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# NOTES

## Role of the *Entamoeba histolytica* Cysteine Proteinase in Amebic Liver Abscess Formation in Severe Combined Immunodeficient Mice

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**Evidence from in vitro studies suggest that the *Entamoeba histolytica* cysteine proteinase plays a role in the tissue lysis and cytopathic effects seen in invasive amebiasis. We used affinity-purified antibodies against a recombinant *E. histolytica* cysteine proteinase to demonstrate that the proteinase is present extracellularly in amebic liver abscesses in mice with severe combined immunodeficiency (SCID mice). Treatment of *E. histolytica* trophozoites with specific cysteine proteinase inhibitor E-64 blocked or greatly reduced liver abscess formation at 48 h in SCID mice. Our study suggests an important role for a functional cysteine proteinase in amebic liver abscess formation.**

The hallmark of invasive *Entamoeba histolytica* infection of the intestine or liver is tissue lysis and destruction. One of the key molecules in this process may be the amebic 27-kDa thiol-dependent cysteine proteinase (11, 12, 19). In vitro studies from a number of laboratories have implicated *E. histolytica* cysteine proteinase in cytopathic and/or cytotoxic effects on mammalian cells (3, 4, 7–10), degradation of extracellular matrix components such as fibronectin and laminin (15, 16), and activation of the host complement system (14). Patients with invasive amebiasis produce antibodies that recognize the cysteine proteinase, suggesting that it is released in infection (13). In a rat model of disease which featured the subcutaneous inoculation of amebic trophozoites, proteinase inhibitors were effective in decreasing the inflammation and tissue damage associated with the skin lesions (1). However, viable *E. histolytica* trophozoites were not observed in these lesions (which spontaneously resolved within 72 to 96 h), raising questions about the relevance of this model to natural infection (1). Recently, we developed a murine model of amebic liver abscess using mice with severe combined immunodeficiency (SCID mice) (2). SCID mice develop amebic liver abscesses when virulent *E. histolytica* trophozoites are injected directly into the liver. The goal of this study was to determine whether we could localize the *E. histolytica* cysteine proteinase in amebic liver abscesses in SCID mice and whether inhibitors of cysteine proteinase could affect the development of amebic liver abscess in SCID mice.

To localize the amebic cysteine proteinase, we generated antiserum specific for the *E. histolytica* cysteine proteinase. We first expressed nucleotides 271 to 939 (based on the numbering of the cEh-CPp sequence of Tannich et al. [19]) of the gene encoding cysteine proteinase 1 (Eh-CPp1) from the HM1:IMSS *E. histolytica* strain in *Escherichia coli* as a glutathione-*S*-transferase (GST) fusion protein. This recombinant protein was used to immunize rabbits to generate anti-Eh-CPp serum (17). Anti-cysteine proteinase antibodies were affinity purified on an Eh-CPp1–GST–Sepharose column, and then anti-GST antibodies were adsorbed on a GST-Sepharose column, leaving purified anti-Eh-CPp1 antibodies. Specific reactivity of the anti-EhCPp1 antibodies with 27-kDa native cysteine proteinase was confirmed by Western blotting of *E. histolytica* HM1:IMSS trophozoite lysates using $^{125}$I-labeled staphylococcal protein A to detect bound rabbit immunoglobulin (Fig. 1) (17).

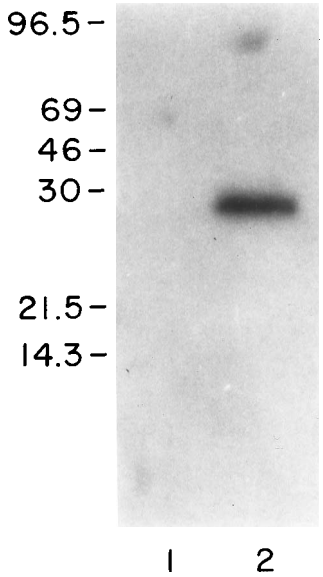


FIG. 1. Anti-EhCPp1 antibodies bind the native 27-kDa *E. histolytica* cysteine proteinase. Lysates from *E. histolytica* HM1:IMSS trophozoites were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and reacted with affinity-purified by GST-adsorbed anti-Eh-CPp1 rabbit antibodies. A single species at 27kDa, the size of the amebic cysteine proteinase, was bound by the anti-EhCPp1 antibodies at a 1:50 dilution (lane 2). No species was recognized by preimmune serum (lane 1). Molecular mass standards in kilodaltons are shown on the left.

