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Mouse exophthalmic chronic orbital inflammatory disease

Induction by human leucocyte intracellular Mollicutes

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Summary. Mollicutes are cell wall deficient bacteria which may be overlooked or confused with viruses because of indistinct light microscopic morphology, poor staining, difficulty in cultivation, and the ability to pass 0.450 micron filters. As they have a distinctive ultrastructural appearance they can be identified using transmission electron microscopy [TEM]. Using TEM vitreous leucocytes from chronic endogenous uveitis patients may demonstrate non-cultivable intracellular 0.005–1.0 micron mollicute-like organisms [MLO], some of which develop into distinctive cell walled cocci, 0.5–0.7 micron in diameter. Inoculation of those MLO containing human vitreous into mouse eyelids produces chronic cardiac and uveal vasculitis with orbital inflammation. Similar MLO are found within the mouse lesional leucocytes. This report describes the chronic orbital inflammation with vasculitis in 67 of 100 of those MLO inoculated mice versus 0 of 200 controls ($P<0.05$). Exophthalmos with inflammation also occurred in 12 of those 67 mice ($P<0.05$). MLO were found within orbital lesional leucocytes of 10 of 10 of those mice using a TEM versus 0 of 10 controls. The results indicate that vasculitis and exophthalmos were important features of this MLO induced mouse orbital inflammation. The implication of these results for human idiopathic chronic orbital inflammatory disease is discussed.

Key words: Intracellular Mollicutes – Leucocytes – Vasculitis – Chronic orbital inflammatory disease – Exophthalmos

Introduction

Mollicutes are cell wall deficient bacteria (Freundt and Edward 1979). As they have an indistinct light microscopic morphology (Boatman 1979), stain poorly with the usual biological stains (Boatman 1979), are difficult to cultivate (Rodwell and Mitchell 1979), and pass 0.045 micron filters (Boatman 1979) they are often overlooked or confused with viruses (Starr 1979). Because of their typical ultrastructural morphology, however, they can be readily identified using transmission electron microscopy [TEM] (Boatman 1979; Horne 1972). Extracellular Mollicutes produce disease in animals (Cassell and Hill 1979), plants (Saglio and Whitcomb 1979), and humans (Clyde 1979) by attaching to cell surfaces and releasing cytotoxic substances (Barile 1979). Intracellular non-cultivable mollicute-like organisms [MLO] cause plant vascular disease (Arora and Sinha 1985; Bove 1981; Saglio and Whitcomb 1979; Horne 1972).

Uveitis is form of vasculitis (Green 1986) associated with leucocytic exudation into the surrounding ocular structures and fluids (Green 1986; Spencer 1985; Jakobiec and Font 1986). In uveitis patients with significant vitreous exudation, vitrectomy clears the vitreous (Michels 1981) and provides material for laboratory investigation (Johnson and Wirostko 1986). Most uveitis is idiopathic or "endogenous" and chronic (Friedman et al. 1982; O'Connor 1983). On TEM vitreous leucocytes from chronic endogenous uveitis patients may demonstate intracellular MLO (Johnson and Wirostko 1986; Johnson et al. 1987; Wirostko et al. 1987; Wirostko et al. 1988). The leucocyte cytoplasm is filled with nuclear anchored 0.005–0.010 micron diameter branching filaments that enlarge into typical pleomorphic 0.01–1.0 micron tubulo-spherical MLO. A unique feature of these MLO is the development of distinctive "spore-like" cell walled 0.5–0.7 micron cocci by some of the spherical MLO. This occurs by deposition of electron dense material in the trilaminar outer membrane, and is particularly prominent

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within polymorphonuclear leucocytes parasitised by this MLO.

Like plant MLO these human leucocyte intracellular MLO could not be cultivated (Johnson and Wirostko 1986) but they could be transmitted to mice (Wirostko et al. 1986). Inoculating them into mouse eyelids produced macroscopic and histological chronic orbital inflammation with uveitis (Wirostko et al. 1986). MLO indistinguishable from the human MLO could be detected within the mouse ocular disease leucocytes using TEM (Johnson et al. 1987; Wirostko et al. 1987; Wirostko et al. 1988). These mice also displayed delayed onset accelerated mortality and widely disseminated systemic lesions (Wirostko et al. 1986). Intracellular MLO could also be detected within systemic lesion leucocytes using TEM (Wirostko et al. 1986; Johnson et al. 1988). The following report provides the details of the exophthalmic chronic orbital inflammatory disease in those mice.

