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Epithelioid Cell Granulomas Experimentally Induced by Prototheca in the Skin of Mice: A Light Microscopic Study

Yasuhiro HORIUCHI and Mikio MASUZAWA

Department of Dermatology, Kitasato University School of Medicine, Sagamihara, Japan

ABSTRACT

Prototheca wickerhamii, an achlorophyllous algae, was previously found to induce massive epithelioid cell granulomas in the skin of mice. By means of light microscopy, examination was made of the histological reactions involved in epithelioid cell granulomas induced by intradermal and/or subcutaneous inoculation of *Prototheca wickerhamii* in BALB/c and ICR mice. Six BALB/c mice showed granuloma nodules while only three of six ICR mice did so. Based on the results of the present and previous studies, BALB/c mice may be considered a strain particularly vulnerable to contracting epithelioid cell granuloma and ICR mice, a resistant strain.

In very early lesions at one week following initial prototheca inoculation, cellular infiltration with varying numbers of polymorphonuclear leukocytes, lymphocytes and some macrophages was observed throughout the dermis and subcutaneous fat tissue. In early lesions at one to two months after inoculation, focal granulomas composed of histiocytic cells and/or macrophages were observed. Mast cells were occasionally present among the histiocytic cell infiltrates. In the granulomatous lesions at two to three months, scattered eosinophils and some lymphocytes were seen. Central necrosis, with numerous neutrophils and many endospores surrounded by the granuloma, was often observed. In late stage lesions at six months, massive lymphocyte and plasma cell infiltration surrounding and/or intervening between vacuolated epithelioid cell clusters was evident. Histological reactions in epithelioid cell granuloma and the ultimate course of this disease can be staged from the histological point of view as follows: 1) diffuse inflammation, 2) cell proliferation leading to epithelioid cell formation, 3) hypertrophy of epithelioid cells with consequent formation of cell aggregates and/or organized granuloma and 4) degeneration of granuloma. Histological reactions were found in this study to be essentially the same in ICR and BALB/c mice, though a time lag was noted in the course of epithelioid cell granuloma formation.

Key words: *Epithelioid cell granuloma, Prototheca, Mice*

Epithelioid cell granuloma has been studied widely in sarcoidosis and in tuberculosis[17]. In sarcoidosis, the bacillus or other factors causing this disease have not been determined, although certain substances[12] and metals such as zirconium[15] cause a sarcoid type granuloma. Epithelioid cells derive from tissue macrophages (histiocytes)[17], which come from bone marrow precursors via circulating monocytes[16]. Little attention has been directed, however, to the histological reactions involved in the onset of early epithelioid cell granuloma, particularly at the start of characteristic lesion formation, and in the late degenerative phase of granuloma.

Prototheca wickerhamii, an achlorophyllous algae[10], is often found in the milk of cows with mastitis, in marine water and sewage. Human protothecosis is extremely rare[10]. The histological reactions in most reported cases with cutaneous protothecosis are chronic granulomatous inflammation with a mixed inflammatory cell infiltration[10]. A number of human cases with a histology of epithelioid cell granuloma, however, have been reported[14]. Experimental protothecosis in animals appears in some reports, one of which maintains that this condition occurs only in the liver of prednisolone-treated mice as a result of *Prototheca zopfii* strain inoculation[8]. *P. wickerhamii*, isolated from skin specimens of a patient with cutaneous protothecosis, was found in a previous study to induce massive epithelioid cell granulomas in the skin of mice[6]. This may

Reprint requests to: Dr.Y.Horiuchi, Division of Dermatology, Higashi-Matsuyama Medical Association Hospital, 1-15-10 Shinmei-cho, Higashi-Matsuyama City, Saitama 355, Japan.

thus serve as a suitable animal model for studying epithelioid cell granuloma formation. The same lesion was also observed in this study, in which examinations were focused on ascertaining specific histological reactions in the course of development of granulomas experimentally induced by prototheca in the skin of mice.

MATERIALS AND METHODS

Mice

Six female BALB/c mice and six female ICR (Institute of Cancer Research) mice, from SLC Japan CO.Ltd (Shizuoka, Japan) were used, in order to determine differences in histological reactions in the course of epithelioid cell granuloma formation between BALB/c mice with specific immunity sensitive to X-ray radiation and ICR mice with normal immunity. Pelleted food was made available throughout the study.

