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Transvascular migration of L2C leukemic cells studied in the liver of the guinea pig

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Summary. The possible routes of transvascular migration of leukemic cells in the liver were studied in guinea pigs with an L2C lymphoblastic cell-line inoculation leukemia. Invasion of the hepatic parenchyma theoretically can occur in three ways:

1. Through the intact sinusoidal endothelium, utilizing either preexistent gaps (normal in the liver), or newly created pores, whether interendothelial or intraendothelial. We could not convincingly demonstrate this, but could not wholly exclude this either.

2. After destruction or retraction of the endothelium, either on account of the remarkable sinusoidal engorgement and distension by masses of leukemic cells, or by direct assault on the endothelium by the leukemic cells. We can clearly demonstrate the former, and hold it to be the major cause of hepatic infiltration. Evidence for a direct endotheliolytic effect was not uncovered in our studies.

3. Secondary infiltration from the portal triads. Heavy leukemic infiltration of the triads, whether from the portal or hepatic veins, or from the lymphatics, is indeed and early an consistent feature – but the infiltration of the hepatic lobule shows no peripheral, or any other zonal preference.

In both portal and hepatic veins, leukemic cells transverse the endothelium through a cytoplasmic "pore", adjacent to cell junctions, without obvious damage to the endothelium.

Key words: Leukemia – Liver – Ultrastructure – Endothelium – Guinea pig

Introduction

Considering the importance of the metastatic process, it is surprising that relatively few ultrastructural studies have been concerned with the route followed by the neoplastic cells in crossing the blood vessel wall (Ludatscher

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et al. 1967; Mladenov et al. 1967; Locker et al. 1970; Chen et al. 1972; Dingemans 1973; Dingemans 1974; Campbell 1975; Sindelar et al. 1975; Carr et al. 1976; DeBruyn et al. 1977; Roos et al. 1977; Dingemans et al. 1978). The usual experimental procedure consists of the injection into the vascular system of cell suspensions obtained by fragmentation of solid tumors. The procedures used to obtain such suspensions as well as the method of introducing such cells in a single bolus into the circulation leaves such studies open to serious logical objections. Our ultrastructural observations in the guinea pig describe the transvascular metastatic route followed by leukemic cells, two weeks after the subcutaneous inoculation with a known (L2C) leukemic cell line.

Materials and methods

A suspension of 7.0×10^5 to 2.8×10^6 L2C cells in pyrogen free saline was inoculated under the skin of the flank of eleven strain II guinea pigs of 300–350 gm each. The fresh L2C cells are obtained from a terminal donor guinea pig previously inoculated with L2C frozen cells obtained from the laboratory of Dr. Ira Green, National Institutes of Health. Two further guinea pigs serve as normal controls. At intervals of one to fifteen days (5 in the first eight days, the other 6 at 12–15 days) after inoculation, the animals are anesthesized with an intraperitoneal injection of Nembutal Sodium solution, 20–40 mg per kg body weight. Through cardiac puncture an aliquot of blood is obtained for total and differential blood cell counts. Under 1% Procaine anesthesia tracheostomy is performed, followed by thoracotomy. Ventilation is maintained manually with a rubber cuff, controlled by direct visualization of pulmonary expansion. The animals are then perfused through the ascending aorta with a chilled, double-aldehyde fixative consisting of 2.5% Glutaraldehyde with 1.5% Paraformaldehyde in 0.08 M Cacodylate buffer (pH 7.2) with 0.15% Calcium Chloride added (osmolarity 1.285 + 15 milliosmol). In some 15–20 min 1,000 ml of this solution is used. A constant flow is maintained by adjusting the fluid pressure with a sphygmomanometer cuff around a plastic bag (pressure 120–150 mm of mercury). At autopsy, tissue blocks of every major organ including the brain and the area of inoculation, are obtained. In the 5 pre-leukemic animals (1–8 days), no hepatic infiltration was seen (16 blocks, thick sections). Of the 6 leukemic animals (12–15 days), all 24 liver blocks revealed infiltration. Six blocks were further trimmed down and utilized for thin sections. The tissue is further fixed for two hours in the same double-aldehyde solution, then post-fixed in 2% Osmium Tetroxide for two hours. Then the tissues are dehydrated in a graded series of chilled ethanol, passed through Propylene Oxide, placed in a 1:1 mixture of Propylene Oxide and Epon 812 for 45 min and finally embedded in Epon 812. One micron thick sections are stained with a 1% solution of Toluidine Blue. From these sections specific areas are chosen for electron microscopic examination. The blocks are trimmed and the area remaining is thin sectioned on a LKB ultramicrotome. The thin sections are collected on 200 mesh copper grids (ultrahigh transmission grids, Polysciences, Inc., Warrington, PA, USA, stained with Uranyl Acetate and Lead Citrate and viewed at 60 KV in a Philips 300 electron microscope.

