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Inflammatory Reaction in Experimental Hepatic Amebiasis

An Ultrastructural Study

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One of the hallmarks of tissue necrosis produced by the human protozoan parasite *Entamoeba histolytica*, the causative agent of human amebiasis, appeared to be the lack of inflammatory reaction to the invading trophozoites. Recent evidence suggests, however, that inflammatory cells do appear during early stages of amebic destructive lesions and that they contribute to the establishment of foci of tissue necrosis in intestinal and liver lesions. The present analysis of the fine-structural changes that take place during early stages of amebic liver abscesses induced in hamsters after the intraportal inoculation of axenic amebas has shown that large numbers of polymorphonuclear leukocytes (PMNs) are recruited around invading amebas. These leukocytes lyse as a consequence of contact-mediated damage induced by the trophozoites. Amebas were also capable of ingesting apparently intact PMNs. Macrophages and eosinophils were also recruited at the foci of inflammation. At all times examined, trophozoites of *Entamoeba histolytica* survived in spite of being in close contact with PMNs or degranulating eosinophils. The ultrastructural observations have also shown the lack of direct contact between amebas and liver parenchymal cells during the initial stages of the focal liver necrosis induced by the parasite, therefore supporting the view that hepatic damage may be effected indirectly through lysis of inflammatory cells. The results also provide a basis for the understanding of the induction of experimental protective immunity against invasive amebiasis, a process which seems to be mostly dependent on cellular mechanisms. (Am J Pathol 1988, 130:112–119)

IT HAS BEEN traditionally accepted that the invasive lesions of human amebiasis produced by the protozoan parasite *Entamoeba histolytica*, mainly the ulceration of the cecal mucosa and liver abscess, develop without evidence of inflammatory reaction.[1–4] Recent experimental studies, however, suggest that the apparently sparse cellular response may be, rather, the result of a rapid destruction of inflammatory cells by the invading amebas. On the one hand, the *in vitro* interaction between inflammatory cells and trophozoites of *E histolytica* produces the lysis of polymorphonuclear luekocytes (PMNs) and macrophages.[5–8] On the other hand, ultrastructural observations in human[9] and in experimental intestinal amebiasis[10–12] have suggested that the destruction of inflammatory cells may be relevant in the pathogenesis of tissue necrosis, the hallmark of invasive amebiasis. Furthermore, the participation of inflammatory cells during the early stages of experimental amebic liver abscess formation in hamsters has been recently demonstrated at the light-microscopic level.[13,14]

Thus, it appears that the accepted dogma concerning the absence of cellular inflammatory reaction in invasive amebiasis may be a misconception based on the study of very late stages of tissue damage induced

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by the parasite and the difficulty in identifying lysed PMNs, which are rapidly killed upon contact with virulent amebas.

As a continuation of our study on the development of experimental amebic liver abscess,[14] we now report the results of the fine-structural analysis of the interaction between pathogenic amebas and leukocytes during the period that precedes the establishment of liver necrosis in the hamster. A light-microscopic quantitative analysis of the inflammatory cells present in amebic lesions was also performed. These observations have confirmed that, *in vivo*, large numbers of PMNs lyse in contact with amebas, initiating the establishment of focal liver necrosis.

Materials and Methods

Amebic Cultures

Trophozoites of *E histolytica* strain HM-1 were maintained axenically in BI-S33 medium formulated by Diamond et al.[15] The inoculum was prepared from 72-hour amebic cultures in the logarithmic phase of growth and adjusted to a concentration of 1×10^6 trophozoites/ml of culture medium. Each animal received 2.5×10^5 amebas in 0.25 ml of medium.

Animals

Forty male golden hamsters (*Mesocricetus auratus*) aged 10–12 weeks were selected from a previous experimental group of animals inoculated intraportally with amebas and utilized for the sequential histologic study of abscess formation. The surgical procedures and other experimental data were reported elsewhere.[14] Livers selected for the present study were those from animals with small liver lesions detectable with the dissecting microscope 3 and 6 hours after inoculation, as well as those macroscopic lesions appearing between 9 and 24 hours after inoculation.