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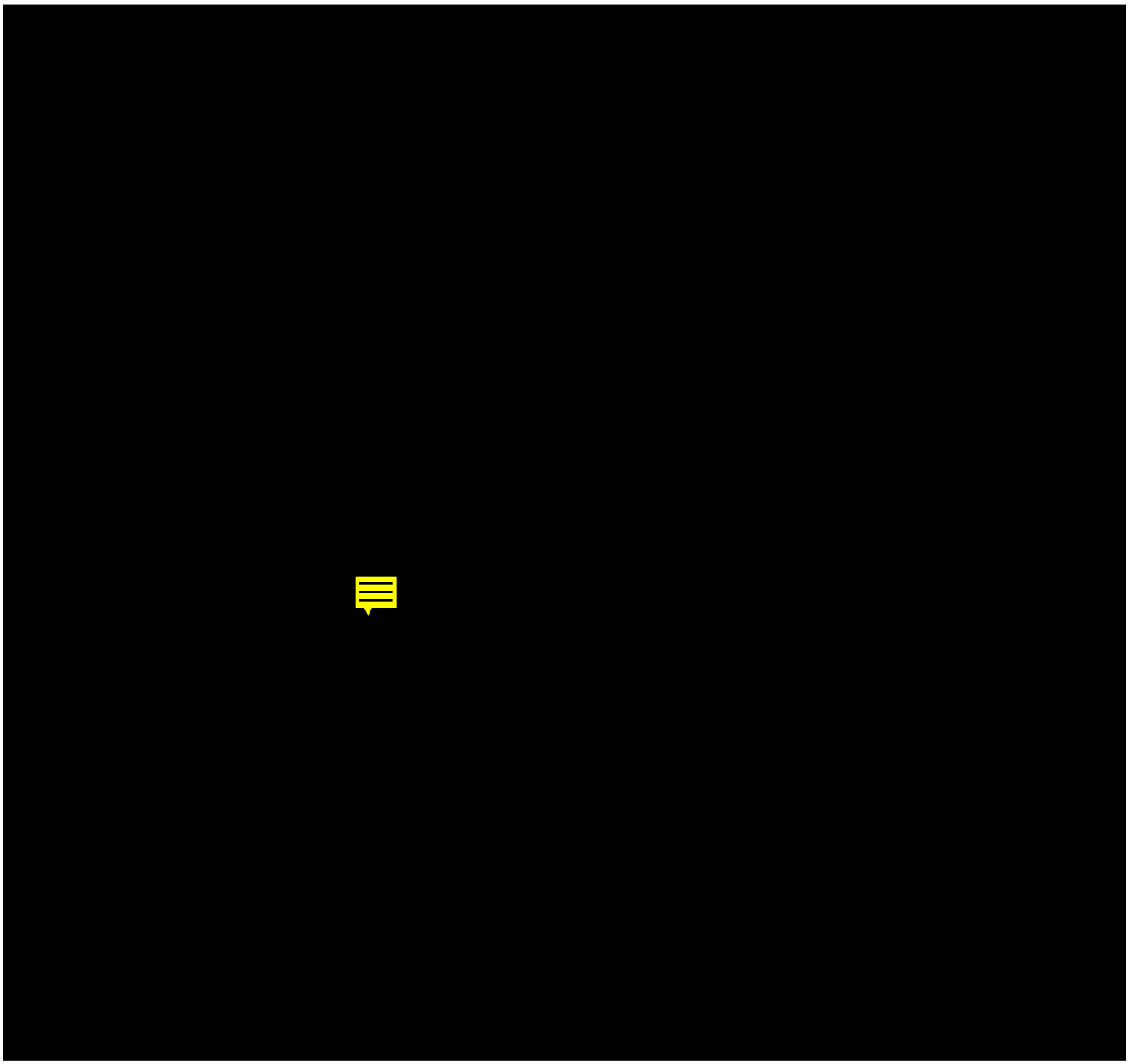

FIG. 2. Photomicrograph of a section of a SCID mouse amebic liver abscess immunostained for *E. histolytica* cysteine proteinase. (A) Diffuse staining (brown color) of the abscess by anti-EhCPp1 antibodies is clearly visible, as is staining of individual amebic trophozoites. The section was counterstained with hematoxylin (magnification, ×100). (B) High-power view of the boxed area showing in greater detail the staining of individual trophozoites and the extracellular foci (note the lower left and lower center portions of the micrograph) of intense anti-EhCPp1 staining (magnification, ×600).