Results

The L2C leukemic cells are characterized by small amounts of cytoplasm. The surface is covered by numerous, widely spaced microvilli. There are large numbers of ribosomes, many in rosettes, also a few cisternae of the rough endoplasmic reticulum and often viral particles, almost in every cell (none were found in normal white blood cells), none of which will be described (Nadel et al. 1967). Other organelles are inconspicuous. The nuclei

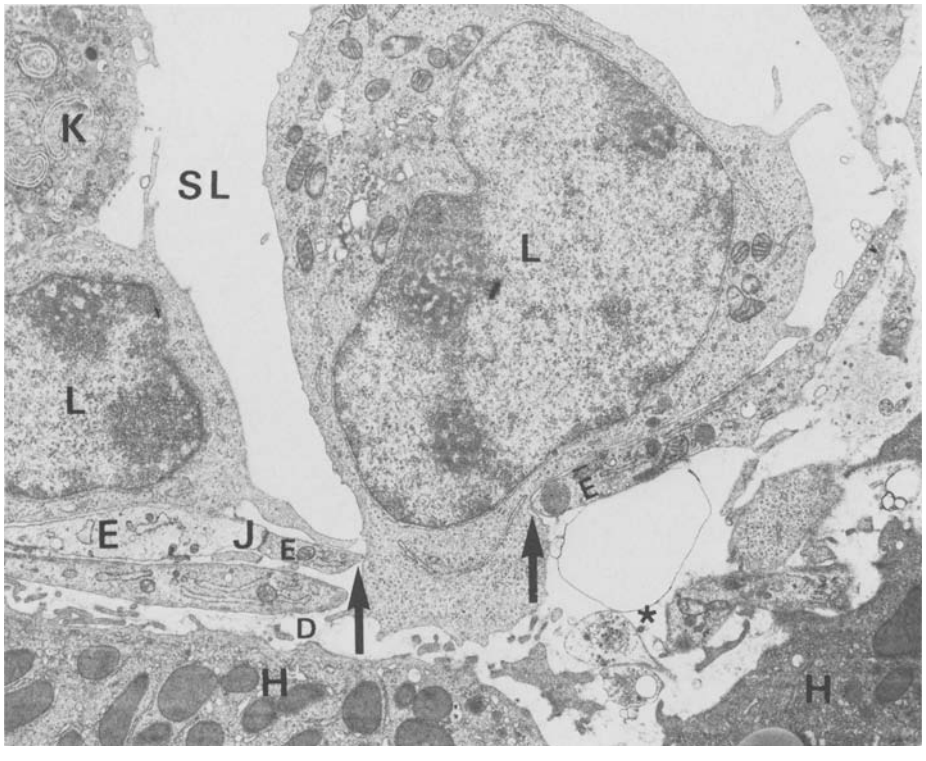


Fig. 1. Liver Sinusoid: A blunt process of a leukemic cell projects through an endothelial "pore" (*arrow*) into the space of Disse. *L*=leukemic cell, *SL*=sinusoid lumen, *E*=endothelium, *J*=junction, *K*=Kupffer cell, *H*=hepatocyte, *=nerve endings. ×6,547

are usually indented and contain ordinary amounts of finely dispersed chromatin with some condensation on the nuclear membrane. Using these criteria, differentiation of leukemic cells from other formed elements in the blood or from endothelial cells is no problem.

All six leukemic animals showed extensive infiltrates by such leukemic cells. According to the site of infiltration, we can have:

1. Leukemic cells in the hepatic sinusoids;
2. Leukemic cells in the hepatic parenchyma;
3. Leukemic cells in the portal spaces.

1, 2. Sinusoidal accumulations and parenchymal infiltrates

We will define as *parenchymal* those infiltrates in which the leukemic cells have crossed the endothelia of the sinusoid, have invaded the space of Disse and are in contact with hepatocytes. Four grades can be recognized.

Grade I. The general configuration of the sinusoid and the adjacent liver plates (cords) is well preserved. The endothelial lining is mostly intact, with occasional gaps through which leukemic cells send blunt processes into the

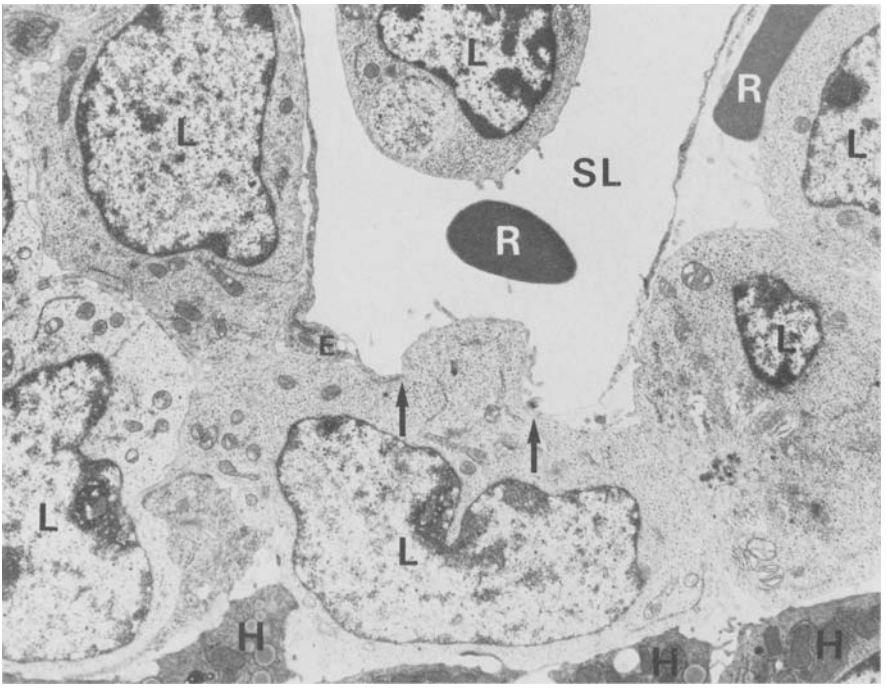


Fig. 2. Liver Sinusoid: Several leukemic cells are between the hepatocytes and the sinusoid. In the center a blunt process of a leukemic cell protrudes into the lumen through an endothelial gap (*arrows*). *L* = leukemic cell, *SL* = sinusoid lumen, *E* = endothelium, *R* = red cell, *H* = hepatocyte. × 4,103