Preparation and Inoculation of Prototheca

Prototheca wickerhamii organisms[6], obtained from skin biopsy specimens of a patient[10] with cutaneous protothecosis, were cultured in Sabouraud's medium (peptone, 10g and glucose, 20g/liter). A 0.5ml aliquot of the organisms of 4.0 to 8.0×10^6/ml was washed once with sterile saline and resuspended in 0.5ml saline. The prototheca suspension was divided into three portions: 1) for biopsy at one week following initial inoculation or at one week following a period of inoculation of one or two months subsequent to initial inoculation, 2) for biopsy at one week following a period of inoculation of three months subsequent to initial inoculation and 3) for biopsy at one or two months following a period of inoculation of six months subsequent to initial inoculation. The suspension was inoculated into the dermis and/or subcutaneous tissue of the back (three separate sites) of each mouse once every week. On a specified day after inoculation, biopsy specimens were obtained and fixed in 10% formalin solution and stained with hematoxylin-eosin, periodic acid-Schiff (PAS), Giemsa solution and toluidine blue.

RESULTS

All six BALB/c mice developed a nodular granuloma by one to three months following the initial prototheca inoculation. Only three of the six ICR mice showed the nodular granuloma at two to six months. As a result, BALB/c mice were considered a strain particularly vulnerable to contracting epithelioid cell granuloma and ICR mice, a resistant strain.

Very early lesions (One week after initial inoculation)

In both the BALB/c and ICR mice, polymorphonuclear leukocytes, some mast cells and eosinophils were seen sparsely but diffusely scattered throughout the deep dermis or subcutaneous fat tissue (Fig. 1a,b). No granulomatous reactions could be detected. A few large macrophage-like cells were occasionally present (Fig. 1a). PAS