Tissue Collection and Processing

Selected liver fragments less than 1 mm thick were fixed by immersion in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 hours at room temperature, postfixed with 1% osmium tetroxide in the same buffer for 2 hours, and embedded in Epon 812. Sections 0.5 μ thick stained with alkaline toluidine blue were used for localization of amebic lesions with a light microscope. Thin sections of representative regions were obtained with diamond knives and stained with uranyl acetate and lead citrate. At least 8 lesions from each postinoculation time were examined with a Zeiss EM-10 electron microscope operating at 60 kv.

A quantitative analysis of the different types of inflammatory cells present in lesions 3, 6, and 9 hours after inoculation of the parasites was performed with the use of light photomicrographs of at least three representative lesions at each time point. Photomicrographs were enlarged to a final magnification of ×3000.

Results

Quantitative Analysis of Inflammatory Cells

The relative percentage of different types of inflammatory cells after intraportal inoculation of trophozoites was determined in light photomicrographs at 3, 6, and 9 hours; the total number of observations at each time was 622, 278, and 182, respectively. From these, PMNs represented 78% (485), 56% (155), and 27% (49) of undamaged cells present in sections of foci of acute inflammation. Macrophages were found to comprise 20% (125), 44% (122) and 73% (132), respectively, while eosinophils comprised 2% (12) of cells in lesions at 3 hours. Amebas were found in contact with PMNs or their remnants, whereas macrophages always had a more peripheral distribution. As the lesion increased in size, lysed or damaged inflammatory cells were more abundant and occupied larger areas. No direct contact between the parasite and liver cells was found.

Interaction of Amebas With PMN Neutrophils

At 3 hours after inoculation of virulent trophozoites in the portal vein of hamsters, the liver surface presented whitish spots 1–3 mm in diameter. These constituted the earliest lesions studied by electron microscopy. At low magnification each lesion was formed by a focal acute inflammatory reaction around centrally located amebas. As shown in Figure 1, many PMNs formed a rosette around single amebas. Amebas were found in apposition to PMNs; the adjacent plasma membranes showed an undulating profile. The cytoplasm of the parasites had multiple randomly distributed cytoplasmic vacuoles, as well as vacuoles containing membrane debris and dense granules. The latter were more abundant near the cell surface of the trophozoites (Figure 2). No evidence of membrane fusion between parasites and target cells or of surface discharge from amebic cytoplasmic vacuoles was obtained. PMNs had typical multilobulated nuclei and specific granules; the surface contacting the parasite showed pseudopodia stretching out laterally, formed by cytoplasmic re-