space of Disse (Fig. 1). (Even in normal controls the endothelial lining shows numerous discontinuities.)

Grade II. The general configuration of the sinusoid in adjacent liver cords now is distorted; numerous leukemic cells choke a markedly enlarged space of Disse (Fig. 2). The endothelial cells are still largely intact.

Grade III. The general configuration of the sinusoids is markedly distorted. In contradistinction to Grade II, the endothelial cells are largely lost and leukemic cells establish extensive contacts with the hepatocytes.

Grade IV. The parenchyma is replaced by sheaths of densely packed leukemic cells with only occasional islands of hepatocytes. Such hepatocytes are distorted, often stretched out to thin ribbons of cytoplasm, but the epithelial continuity is still maintained by cell junctions adjacent to the bile canaliculus with its closely spaced typical microvilli (Fig. 3).

3. Portal space infiltrates

Infiltration of the portal spaces by leukemic cells is extensive. The bile ducts and the arteries, although surrounded by sheaths of leukemic cells,

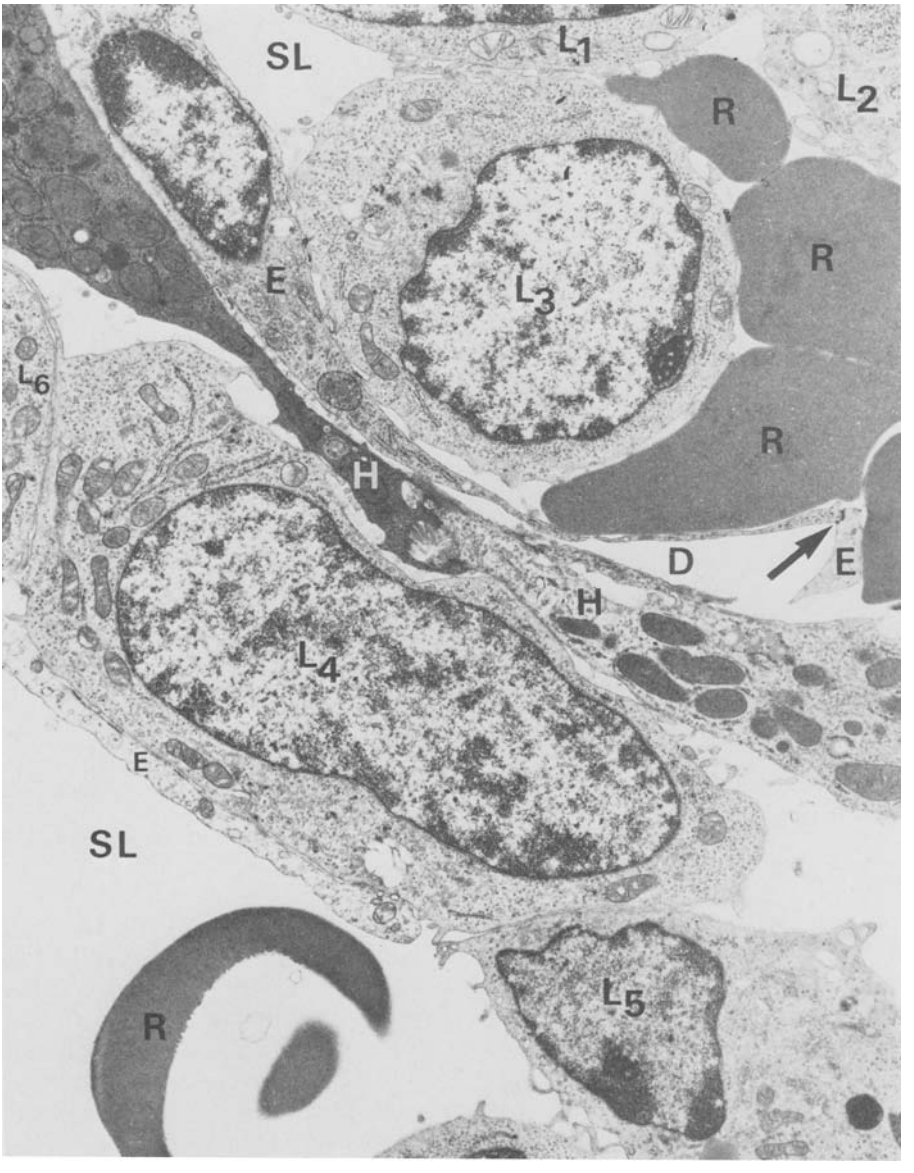


Fig. 3. Two Liver Sinusoids: Leukemic cells number 4 and 6 are compressing two adjacent hepatocytes. Note the bile canaliculus (*center*). *SL* = sinusoidal lumen, *L* = leukemic cell, *H* = hepatocyte, *E* = endothelium, *D* = space of Disse, *Arrow* = junction. × 5,088

are normal. Extensive leukemic infiltration, however, are seen in the veins, both portal and hepatic (Figs. 4 and 5). We will classify these leukemic cells according to their location as follows: intravascular, intramural and migrating.