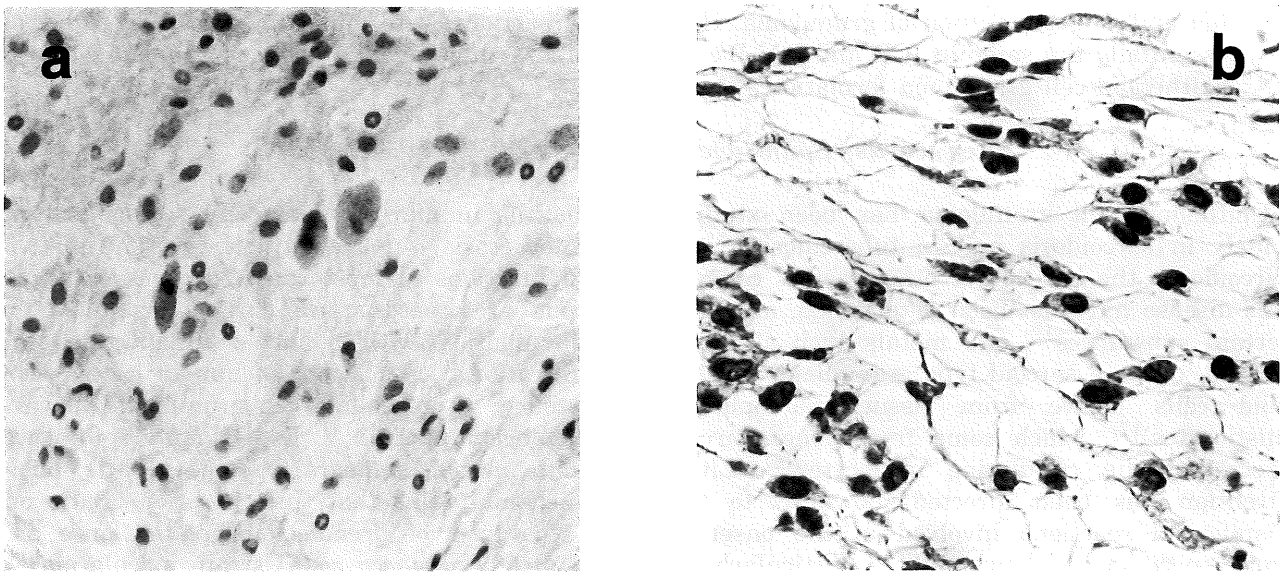

Fig. 1. Very early lesions (one week after initial inoculation). a: In the BALB/c mice, mononuclear cell infiltration, primarily polymorphonuclear neutrophils, eosinophils, histiocytes, macrophages and a few mast cells can be seen in some places throughout the deep dermis. Note three large macrophages mixed with mononuclear cell infiltrates. (Hematoxylin & eosin, ×200) b: In the ICR mice, mononuclear cell infiltration, histiocytes, neutrophils and eosinophils are evident throughout the subcutaneous fat tissue. (Hematoxylin & eosin, ×400)

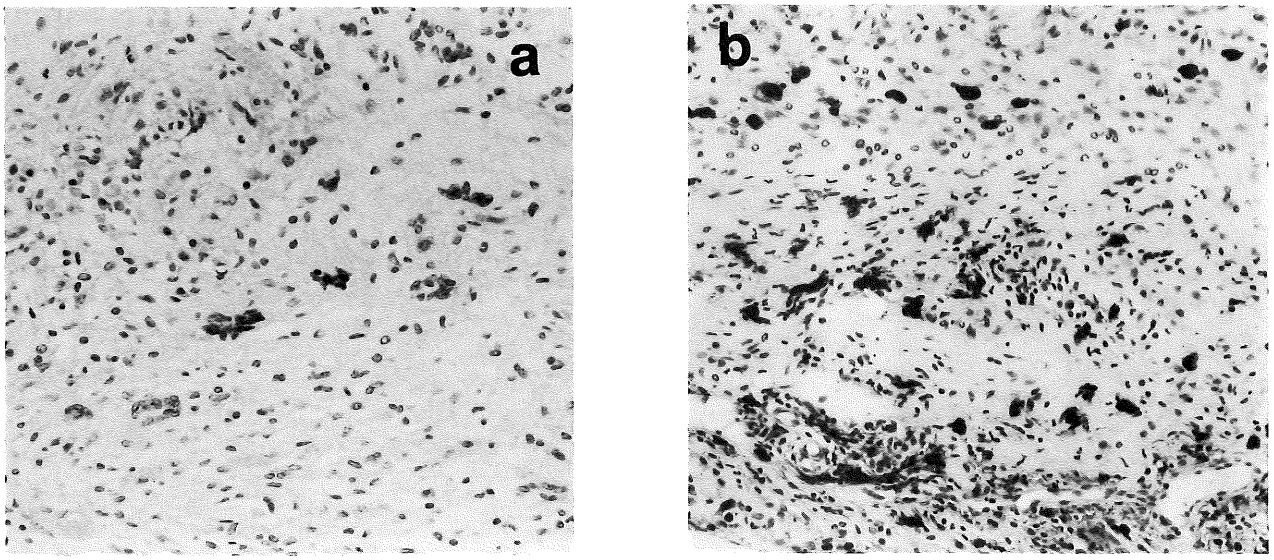

Fig. 2. Early lesions (one month after initial inoculation). a: In the BALB/c mice, extensive histiocytic infiltration with scattered multinucleated giant cells and macrophages are apparent.(Hematoxylin & eosin, ×100) b: Scattered mast cells stained with Giemsa can be seen among mononuclear/histiocytic infiltrates. (Giemsa stain, ×100)

staining failed to show algal organisms.

Early lesions (One to two months following initial inoculation)

In the BALB/c mice, the extensive histiocytic infiltration contained epithelioid-like cells. Aggregates of histiocytic cells with multinucleated giant cells were occasionally noted in the dense histiocytic infiltrate (Fig. 2a). Thereafter, the multinucleated giant cells ceased to be evident in the late phase of granuloma. Scattered mast cells staining with Giemsa solution and toluidine blue were seen among histiocytic/macrophage-like cells in the deep dermis (Fig. 2b). Some prototheca organisms still intact were present among and within some cells. In the ICR mice, extensive histiocytic cell infiltration was apparent, and some macrophage-like cells could be seen along the edge of dense histiocytic cell infiltrates (Fig. 3a). Focal aggregated histiocytes, macrophages and developing epithelioid-like cells were noted among the cellular infiltrates (Fig. 3b). Epithelioid cells formed cellular nests or clusters (Fig. 3b, c). In some cases, plasma cells were situated quite close to the epithelioid cell nests (Fig. 3c). Small pieces of PAS positive material were present in the cytoplasm of the histiocytic cells and/or macrophages.