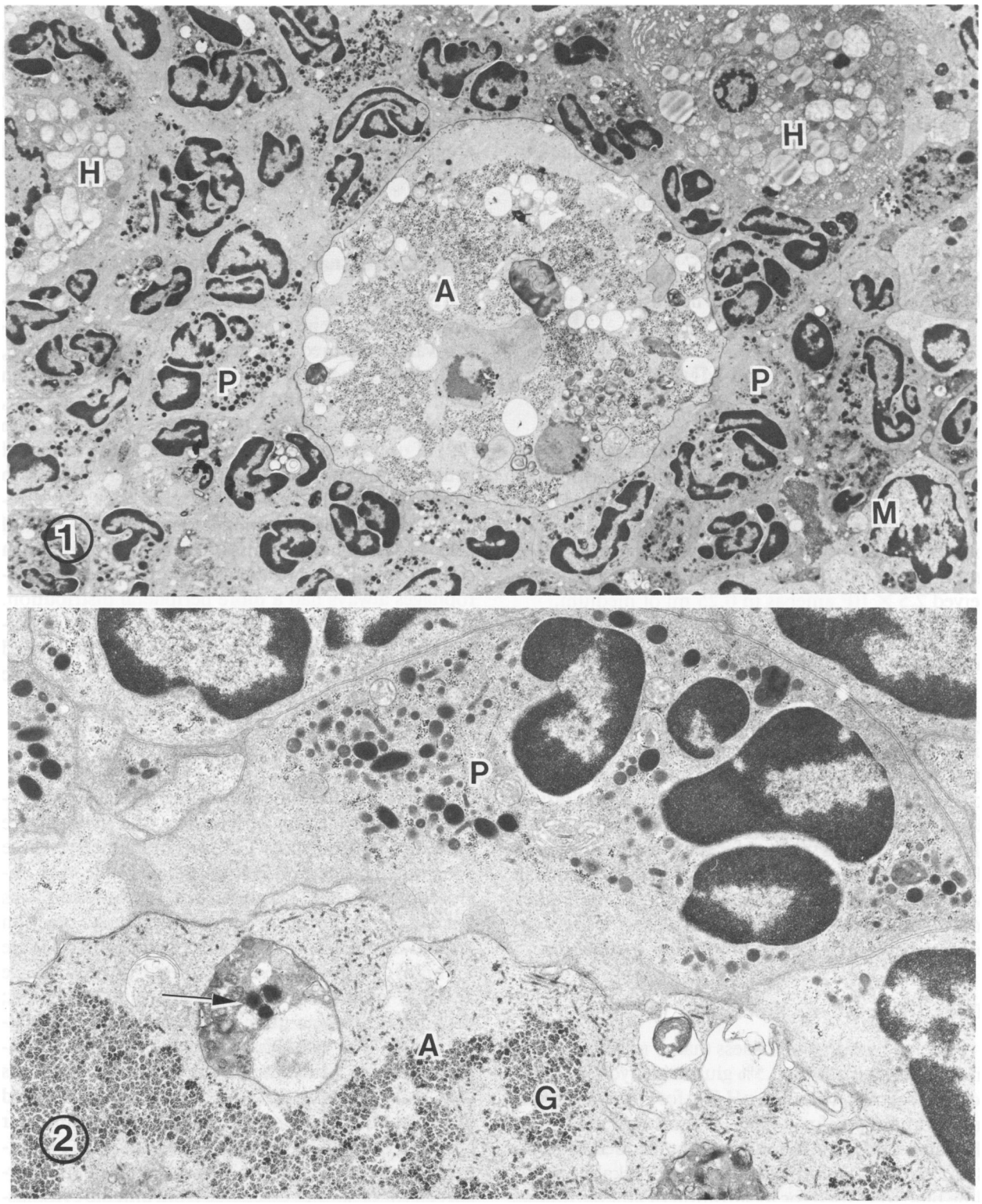

Figure 1—Hepatic amebic lesion 3 hours after inoculation. A centrally located ameba (*A*) is surrounded by large numbers of polymorphonuclear neutrophils (*P*). At the periphery of the lesion a macrophage (*M*) and vacuolated hepatocytes (*H*) are seen. (×3000) **Figure 2**—A closer view at the interface between a polymorphonuclear neutrophil (*P*) and a contacting ameba (*A*). At the undulating region of contact the peripheral cytoplasm of the PMN is devoid of granules, while in the ameba cytoplasmic vacuoles contain granules (*arrow*) similar in appearance to those of PMNs. *G*, glycogen. (×13,000)

gions containing a finely fibrillar material, devoid of granules and membranous organelles (Figure 2). Morphologically intact PMNs were also found inside trophozoites, located within large phagocytic vacuoles (Figure 3). The peripheral location of macrophages was confirmed at the ultrastructural level. Scanty vacuolated or degenerated hepatocytes were also seen.

From 6 to 12 hours after inoculation drastic changes were noticed in the cytoplasm of PMNs contacting the amebas. They lacked specific granules and showed prominent vacuoles containing membranous and granular debris (Figure 4) as well as irregularly swollen cytoplasmic regions along the contacting surface, with displacement of the nucleus and cytoplasmic organelles to the opposite side of the leukocyte (Figure 5). In turn, the amebic cytoplasm close to the contacting zone with PMNs displayed vacuoles containing either PMN granules (Figure 4) or devoid of particulated components (Figure 5).

From 12 to 24 hours after inoculation, as the size of the hepatic lesions progressively increased, the inflammatory reaction immediately surrounding the amebas consisted of abundant remnants of lysed leukocytes (Figure 6). Evidence of PMN necrosis included the granular appearance of nuclei, loss of nuclear membranes, and the disruption of the normal cytoplasmic structure that left only few remaining peripheral cytoplasmic granules (Figure 6). Hepatocytes bordering the inflammatory reaction showed degenerative changes characterized by abundant swollen mitochondria, cytoplasmic vacuolation, and nuclear degeneration (Figure 7).