a) *Intravascular*. Only a few leukemic cells are present, randomly distributed throughout the blood vessel lumen. We must keep in mind, however, that

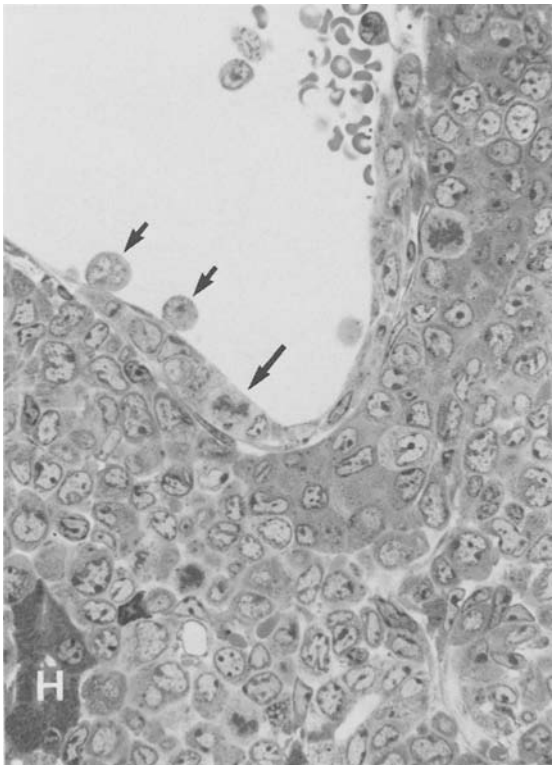


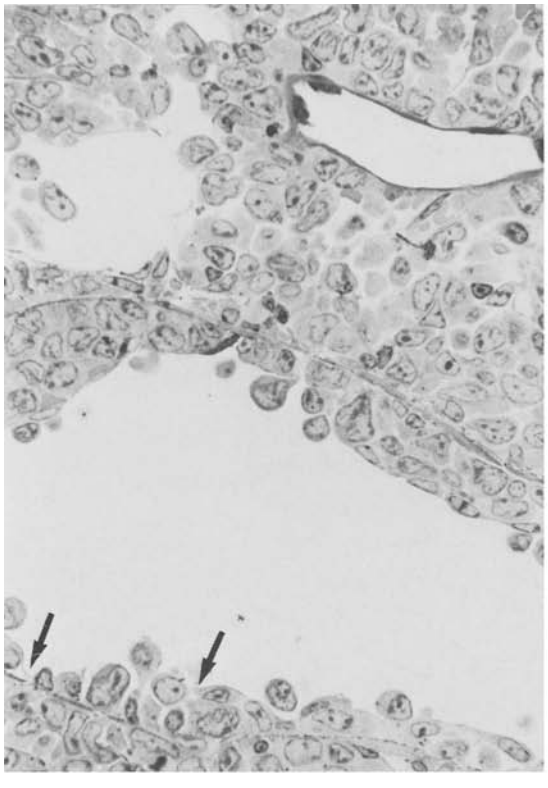
Fig. 4. Extensive leukemic infiltrates of a portal space extending to the adjacent liver parenchyma where only an island of hepatocytes is still present (*H*). Notice subendothelial leukemic cells; one of them is in mitosis (*large arrow*). Two leukemic cells appeared marginated (*small arrow*). One micron section, Epon, Toluidine Blue stained. ×812

these are perfused preparations where the central portion of the vascular lumen has been cleansed. Most of the cells are in close proximity to the endothelium, we define such cells as *marginated* if they establish contact with the endothelium for one-third or more of the total cell perimeter. Whereas the free cells in the lumen are round in cross section, marginated cells are flattened against the vascular outline. Moreover, in the flattened portion of the marginated cells the cytoplasmic membrane looses its customary microvilli. The slit defined by the cytoplasmic membrane parallel to the endothelium will be from 15–50 nm wide.

In a few instances a leukemic cell is completely surrounded by a thin endothelial cytoplasmic layer (Fig. 6), the space between the leukemic cells

Fig. 5. Extensive leukemic infiltrates in a portal space. There is massive subendothelial accumulation of leukemic cells. Between arrows is an area lacking endothelial lining; the leukemic cells are directly apposed to the basal lamina. ×850

Fig. 6. Portal Vein: A leukemic cell is completely encircled by a thin endothelial cytoplasmic layer. *LU*=lumen, *J*=junction, *E*=endothelium, *L*=leukemic cells, *SM*=smooth muscle. ×3,300