Organized granuloma lesions (Three months following initial inoculation)

At this stage, epithelioid cell granulomas were evident in the dermis and subcutaneous fat tissue of both mice species (Fig. 4a). In the BALB/c mice, a central necrotic area with cytolytic neutrophils mixed with numerous prototheca organisms (Fig. 4b, c, PAS stain) surrounded by histiocyte and epithelioid cell clusters could frequently be seen (Fig. 4b). Epithelioid cell nests (Fig. 4a, b) were particularly evident in the BALB/c mice, and appeared to be tumorous (Fig. 4b). The mature epithelioid cells had large, pale oval nuclei with prominent nucleoli and abundant eosinophilic cytoplasm (Fig. 4d). In the ICR mice (photographs not shown), dense histiocytic cell infiltrates mixed with scattered neutrophils and eosinophils were noted, along with a number of small epithelioid cell nests. Hardly any prototheca organisms among or within the cellular infiltrates could be found, even following staining with PAS stain. At this stage, some scattered mast cells were seen along the edge of epithelioid granuloma islands in some cases. Extensive reticular fibrosis was evident among the epithelioid cell clusters in both mice strains (Fig. 4a).

Late stage lesions (Six months following initial inoculation)

In both strains of mice, dense inflammatory infiltrates (Fig. 5a), primarily lymphocytes and plasma cells (Fig. 5c) were seen among the epithelioid cell nests, particularly where granuloma still persisted. Scattered multinucleated giant cells (Fig. 5b) were present, particularly in the case of epithelioid cell nests which had vacuolated or degenerated. In some cases, small lymphocytes and leukocytes were diffusely infiltrated among the epithelioid cells. Even in the ICR mice, still intact organisms were frequently seen, particularly where the granuloma still persisted.