Interaction of Amebas With Macrophages

The presence of macrophages at the foci of inflammatory reaction was seen starting at 3 hours after inoculation (Figure 1). At the earliest stages macrophages were scarce and were not in direct contact with the amebas. At periods later than 9 hours after inoculation, macrophages were abundant, showed a more random distribution, and frequently contained phagocytized PMN remnants in their cytoplasm (Figure 8). Even at periods up to 24 hours, direct contact of macrophages with amebas was very seldom seen; and when found, no obvious ultrastructural changes were observed either in the macrophages or in the trophozoites.

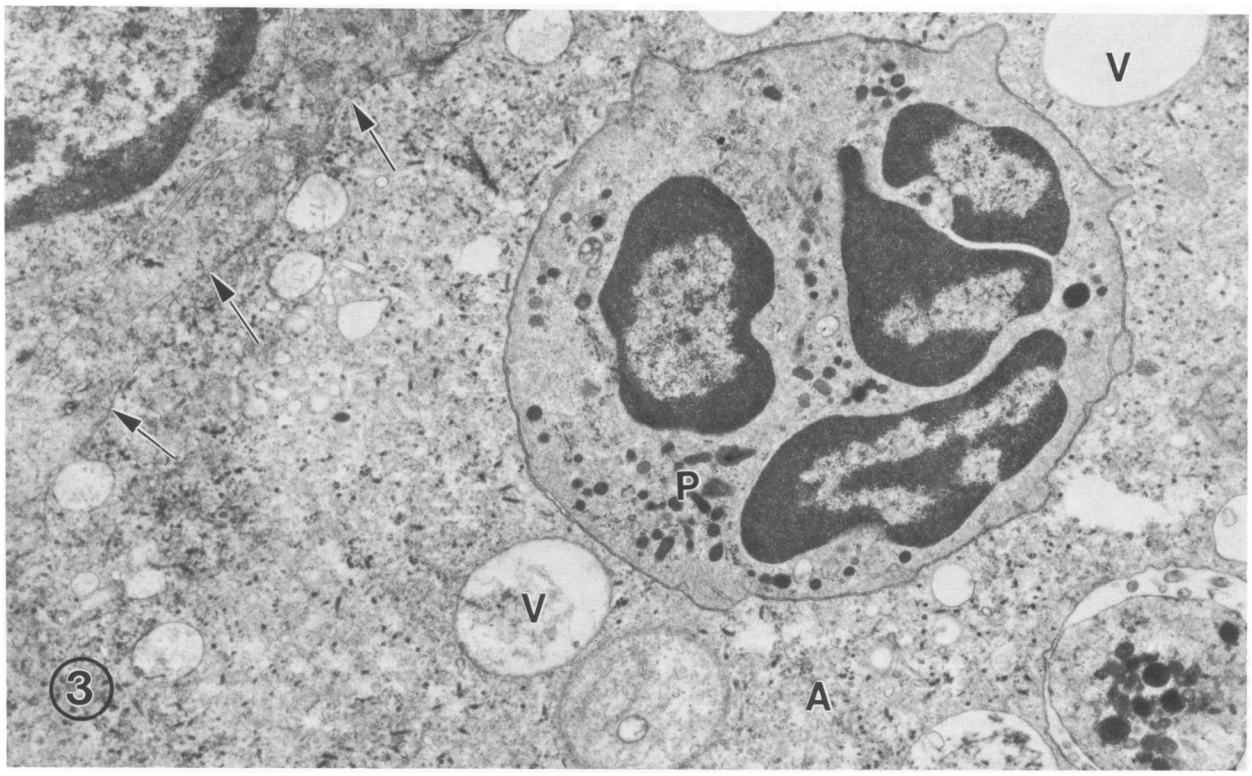

Figure 3—At 4 hours after inoculation an intact PMN (*P*) is located within an autophagic vacuole of an ameba (**A**). Smaller cytoplasmic vacuoles (*V*) are also found. In one region the ameba is in contact with another PMN (*arrows*). (×12,000)