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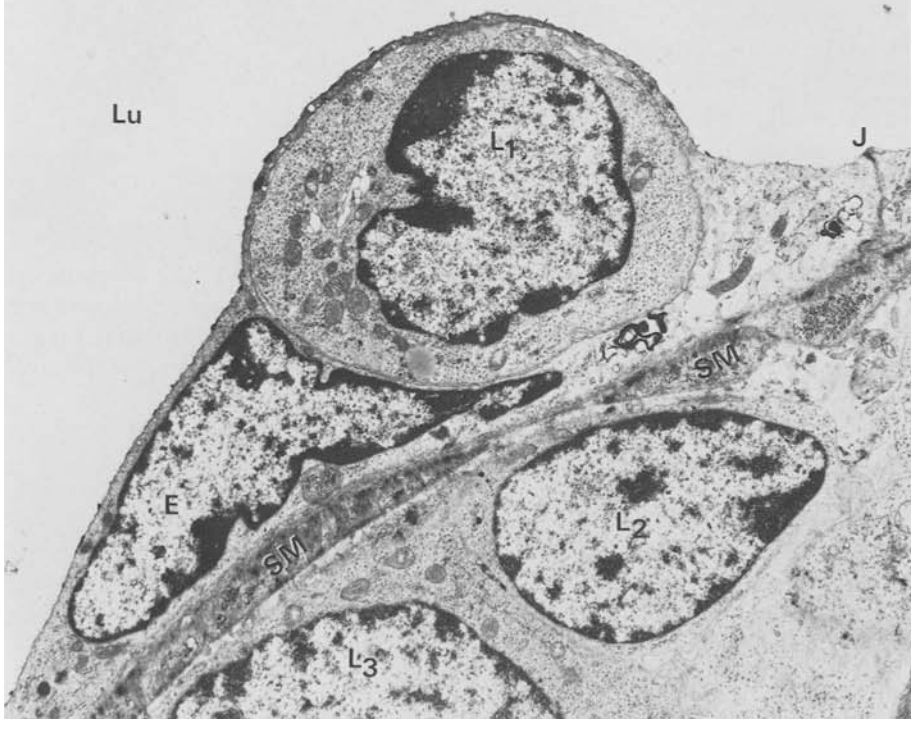

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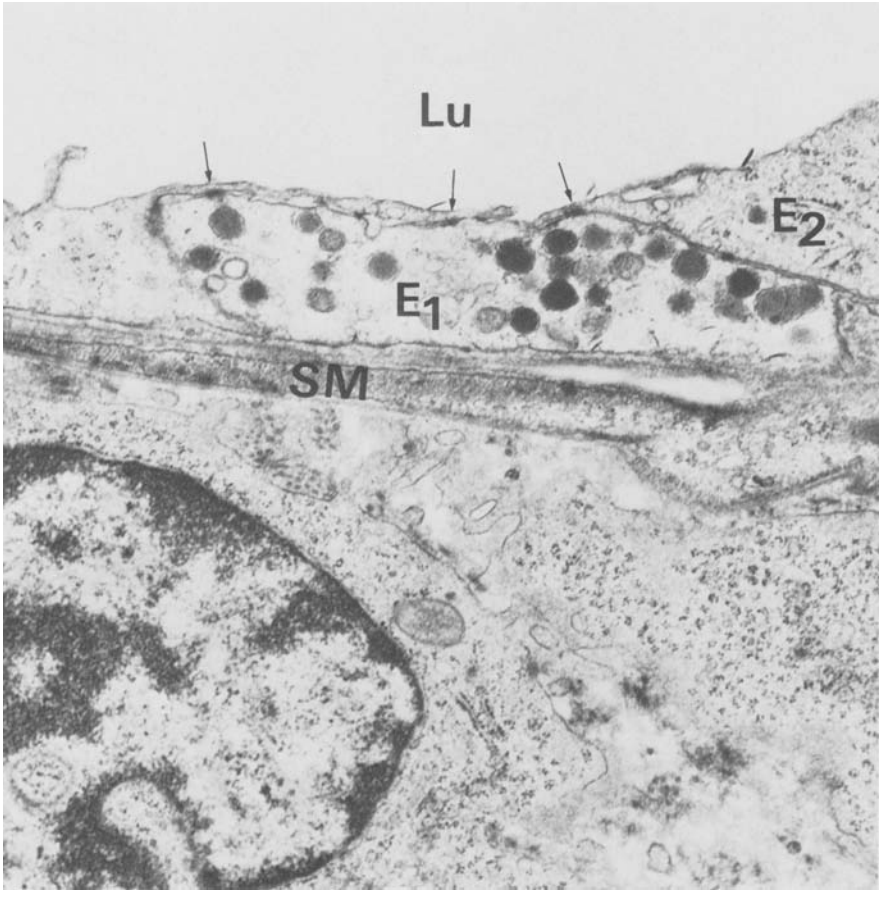

Fig. 7. Portal Vein: The cytoplasm of endothelial cell number 1 contains numerous membrane-bound osmiophilic inclusions. *E* = endothelium, *SM* = smooth muscle, *LU* = lumen. *Arrows* mark cell junctions. × 18,628

and the endothelium now is quite narrow, the leukemic cells is rounded in outline and microvilli are almost totally absent. We could not determine whether such cells are still at some point connected with the vascular lumen or are completely enclosed within the endothelial cells. Even serial sectioning did not resolve the issue.

b) Intramural. Most of the leukemic cells here are found between the adventitia and the muscular coat. Such cells are mostly devoid of microvilli, their shape is variable, adapting to pre-existent spaces within the wall of the vein. Not infrequently such a cell will insinuate itself between the smooth muscle cells and will extend processes which may perforate the basal membrane, reaching the subendothelial space. The endothelium then will be slightly raised from its basal membrane.