Materials and method

The human MLO containing materials were obtained from 4 patients presenting with recent acute exacerbations of chronic endogenous uveitis with severe vitritis (Johnson and Wirostko 1986; Wirostko et al. 1986). Using an Institutional Review Board exempt protocol, each specimen was obtained by vitrectomy (Johnson and Wirostko 1986). The specimen volumes varied from 20–35 cc, and consisted of an admixture of vitreous and sterile saline. Despite using a wide variety of microbiological culture techniques, including those capable of supporting the growth of human extracellular Mollicutes, and tissue culture cell lines, no growth occurred from aliquots of these specimens and no cytopathogenic effect was observed (Johnson and Wirostko 1986). Using TEM the vitreous from each of the 4 patients demonstrated MLO within polymorphonuclear leucocytes (Johnson et al. 1987), lymphocytes (Wirostko et al. 1987), and monocytes (Wirostko et al. 1988). All 4 specimens were stored at 4 degrees Celsius until animal inoculation.

300 DC-1 male mice, 12–16 weeks old, and weighing 15–20 g were inoculated on the same day. Using an Animal Care Committee approved protocol each mouse received a subcutaneous 0.1 cc inoculum in the lateral part of each lower eyelid. The vitreous specimen from each patient was inoculated into 25 mice, for a total of 100 mice. Vitreous from each of 10 eye-bank eyes was inoculated into 10 mice and 100 mice received sterile saline, for a total of 200 control mice. The mice were observed twice daily, 5 days a week, for 12 months for persistent exophthalmos and spontaneous mortality. All mice, both those dying during the observation period and those living at the end, were subjected to a complete autopsy. The eyes and orbital tissues of all mice were eviscerated and fixed in 150 mM cacodylate pH 7.4 buffered 2% formaldehyde/2% glutaraldehyde. After fixation one tissue section taken through the lower lid inoculation site of each eye and orbit was processed using routine histological techniques, embedded in paraffin, and stained with haematoxylin-eosin. Three uveitis vitreous and 3 eye-bank vitreous control inoculated mice sacrificed during the observation period were processed by a different technique (Wirostko et al. 1986).

Table 1. Exophthalmos incidence by orbital infiltrate extent

Orbital infiltrate		
Extent groups	Number of mice	Number of mice with exophthalmos
IV	3	3
III	9	9
II	24	0
I	31	0
Total	67	12

Table 2. Orbital infiltrate extent by mouse survival time

Mouse survival time (months)	Number of mice in infiltrate extent groups				
	I	II	III	IV	Total
0– 3	1	0	0	0	1
4– 6	13	9	1	0	23
7– 9	9	8	3	1	21
10–12	8	7	5	2	22
Total	31	24	9	3	67

Using a light microscope the histological features and intraorbital extent of all inflammatory lesions in both orbits of all mice were recorded. Each lesion was placed into 1 of 4 inflammatory infiltrate histological extent groups using the following criteria: Group I-inflammatory infiltrate confined to the inoculation site; Group II-deeper and multifocal inflammatory infiltrates; Group III-wide spread diffuse infiltrates but extra-ocular muscles, nerves, and blood vessels still recognizable; Group IV-entire orbital section diffusely infiltrated and no normal orbital structure recognized. Using the histological section with the more extensive infiltrate as the criterion, infiltrate extent was correlated with visible exophthalmos incidence and mouse survival times.

Orbital inflammatory lesions from 10 uveitis vitreous inoculated mice were excised from the paraffin blocks, deparaffinized, reembedded in araldite, ultra-thin sectioned, and stained with uranyl acetate-lead citrate. Using TEM all leucocytes in multiple grid sections per lesion were studied for intracellular MLO. Analogous orbital structures from 10 eye-bank vitreous inoculated control mice were similarly prepared and studied.