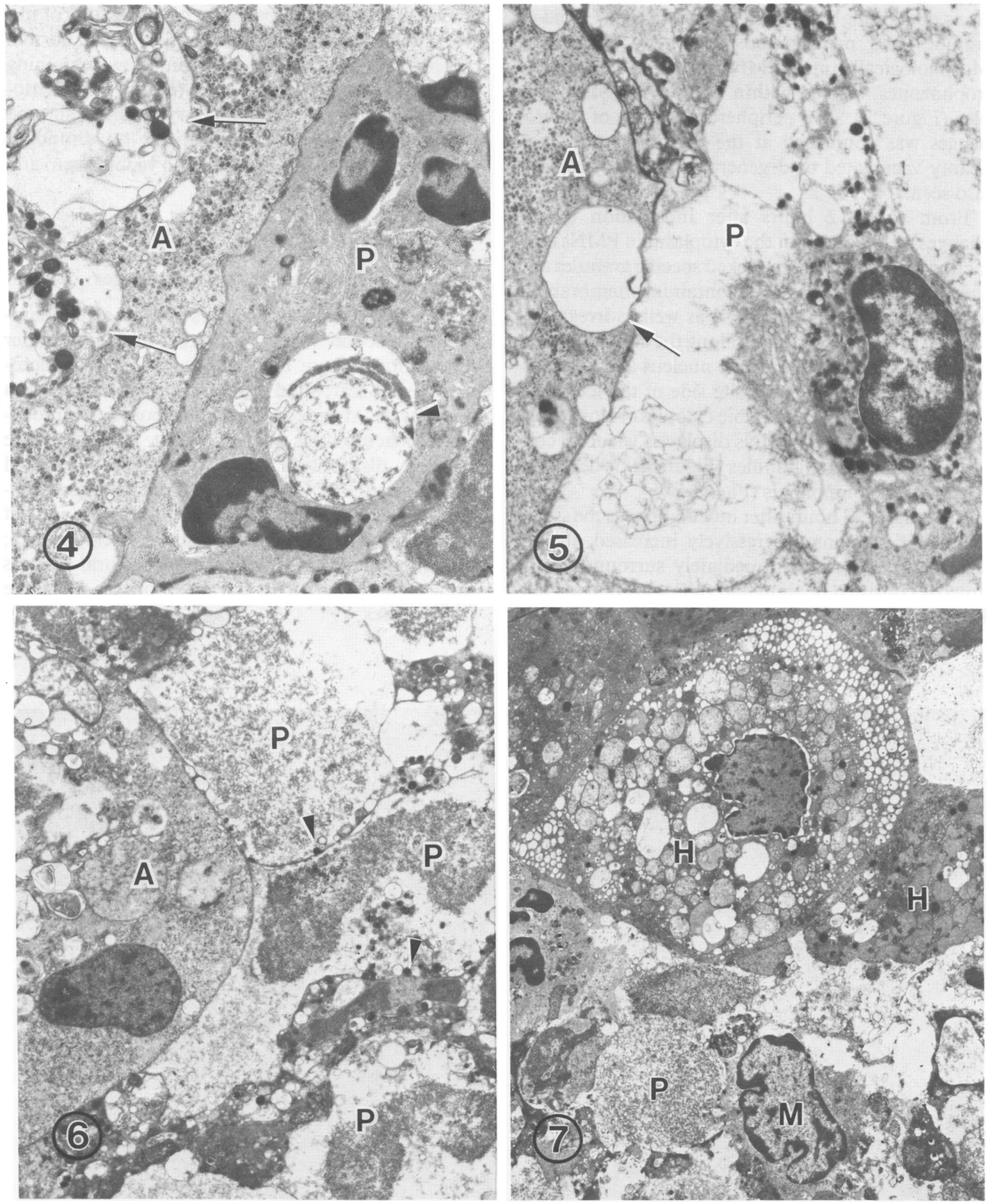

Figure 4—At 6 hours after inoculation a PMN (*P*) shows a large degenerative vacuole (*arrowhead*) containing flocculent material and lack of cytoplasmic granules. The trophozoite (*A*) contains large vacuoles with granules, possibly of PMN origin (*arrows*). (×7500) **Figure 5**—At 12 hours after inoculation, a swollen PMN (*P*) with an essentially empty cytoplasm is seen in contact with an ameba (*A*). Organelles and the nucleus are displaced. In turn, the adjacent cytoplasmic region of a trophozoite contains an empty vacuole, in close contact with the cell surface (*arrow*). (×7500) **Figure 6**—Twenty-four hours after inoculation. Most PMNs (*P*) are lysed and show few cytoplasmic granules (*arrowheads*) associated with membrane remnants and nuclear debris. (*A*) ameba. (×6000) **Figure 7**—Twelve hours after inoculation. The electron micrograph shows a region of interaction between lysed PMNs (*P*) and hepatocytes (*H*). Both hepatocytes are severely damaged. Remnants of macrophages (*M*) are also found. (×3000)

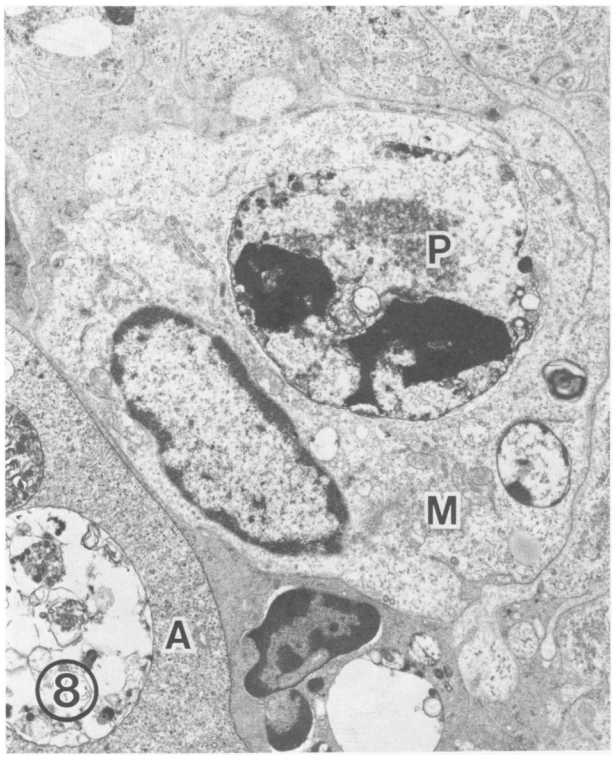

Figure 8—At 18 hours after inoculation a macrophage (*M*) in close contact with an ameba (*A*) contains a partially degraded PMN (*P*). (×6000)

Interaction of Amebas With Eosinophil Leukocytes

Scarce eosinophils were observed in foci of acute inflammatory reaction of the liver starting 3 hours after inoculation of virulent amebas. Direct contact of these cells with trophozoites was not common. However, at later stages eosinophils were closely apposed to trophozoites (Figure 9). Eosinophils in contact with the parasite surface showed a dense material in the narrow extracellular space between converging