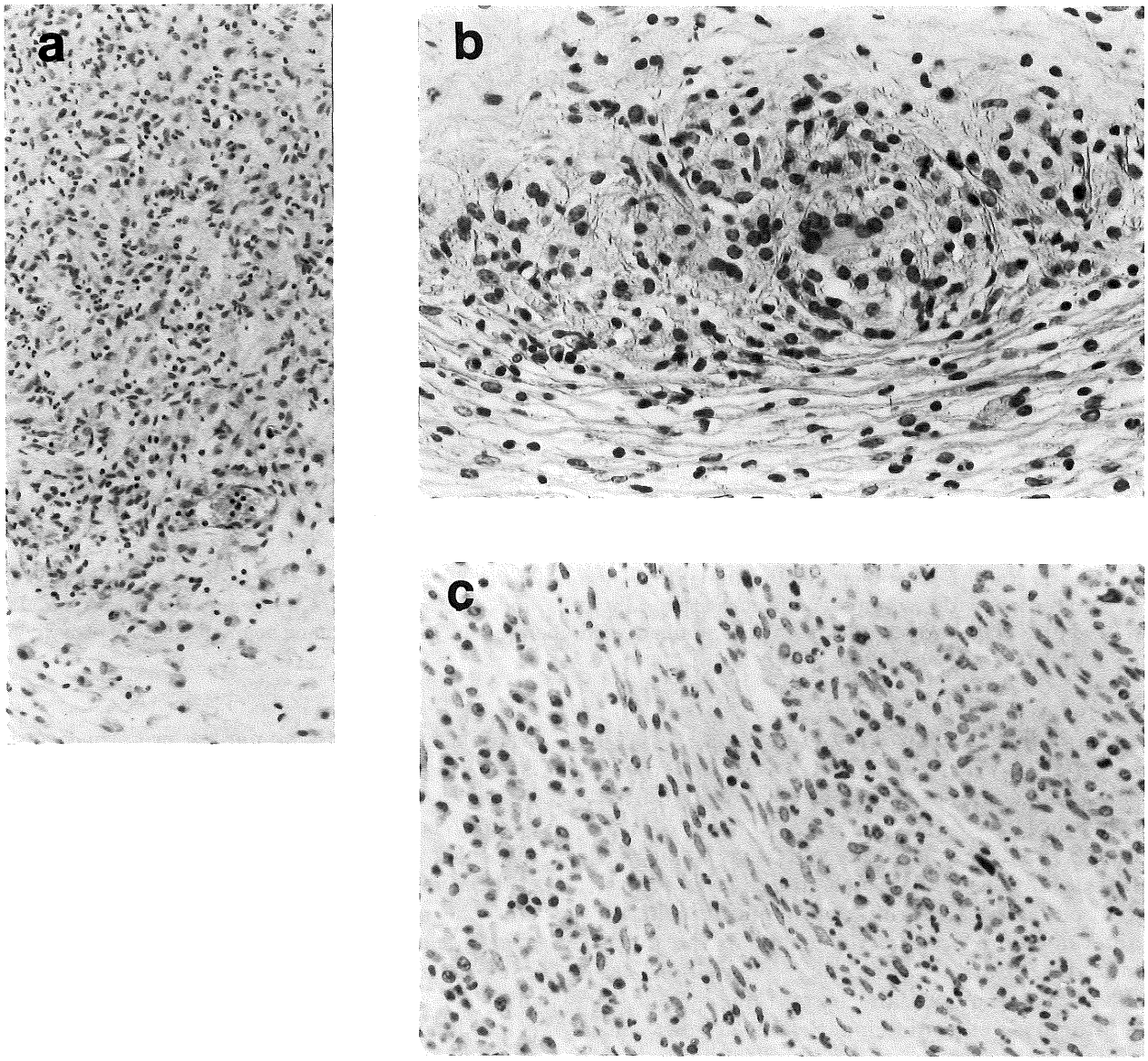

Fig. 3. Early lesions (two months after initial inoculation). a: In the ICR mice, extensive histiocytic cell infiltration is apparent in the dermis and some macrophages are present along the edge of dense histiocytic cell infiltrates. (Hematoxylin & eosin, ×100) b: Epithelioid cell-like aggregates are present. (Hematoxylin & eosin, ×200) c: Outside of epithelioid cell clusters, there are many plasma cells. (Hematoxylin & eosin, ×200)

Distinctive features at this stage were dense inflammatory infiltrates with lymphocytes and plasma cells, and abundant foamy and/or vacuolated and degenerated epithelioid cells (Fig. 5b).

DISCUSSION

In the formation of epithelioid cell granuloma such as sarcoidosis[2], there appear to be three stages: 1) diffuse inflammation, 2) cell proliferation leading to epithelioid cell formation and 3) hypertrophy of the epithelioid cells with consequent cell aggregate formation. The present results support this mode of staging. A late stage in which the epithelioid cell granuloma has degenerated was also noted.

At a very early stage, some histiocytes and macrophages in the dermis and subcutaneous adipose tissue at the site of inoculation could be seen among diffuse polymorphonuclear leukocyte infiltrates. A granuloma is formed first by a proliferation of monocytes[1]. The differentiation of monocytes into epithelioid cells during epithelioid cell granuloma formation and subsequent development may be considered to occur in five morphological stages: monocytes, immature macrophages, macrophages, immature epithelioid cells, and epithelioid cells[1]. The morphological recognition of monocytes by such means as light microscopy has so far been difficult, although infiltrating mononuclear cells with round nuclei