We examined whether the *E. histolytica* cysteine proteinase could be detected in both amebic trophozoites and host tissues during the early phases of abscess formation in SCID mice. SCID mice were intrahepatically inoculated with $10^6$ virulent *E. histolytica* HM1:IMSS trophozoites by using our standard protocol (2). Mice were sacrificed 48 h later, their livers were removed and inspected for the presence of an amebic liver abscess, abscesses and livers were weighed, the percentage of liver abscessed was calculated, and the livers were prepared for histopathology by fixation in Bouin's solution overnight. After fixation, livers were frozen, sectioned, and stained with a 1:2,000 dilution of the anti-Eh-CPp1 antibody. Antigen-antibody complexes were detected with gold-labeled goat anti-rabbit secondary antibody (AuroProbe LM GAR) and silver enhancement (Amersham Corporation, Arlington Heights, Ill.) as previously described (15). A representative section from a SCID mouse amebic liver abscess at 48 h immunostained for the amebic cysteine proteinase is shown in Fig. 2A. A number of amebic trophozoites are present, and many are stained (seen as a brown color) by the anti-Eh-CPp1 antibody. Staining is not confined to amebic trophozoites, however, as there is diffuse staining throughout the liver abscess. This is highlighted in the detail of the high-power section (Fig. 2B) which clearly shows foci of extracellular cysteine proteinase staining that are not associated with amebic trophozoites. No cysteine proteinase staining was seen in portions of normal liver distant from the abscess. Addition of a 5-μg/ml solution of the recombinant Eh-CPp1–GST fusion protein to the anti-Eh-CPp1 antibodies

during the staining process completely eliminated staining of trophozoites and extracellular staining in the liver abscess sections (data not shown), confirming that only amebic cysteine proteinase was being stained by the anti-Eh-CPp1 antibodies. The immunohistochemical findings provide strong evidence that significant quantities of extracellular *E. histolytica* cysteine proteinase are present during amebic liver abscess formation. Whether the extracellular proteinase is actively secreted by the trophozoite or represents release from dead or damaged trophozoites is unclear, but viable *E. histolytica* trophozoites are present and can be cultured from these lesions (2). In addition, SCID mouse amebic liver abscess sections stained with a monoclonal antibody to the serine-rich *E. histolytica* protein (SREHP) (which is primarily surface membrane located but can also be detected in a focal distribution within permeabilized trophozoites) (18) did not reveal extracellular SREHP in the abscess tissue (data not shown), suggesting that it is unlikely that the source of extracellular cysteine proteinase is solely dead or damaged trophozoites.

Having established that the *E. histolytica* cysteine proteinase can be detected in 48-h amebic liver abscesses in SCID mice, we set out to determine whether inhibition of the amebic cysteine proteinase by the specific cysteine proteinase inhibitor, L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) could alter abscess formation. We chose a concentration of E-64 (0.2 mM) which completely blocks amebic cysteine proteinase activity (as measured by gelatin substrate gel) at 35°C (data not shown) but does not affect trophozoite viability or growth. This was confirmed by inoculating cultures with approximately 5,000 trophozoites into standard medium containing 0.2 mM E-64 and comparing the growth (by counting trophozoite numbers) and viability (as measured by trypan blue exclusion) of E-64-treated amebae with an identical number of trophozoites that were inoculated in standard medium alone. After 26 h the numbers of amebae were 46,030 ± 2,313 in the E-64-treated cultures (95% ± 6% viability) and 45,555 ± 3,142 (95% ± 3% viability) in control cultures. The failure of E-64 to show direct cytotoxic effects on the amebae at the 0.2 mM concentration is also consistent with a recent study in which we examined the ability of *E. histolytica* trophozoites to decrease the transepithelial resistance of monolayers of the intestinally derived Caco-2 cell line (6). In those experiments, treatment of *E. histolytica* trophozoites with 0.2 mM E-64 did not abolish their ability to lower Caco-2 transepithelial resistance, suggesting that cysteine proteinase activity is not involved in the effects on monolayer resistance and that treatment with E-64 did not significantly affect the short-term viability and some of the pathogenic properties of the amebae. Thus, any effects of E-64 on liver abscess formation should be due to inhibition of cysteine proteinase activity rather than to a direct toxic effect on amebae.

SCID mice were inoculated with either $10^6$ *E. histolytica* trophozoites in 100 μl of TYI-33 medium (total $n = 10$, one trial with four mice, a second identical trail with six mice, or $10^6$ *E. histolytica* trophozoites in 100 μl of TYI-33 medium–0.2 mM E-64 ($n = 10$, divided as above). After 48 h, SCID mice were sacrificed, and livers were processed as described above. All 10 control animals had amebic liver abscesses, with a mean size of 36% ± 6% (mean ± standard error of the mean) of the total live abscessed. Two of the 10 SCID mice receiving amebae coincubated with E-64 had no liver abscess detected; the mean abscess size in the remaining eight SCID mice was 6% ± 2% of the liver. Thus, the amebic liver abscesses were significantly smaller ($P < 0.001$) in SCID mice inoculated with *E. histolytica* trophozoites that had been coincubated with E-64.