plasma membranes (Figures 9–11). The amebas interacting with these leukocytes had always a normal ultrastructural appearance, except for the occasional presence of vacuoles containing PMN remnants (Figure 9) or eosinophil granules. Other eosinophils had a pale cytoplasm with few specific granules, and in regions of extensive leukocyte lysis remnants of eosinophils were identified (Figure 12).

Discussion

In the present ultrastructural study the ameba–leukocyte interactions taking place during the early stages of amebic liver abscess were analyzed. Contact between usually single amebas with large numbers of PMNs resulted in lysis of the inflammatory cells, which sometimes were engulfed intact by the parasites. These morphologic changes indicate the existence of an aggressive mechanism of *E histolytica* trophozoites operative in the development of focal amebic necrosis of the liver, namely, the lysis of PMNs possibly exerted through the contact-dependent cytotoxic activity shown *in vitro* by amebas in contact with leukocytes.[5–8]

The presence of eosinophil leukocytes in the cellular infiltrates of amebic liver lesions is demonstrated for the first time by electron microscopy. In a histologic study, Chadee and Meerovitch[13] previously reported the presence of eosinophils at late stages of experimental amebic liver abscess. In spite of their scarcity, eosinophils were found adhering and degranulating on the surface of adjacent amebas where a characteristic dense extracellular deposit appeared, similar to that seen in thin sections of eosinophils killing metazoan parasites.[16,17] However, in amebic lesions there was no evidence of parasite lysis as a consequence of the degranulation of eosinophils.