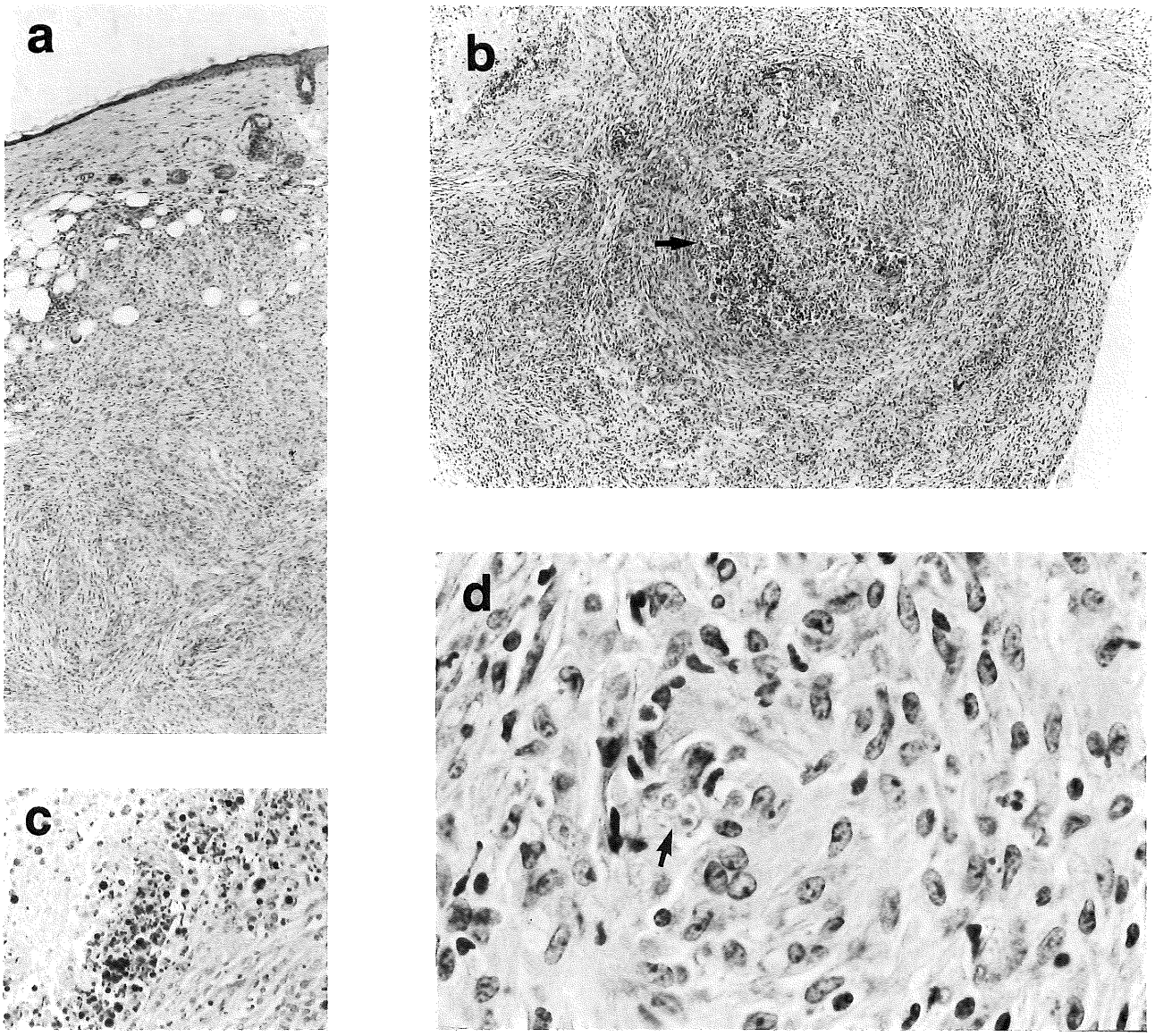

Fig. 4. Granulomatous lesions (three months after initial inoculation). a: In the BALB/c mice, tumorous epithelioid cell granuloma nests can be seen throughout the dermis and subcutaneous fat tissue. (Hematoxylin & eosin, ×40) b: Aggregates of cytolytic neutrophils and many prototheca endospores (arrow) surrounded by epithelioid cell nests. (Hematoxylin & eosin, ×40) c: Prototheca organisms in the central necrotic area with dead neutrophils. (PAS, ×100) d: At high power magification, epithelioid cells have large, pale, oval nuclei with prominent nucleoli and abundant eosinophilic cytoplasm. Note prototheca organisms (arrow) among and within the epithelioid cells. (Hematoxylin & eosin, ×400)

may possibly be monocytes. Clarification in greater detail of specific cellular events in granuloma through the use of cell surface markers should be made. At this stage, no prototheca organisms were present, possibly since they had been digested completely by phagocytic macrophages even in the BALB/c mice.