We propose that the protective effects of E-64 on liver abscess formation are based on the inhibition of the amebic cysteine proteinase. E-64 may block tissue damage (such as fibronectin and laminin digestion and cytopathic effects on host cells) caused by the extracellular amebic cysteine proteinase. The protective effects of E-64 on liver abscess formation may also be based on the inhibition of intracellular amebic cysteine proteinase. If the cysteine proteinase is necessary for amebic digestion of host macromolecules, inhibition of the intracellular enzyme could eventually prove toxic to the parasite.

In summary, we have been able to demonstrate the presence of the *E. histolytica* cysteine proteinase both in amebic trophozoites and extracellularly in amebic liver abscesses in SCID mice. In addition, we have found that treatment of amebic trophozoites with the specific cysteine proteinase inhibitor E-64 at the time of their inoculation into SCID mice results in significantly decreased liver abscess size 48 h later. Our study suggests an important role for a functional *E. histolytica* cysteine proteinase in the development of amebic liver abscess in the SCID mouse.

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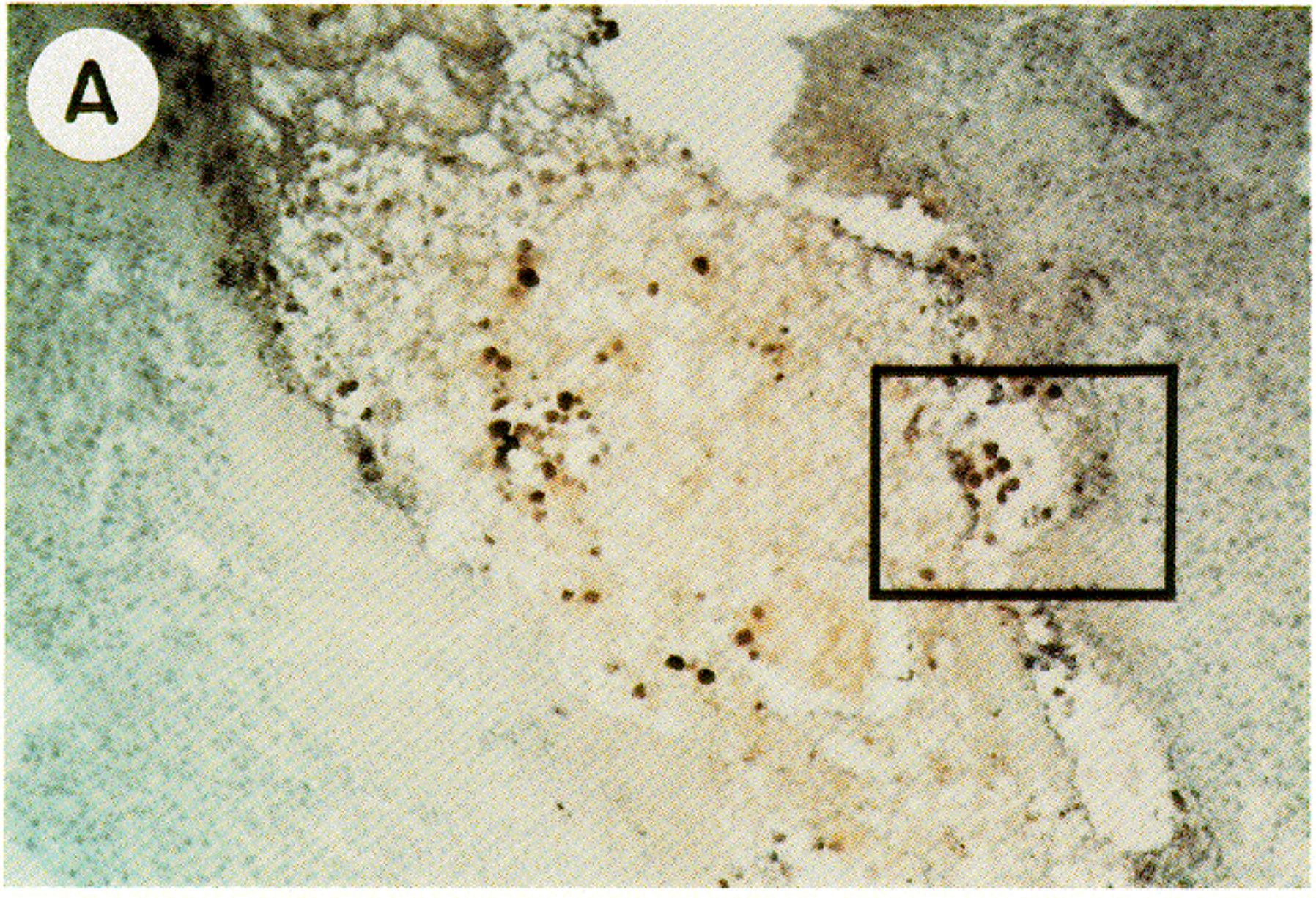
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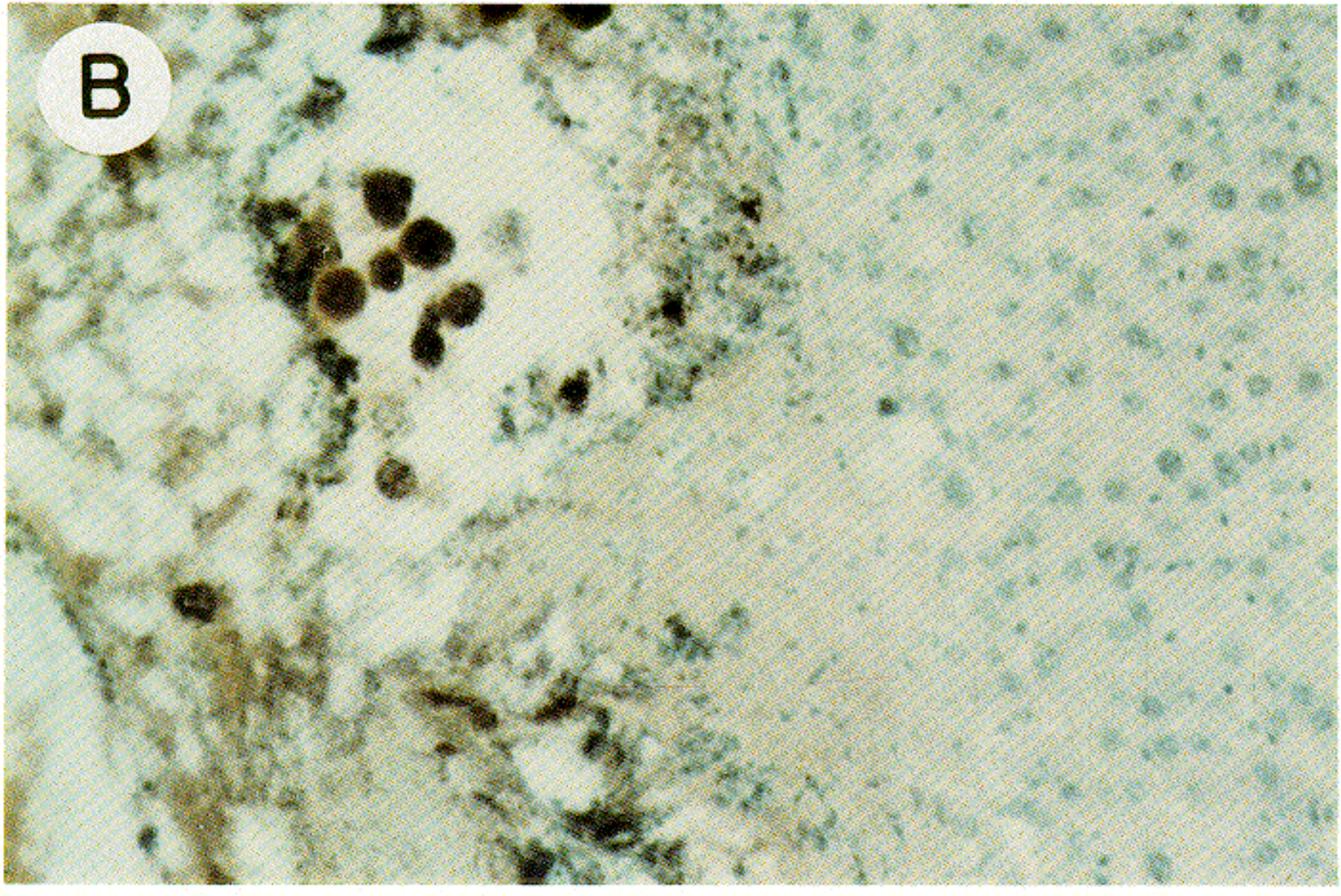
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