The development of amebic liver lesions was also associated with a gradual increase in the number of macrophages, particularly in lesions seen 12 hours or later after inoculation of the parasites. Up to 24 hours, these phagocytic cells were rarely in contact with the amebas. Most of them were distributed at the periphery of the lesions, phagocytizing cell debris. At the early stages of liver abscess development here studied macrophages were not destroyed by the amebas, as may be seen under *in vitro* conditions.[18] However, at later stages when acute inflammation evolves into granulomas, macrophages decrease progressively in number, because granulomas are gradually substituted by focal regions of liver necrosis.[14] These results suggest that macrophage lysis may play a role in the extension of amebic liver abscess, after necrosis is initiated by the destruction of acute inflammatory cells.

The observations also demonstrate at the ultrastructural level the lack of direct contact between amebas and liver parenchymal cells, an observation that supports the view that hepatic damage is initiated by the amebas mainly in an indirect way through lysis of inflammatory cells.[14] Further support was recently given by the results of the interaction of human neutrophils and *E histolytica*. These studies established that *in vitro* lysis of human neutrophils by pathogenic amebas enhance the destruction of liver or Chinese hamster ovary cell monolayers.[19]

In conclusion, the fine-structural analysis of the inflammatory reaction present at early periods of experimental hepatic amebic infection has confirmed the suggestion that lysis of PMNs plays a major role in the initial development of amebic liver abscesses. Moreover, the ultrastructural evidence indicates that