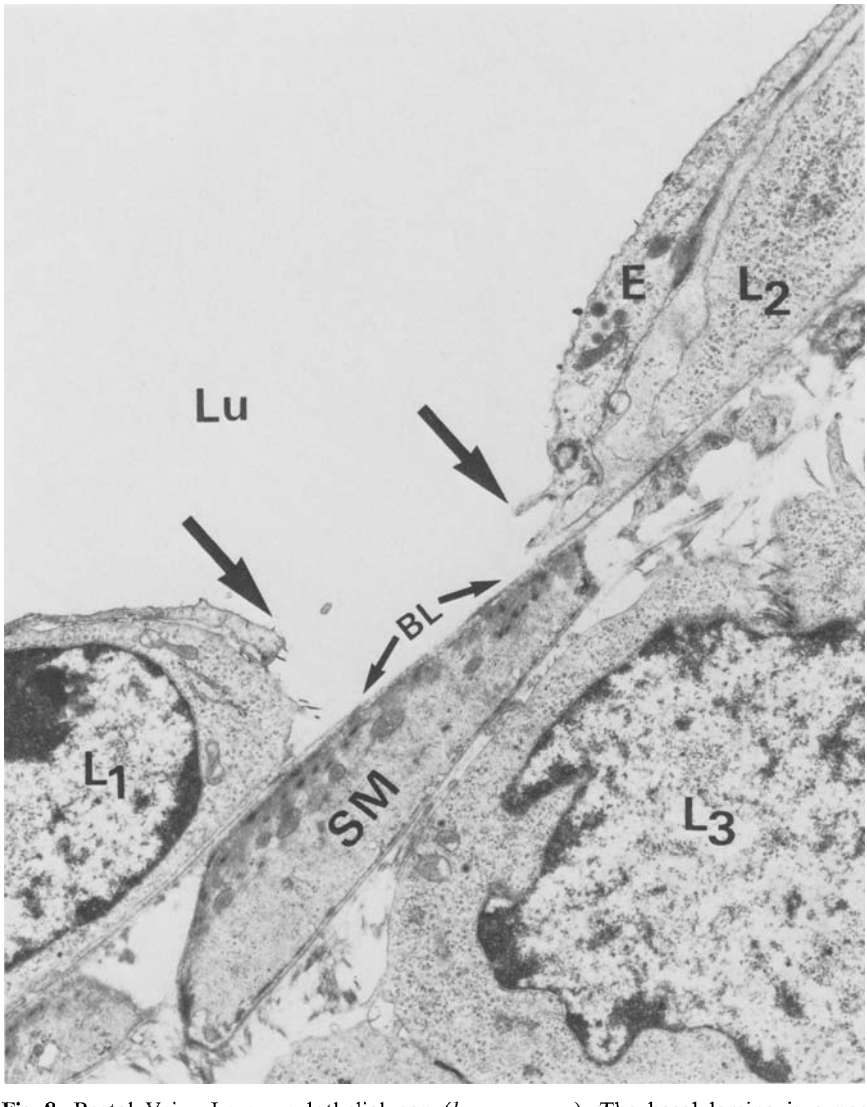

Fig. 8. Portal Vein: Large endothelial gap (*large arrows*). The basal lamina is exposed to the lumen. The leukemic cells number 1 and 2 are under the endothelium. Leukemic cell number 3 is under a smooth muscle cell. *L* = leukemic cell, *E* = endothelium, *BL* = basal lamina, *LU* = lumen. × 29,157

The endothelium in other areas eventually becomes markedly separated from its basal membrane by leukemic cells. The endothelium bulges into the lumen where in addition there are a variety of changes, as compared to the normal flat endothelium of control animals. The most constant finding is the presence of numerous dense, osmiophilic membrane-bound round granules about 180–214 nm in diameter (Figs. 7 and 8). There is a marked