Results

Persistent exophthalmos was observed in 12 uveitis vitreous inoculated mice versus 0 in the 200 controls ($P<0.05$). Histological orbital inflammatory lesions were found in 67 uveitis vitreous inoculated mice versus 0 in the controls ($P<0.05$). The distribution of those lesions in the 4 histological infilatrate extent groups is shown in Table 1. That table also depicts the correlation between visible exophthalmos and infiltrate extent. The correlation between infiltrate extent and mouse survival time is

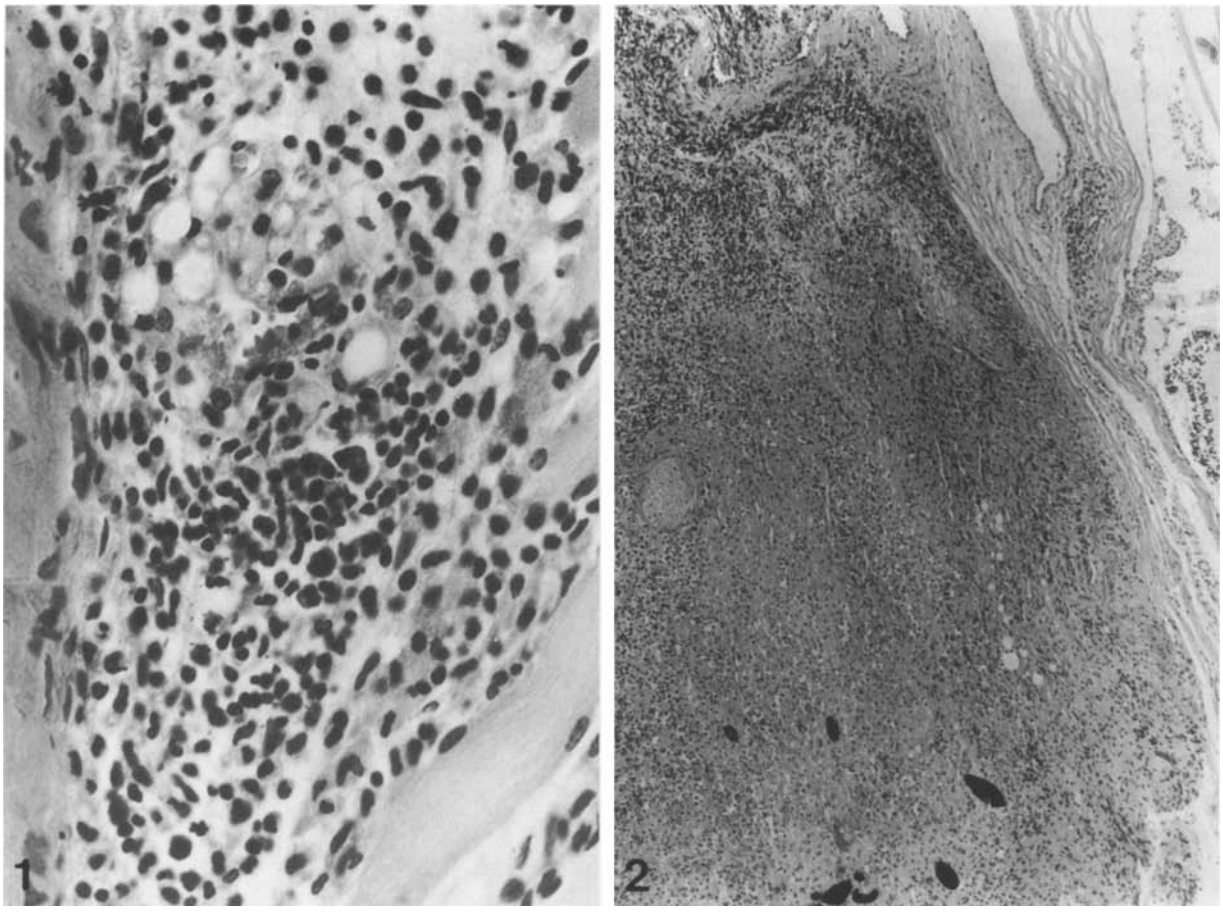

Fig. 1. Orbital episcleral chronic vasculitis. An episcleral vasculitis associated predominantly mononuclear leucocytic orbital infiltrate extends into the sclera (*left*) and extraocular muscles (*right*) in a mouse dying 6 months after uveitis vitreous inoculation. A "granuloma-like" vacuolated cellular aggregate, a few polymorphonuclear leucocytes, and "nuclear-dust" are detectable in this infiltrate. Hematoxylin-eosin stain ×220