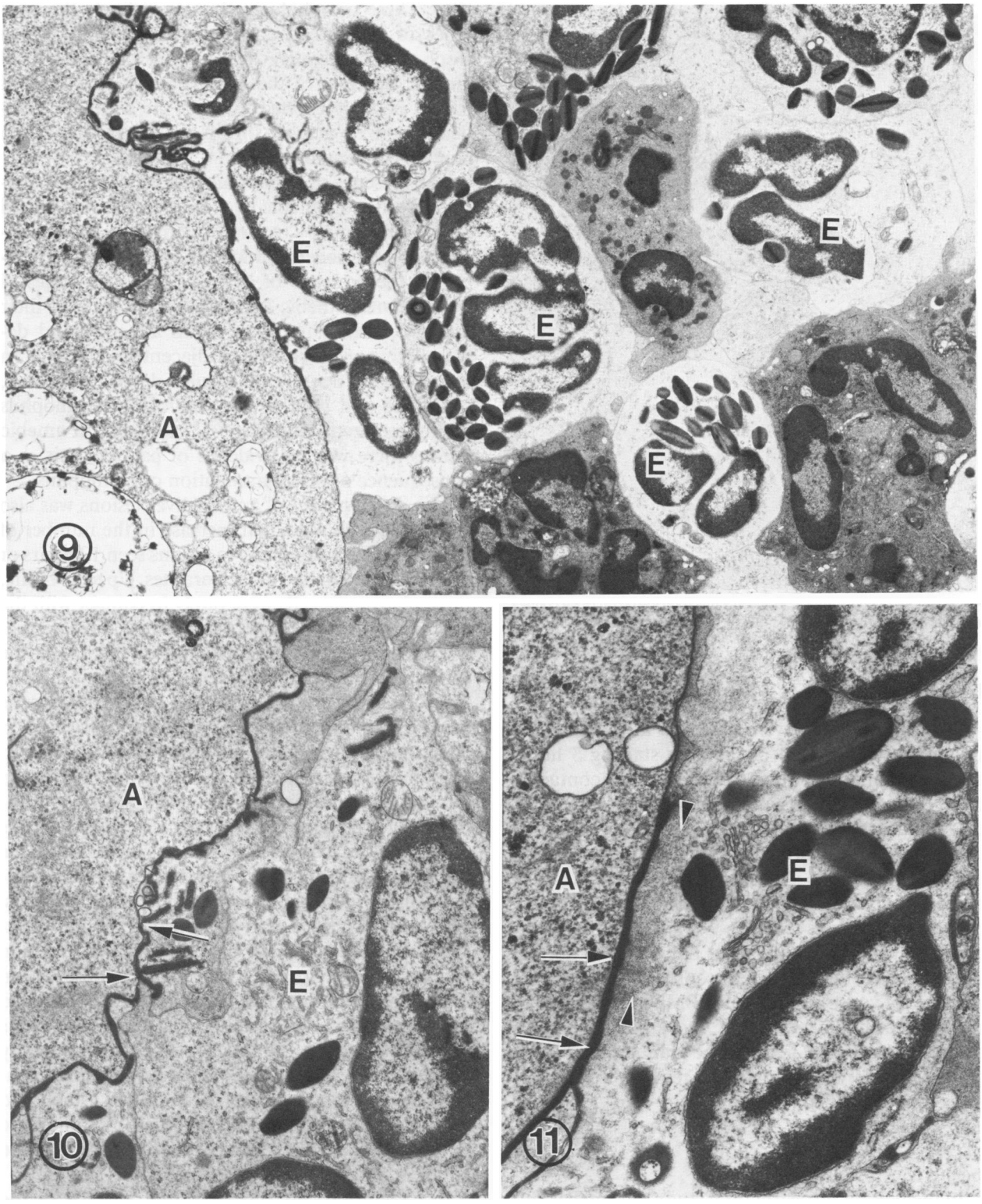

Figure 9—Eighteen hours after inoculation. Several eosinophil leukocytes (**E**) are accumulated close to an ameba (**A**). (×6000) **Figure 10**—A higher magnification of a region of interaction between an eosinophil (*E*) and an ameba (*A*) clearly shows the presence of an electron-dense deposit over the amebic surface. The surface discharge of eosinophil granules is seen at the region of contact (*arrows*). (×10,000) **Figure 11**—Another region of contact between an eosinophil (*E*) and an ameba (*A*). In addition to the extracellular dense deposit (*arrows*), a fibrogranular region is seen in the cytoplasm of the eosinophil at the region of contact (*arrowheads*). (×14,000)

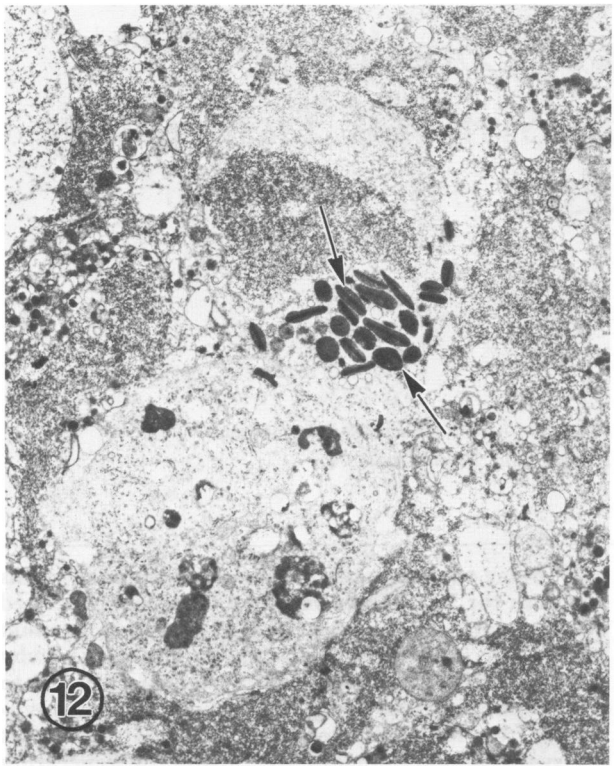

Figure 12—A region of leukocyte lysis contains eosinophil granules (*arrows*). Both PMN and eosinophils degenerate in the presence of amebas. (×6000)

the amebic cytopathic effect is achieved through contact-dependent cytopathic lysis of inflammatory cells. The latter is probably related to the toxins and/or enzymatic action of trophozoites shown by others in *in vitro* studies (reviewed by Martinez Palomo[20]). The use of antibodies against these secreted amebic components will probably confirm the molecular basis of the *in vivo* destructive capacity of the parasite.

The prominent cellular reaction to the presence of invading amebas in the liver provides a basis for the understanding of the induction of experimental immunity against amebic liver abscess, a process that seems to be mostly dependent on cellular, rather than humoral, mechanisms.[21]

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