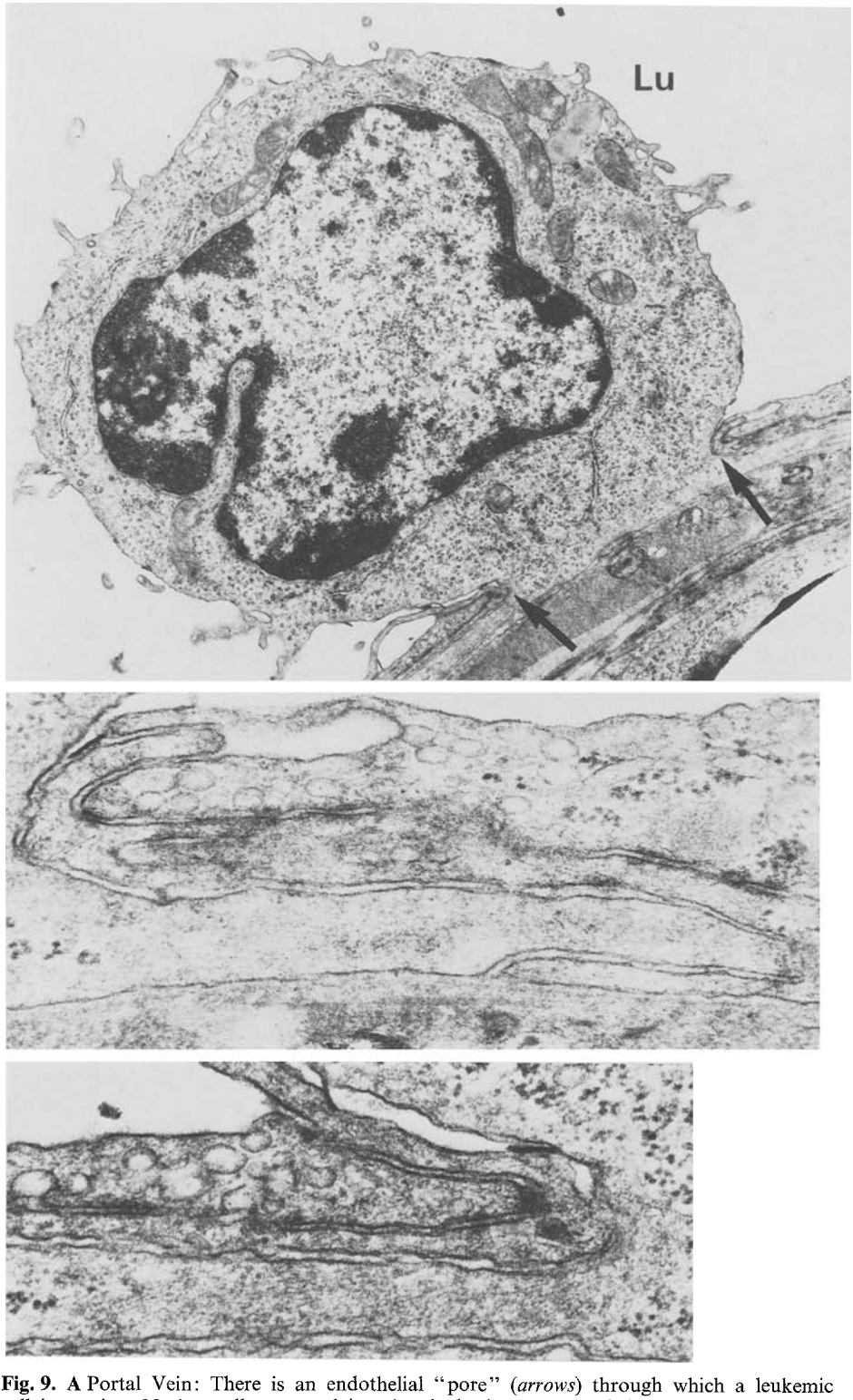

Fig. 9. **A** Portal Vein: There is an endothelial "pore" (*arrows*) through which a leukemic cell is passing. Notice well preserved junction in both extremes of the pores. See detail of the junctions in Fig. 9B and C. *LU*=lumen. ×8,811 **B** Detail of the junction of left side. ×51,494. **C** Detail of the junction on the right side of the pore. ×51,494