Fig. 2. Exophthalmos associated orbital infiltrate. A predominantly mononuclear leucocytic infiltrate extends from the lid inoculation site (*upper left*) and involves the globe (*right*) in an exophthalmic mouse dying 9 months after uveitis vitreous inoculation. This extensive infiltrate obscures the orbital vasculitis. Hematoxylin-eosin stain ×75

depicted in Table 2. Vasculitis was a histological feature in all the lesions (Figs. 1 and 2). This was most easily detected in extent group I and II lesions (Fig. 1). In extent group III and IV infiltrates the exuberant orbital infiltrates made the vasculitis more difficult to appreciate (Fig. 2). All the infiltrates were composed chiefly of mononuclear leucocytes (Figs. 1 and 2). A few polymorphonuclear leucocytes and "nuclear dust" were also found in all the lesions, but their presence was most readily appreciated in Extent Group I and II infiltrates, (Fig. 1). Granuloma-like cellular aggregates were noted in 3 mice, all with either Group I or II infiltrates (Fig. 1). Intracellular MLO parasitised polymorphonuclear leucocytes (Fig. 3), lymphocytes (Fig. 4), and monocytes (Fig. 5), were found in the infiltrates of all 10 uveitis vitreous inoculated mice using TEM. MLO were not found in the orbital tissue samples from any of the 10 control mice.

Discussion

The results indicate that about 1/2 of the mice receiving the uveitis vitreous inocula developed orbital inflammatory lesions. As orbital inflammation was not observed in the controls, this was a significant observation and indicates that the uveitis vitreous produced the inflammation. The orbital inflammatory infiltrates consisted primarily of monocytes and lymphocytes and fewer polymorphonuclear leucocytes. This finding, plus occasional granuloma-like cellular aggregates, indicated a chronic inflammatory process with acute and/or recurrent features (Marchesi 1985). The orbital in-