In the BALB/c mice at about one month after initial inoculation and in the ICR mice, at two months, epithelioid cell-like aggregates and clusters became prominent, suggesting that histiocytes and macrophages may not be able to easily ingest many organisms. In the BALB/c mice, PAS stain at this stage showed some still intact prototheca organisms present among and within the histiocytic cells. At a late stage, even in the ICR mice, there were many prototheca endospores within and among the epithelioid cells, but hardly any could be found in the ICR mice at this stage. Thus, in these mice, organisms may undergo extensive digestion into small pieces[7]. At the time of epithelioid cell granuloma formation, aggregates of cytolytic neutrophils and degenerated or live organisms surrounded by epithelioid cell nests were often present. Thus, for a while, digestion may occur slowly due to the fact

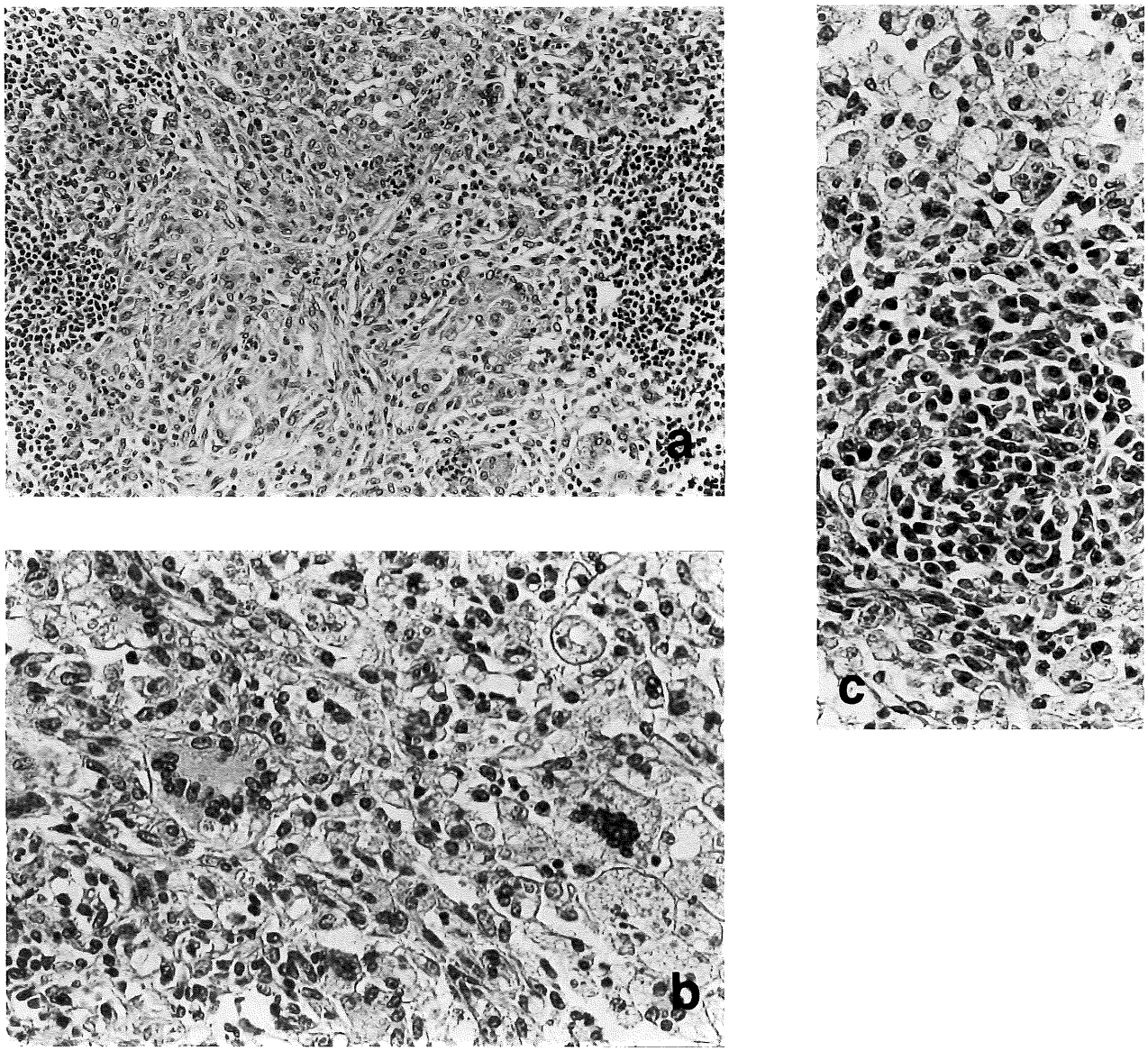

Fig. 5. Late lesions (one month after six months inoculation). a: In the ICR mice, dense lymphocyte infiltration surrounding epithelioid cell nests. (Hematoxylin & eosin, ×100) b: Multinucleated giant cells, and vacuolated and degenerated epithelioid cells. (Hematoxylin & eosin, ×200) c: Aggregates of plasma cells can be frequently seen. (Hematoxylin & eosin, ×200)

numerous organisms are packed together, but thereafter, somewhat more rapidly. At this stage, a few inflammatory cells, primarily lymphocytes and eosinophils, were seen scattered among the epithelioid cell granulomas, and interestingly, on the outside of the epithelioid granulomas. This is in contrast with the late stage, and suggests that granuloma organization may occur through the proliferation and maturation of histiocytes and macrophages into epithelioid cells. The similarity of epithelioid cells in sarcoidosis, leishmaniasis and bacillus granuloma has been demonstrated by light microscopy[2,5]. Morphological differences between the epithelioid cells in the BALB/c and ICR mice were quite difficult to determine by such observation. However, by means of electron microscopy in the BALB/c mice, plasmacytoid epithelioid cells were shown to be the most abundant, while in the ICR mice, fibroblast-like cells[7] were the most abundant. Two different types of epithelioid cells have been reported[4,15].

