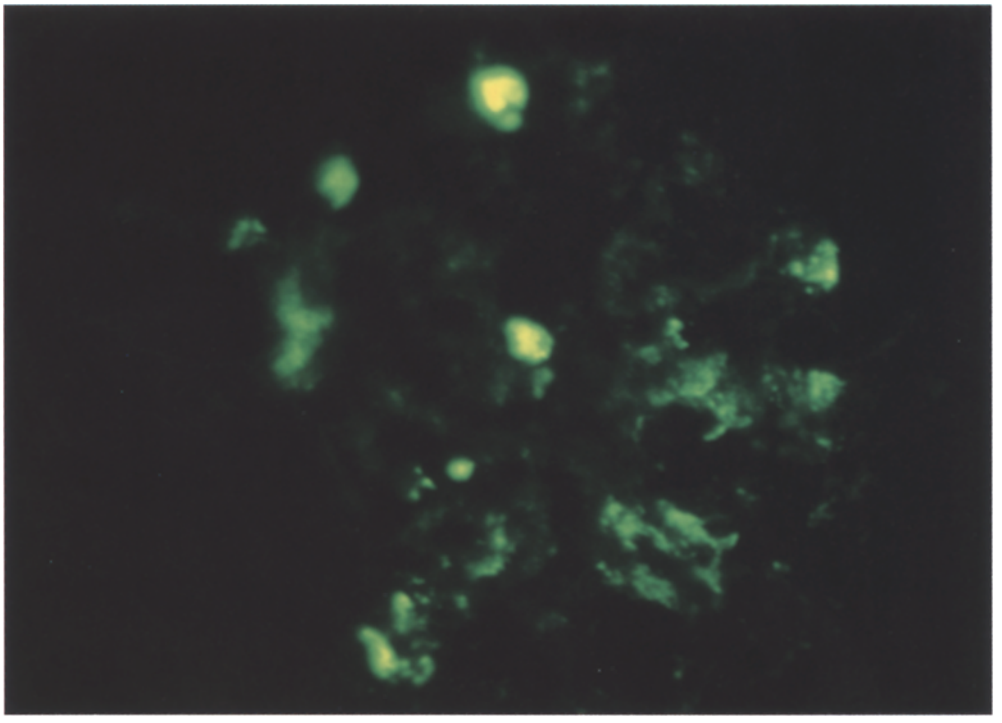

**Fig. 8** Immune complex deposition in kidney (Group C). Immune complexes deposited in granular pattern in glomeruli of the kidney. Rabbit IgGs in immune complexes were stained (Immunofluorescence study, ×400)

Corticosteroids alone do not cause ON reproducibly in experimental studies [1, 3, 4, 5, 6, 7, 12, 14, 15, 16, 17], and there is no evidence of a direct association between steroid therapy and ON. In our previous study [10], ON could be induced in mature rabbits by antigen-antibody reaction in combination with high-dose corticosteroids. However, in the present study, only antigen-antibody reaction by the injection of horse serum twice could induce ON without corticosteroid administration. The effects of corticosteroids on ON remains obscure.

The main location of ON in this rabbit model was not in the epiphysis, but rather in the metaphysis and diaphysis. On in the epiphysis was observed in only one specimen of those having ON in the metaphysis and diaphysis. In recent years, MR imaging has become widely used in the orthopaedic field and metaphyseal ON lesions have been frequently detected in the femur or tibia of patients with intracapital ON [11]. Although collapse subsequent to ON is usually restricted to the weight-bearing area, the early involvement of ON may be more widespread in bone marrow.

In conclusion, the experimental animal model for non-invasive, inducible ON was established using heterologous protein (serum) administration. The model could offer valuable pathological findings in ON especially in its early stages. One could obtain samples before both trabecular and marrow necrosis are demonstrated histologically. Thus, the model would be useful for researchers to have an insight into pathogenesis of ON although it may not be necessarily analogous to what is seen in humans.

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