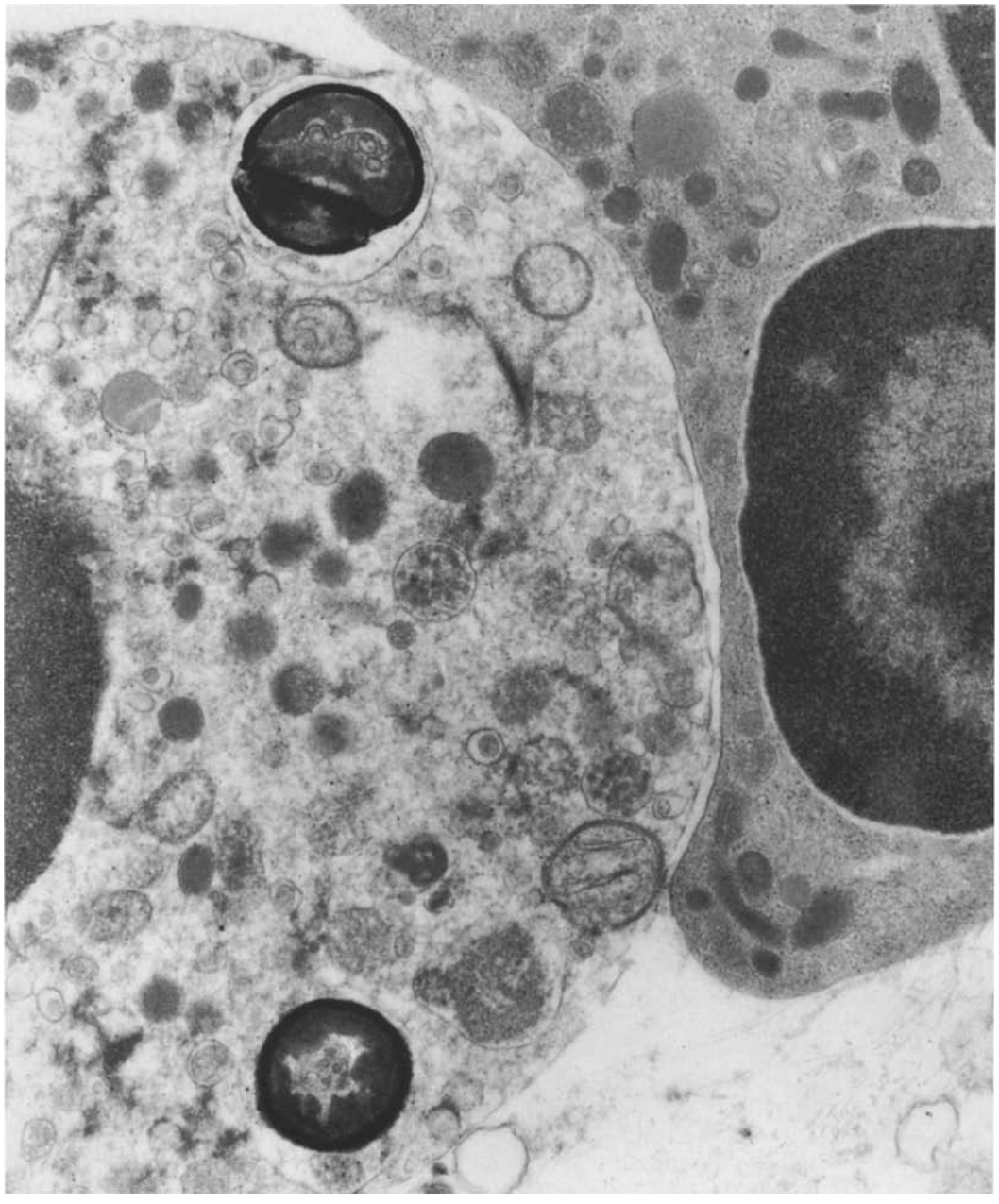

Fig. 3. MLO parasitised orbital infiltrate polymorphonuclear leucocytes. The cytoplasm of the left leucocyte is completely replaced by pleomorphic variably sized and staining tubulo-spherical MLO bodies and distinctive "spore-like" cell walled cocci. The cytoplasm of the right leucocyte contains similar MLO bodies, some of which may be difficult to distinguish from normal leucocyte granules. Uranyl acetate-lead citrate stain × 32,812

flammation was a slowly progressive disease. Mice dying early in the experiment had minimal infiltrates whereas the infiltrates were more extensive in mice living the longest. Exophthalmos was caused by the orbital inflammation and was a visible confirmatory sign of extensive intraorbital disease.

Finding leucocyte intracellular MLO consistently in the orbital infiltrates indicates that they were the cause of the disease. In the present investigation these MLO were indistinguishable from those in the human uveitis vitreous inocula, the mouse uveal tract, and in the mouse cardiac lesions (Johnson et al. 1987; Wirostko et al. 1987; Wirostko et al. 1988; Johnson et al. 1988). It is interesting that the MLO cell wall deficient forms could not be detected using the light microscope. In contrast, the distinctive cell walled 0.5–0.7 micron