At one to four weeks from the initial inoculation, mast cells were occasionally noted in diffuse polymorphonuclear leukocyte and dense histiocytic cell infiltrates, as has also observed by Epstein et al.[5] However, in the initial stage of epithelioid cell granuloma, a few mast cell infiltrates were present. Mast cells, in some cases, have been noted in dermatoses[11], and found to contain many chemical mediators and proteolytic enzymes. These enzymes may thus cause the degeneration of connective tissue. However, their

functions in epithelioid cell granuloma have not been fully clarified.

In this study, in contrast to the late stage of granuloma, the initial stage and the stage of tumorous granuloma showed a number of lymphocyte infiltrates surrounding epithelioid cell aggregates. It would thus appear that lymphocytes may not necessarily be the primary factor in the onset of epithelioid cell granuloma. Delayed hypersensitivity[3 4] has been shown to promote the course of epithelioid cell granuloma. However, granulomas have been reported to be induced without the thymic system[5] and in severe combined immunodeficient mice (SCID)[9] without T and B cell functions. Moreover, massive epithelioid granulomas have come about through the action of a muramyl dipeptide (MDP) in nude rats without T-cell participation[13]. Further examination should be made to determine whether histological reactions in epithelioid cell granuloma actually depend on delayed hypersensitivity. At these stages of granuloma, eosinophils were observed scattered among the epithelioid cells, but their functions at these times remain unclear[5]. In the late stage, dense inflammatory infiltrates consisting mainly of lymphocytes and plasma cells were seen close to the epithelioid cell nests, possibly due to the elimination of granuloma, considering the prominent vacuolation of the cells.

Based on the results of this study, the macrophages and histiocytes of BALB/c mice may be concluded to have little ability to digest prototheca organisms. In three ICR mice, no nodular granuloma could be detected at all, even though the cell membranes of prototheca may consist of materials difficult to digest. The histological reactions requisite for epithelioid cell granuloma formation were found in this study to be essentially the same in BALB/c mice with specific immunity sensitive to X-ray radiation and in ICR mice with normal immunity. A time lag was noted, however, in the course of epithelioid cell granuloma formation.

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