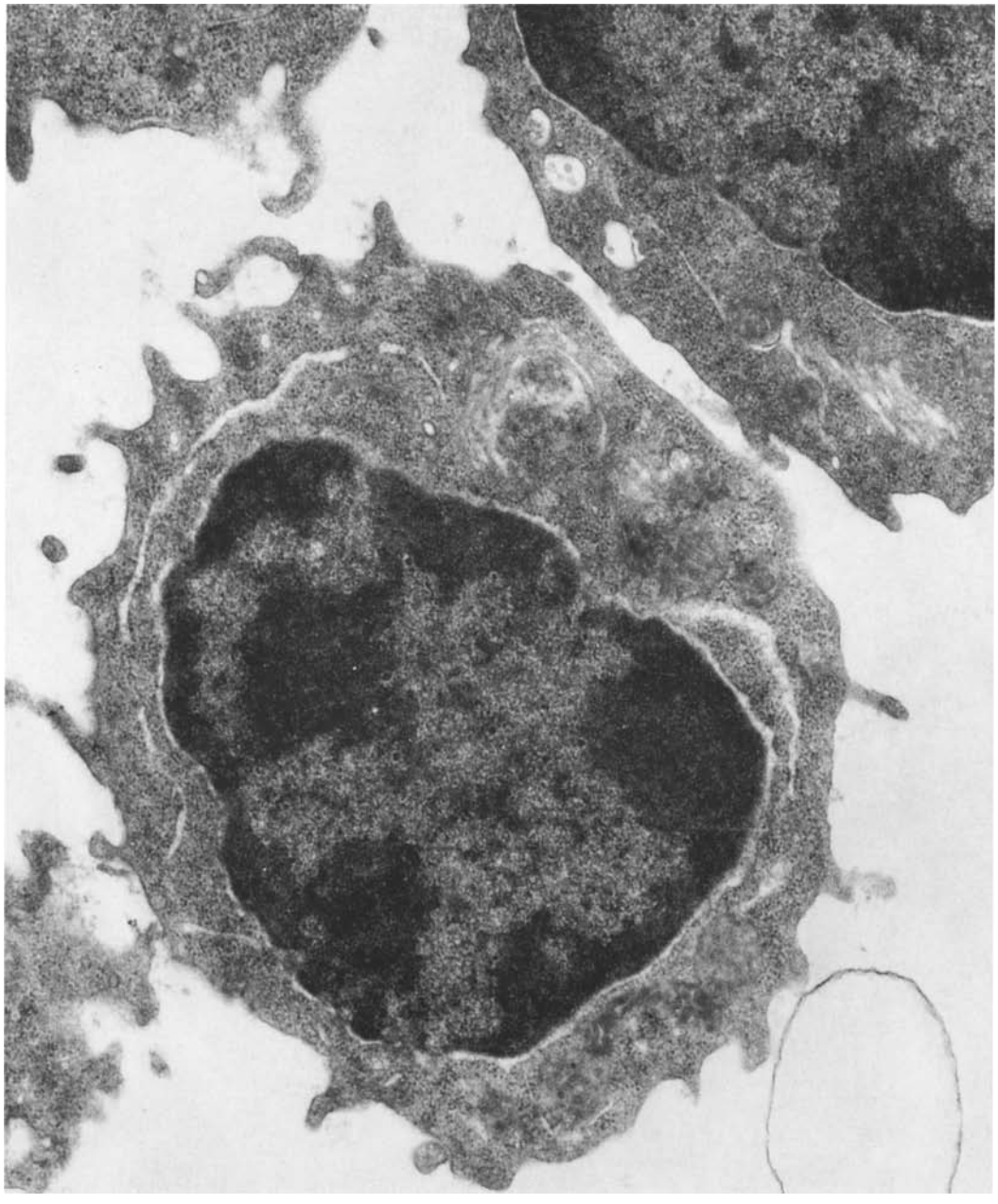

Fig. 4. MLO parasitised orbital infiltrate lymphocytes. Portions of several orbital disease lymphocytes with abnormally increased cytoplasm are shown. Two of them contain intracytoskeletal pleomorphic, branching, undulating, electron dense and lucent tubulospherical MLO bodies. Uranyl acetate-lead citrate stain × 28,780

cocci were detected within the histological infiltrates but their bacterial nature could only be defined using TEM.

Small vessel vasculitis has been a consistent histological feature of diseases produced by this MLO. In the human these MLO caused a uveal vasculitis (Johnson and Wirostko 1986) and they produced a uveal tract vasculitis in these mice (Wirostko et al. 1986). Moreover, the MLO disseminated to the hearts and great vessels of these mice to produce a chronic pancarditis by multifocal small vessel vasculitis (Johnson et al. 1988). The results of the present investigation have demonstrated that this MLO produces chronic orbital inflammation, again by a vasculitis. Since vasculitis can be a histological feature of human idiopathic chronic orbital inflammatory diseases (Jakobiec and Font 1986), this study suggests that MLO

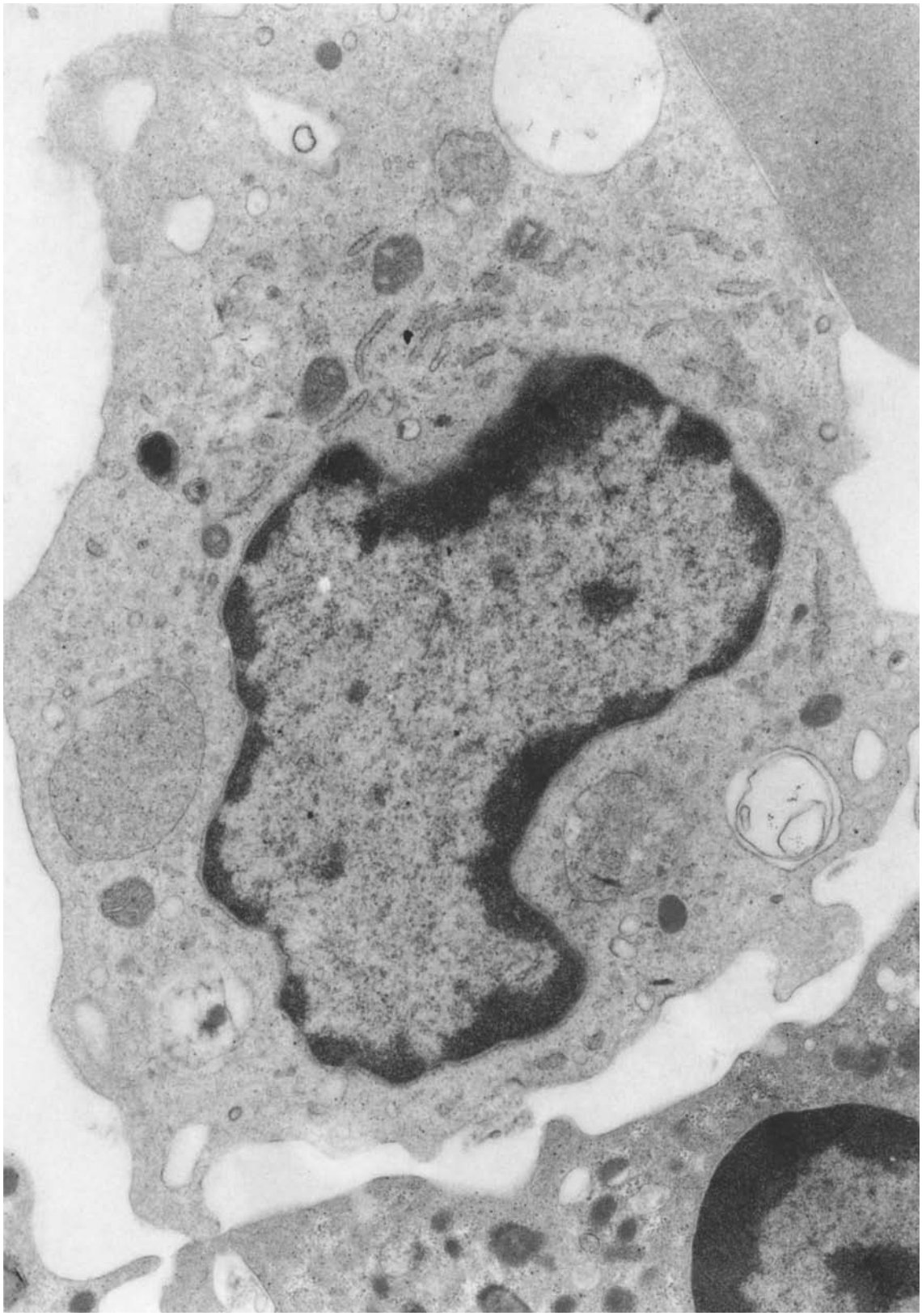

Fig. 5. MLO parasitised orbital infiltrate monocyte. Pleomorphic complex tubulo-spherical trilaminar outer membrane bound MLO bodies are present within the cytoplasm of this orbital disease monocyte. Some of the spherical MLO bodies are filled with internal tubular structures whereas others have an almost empty "bag-like" appearance. Uranyl acetate-lead citrate stain ×28,750

could also play a role in human orbital inflammatory disease. As they cannot be detected using a light microscope, a search for them using TEM seems justified.

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