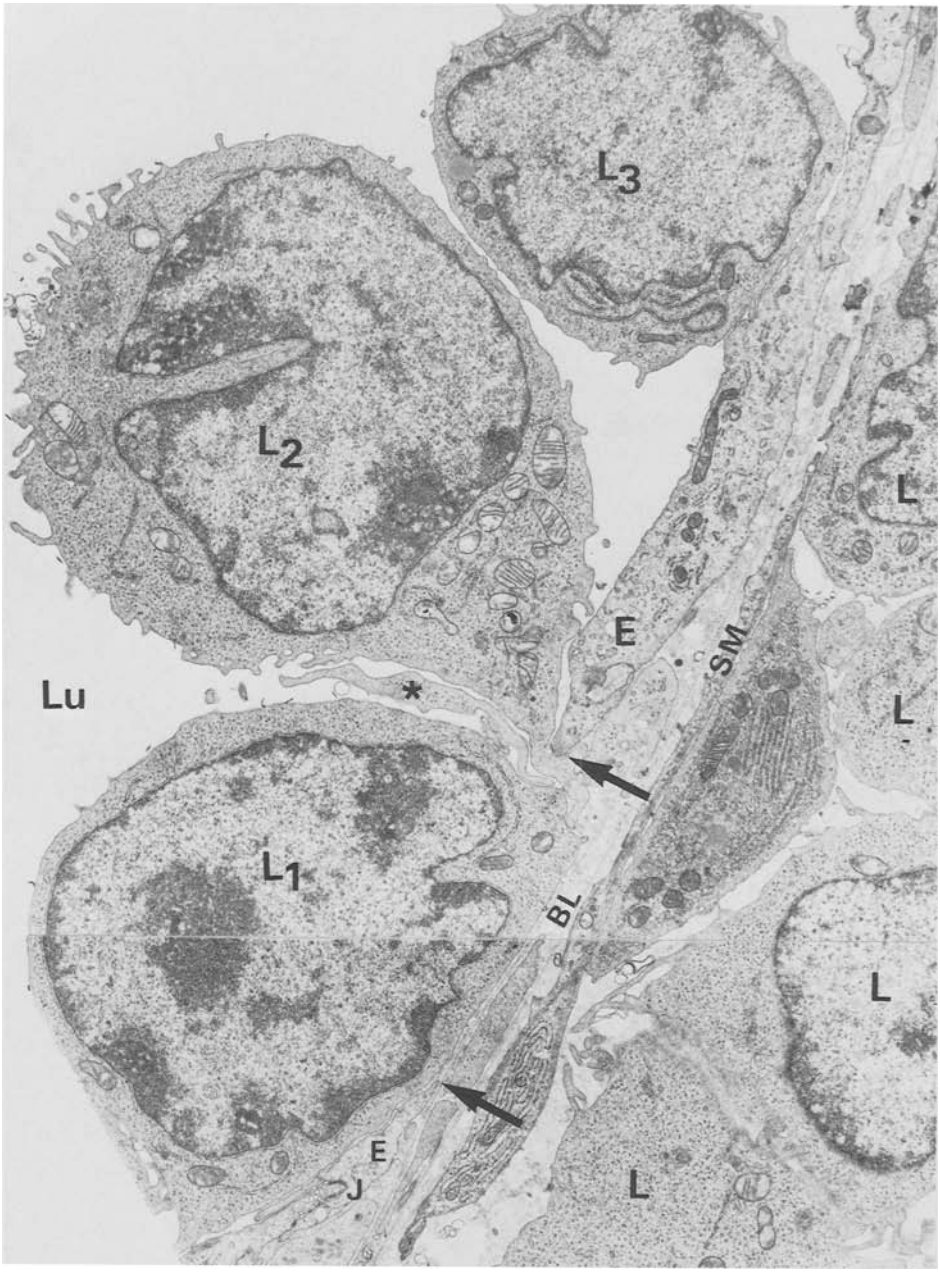

Fig. 10. Portal Vein: Leukemic cells number 1 and 2 are crossing a large endothelial "pore" (*large arrows*) leukemic cell number 3 is marginated. Between cell 1 and 2 there is a cell process (*) of undetermined origin. *L* = leukemic cells, *E* = endothelium, *J* = junction, *SM* = smooth muscle, *BL* = basal lamina. × 5,690

decrease of organelles in some of these endothelial cells; others show extensive disruptions of the cytoplasmic membranes. In some areas the endothelial cells have become completely lost and the basal membrane is exposed to the lumen (Fig. 8).

c) Actual migration of leukemic cells. We will concern ourselves with those images in which portions of the leukemic cell are actually crossing the endothelial barrier. Such passage is generally through a large "pore" of as much as 14 microns across. One or several cells may be observed migrating through a single pore, with the endothelial membrane adjacent to the pores well preserved. Endothelial junctions in the vicinity of such pores are rather elaborate with imbricating configurations (Figs. 9A, 9B and 9C). At least one edge of a pore is always close to such an endothelial junction (Fig. 10). However, on many occasions the pore is literally flanked by two junctions on each side (Fig. 9A), which suggests that such pores occur at the apex of an endothelial cell. Occasional fenestrations, randomly distributed can be seen in the endothelial cells of the portal veins in control animals. A residual cytoplasmic layer is always found in such a fenestration which differentiates it from a pore; pores are never seen in the veins of control animals.

Discussion

This L2C acute B type lymphocytic leukemic arose spontaneously in a female strain-II guinea pig (Congdon and Lorenz 1954). With a potent inoculum of 10^5 cells all injected animals became leukemic. Death takes place 2–4 weeks after inoculation with extensive leukemic infiltration of all viscera and the brain, there is less intense involvement of the bone marrow. Terminally, white blood cell counts in excess of 100,000 cells/mm^3 with 99% blasts in the peripheral smear are obtained.

Neoplastic dissemination in the liver is usually studied by the injection of cell suspensions, either into the general circulation (Chew et al. 1976; Campbell 1977) or into the vena porta (Dingemans 1973; Dingemans et al. 1978). Although this single-dose injection of neoplastic cells is different from the natural situation, it does provide the opportunity to observe the morphological events in a predetermined time sequence. In our natural model malignant cells are continously in circulation and all stages of the metastatic process are going on concurrently.

In an attempt to reconstruct the events we will single out some of the pertinent observations. It should be realized that there are methodologic problems in studying the putative migration of neoplastic cells through the endothelial cells of the hepatic sinusoids, since normally the endothelial lining contain gaps through which a cell could invade. Therefore, in the case of the hepatic sinusoidal endothelia, the creation of new openings is not a necessary precondition to explain the presence of leukemic cells in the Disse space or among the hepatocytes. In the erlier stage of hepatic parenchymal invasion (Grade I) the leukemic cell is marginated and may

project a blunt process through an endothelial gap. Whether this is passage through the endothelial cell (transcytoplasmic diapedesis, Dingemans 1973; Dingemans 1974; Roos et al. 1977) or through a widened intercellular gap (intercellular extravasation, Sindelar et al. 1975) is hard to say. Typically liver sinusoids show cytoplasmic fenestrations (Motta and Porter 1974; Grisham et al. 1975; Nopanitaya and Grisham 1975; Wisse 1970; Brooks and Haggis 1973) and intercellular gaps (Wisse 1983). In addition when the sinusoids are filled with leukemic cells, such leukemic cells may well protrude somewhat through pre-existing gaps. To interpret such a protrusion as a beginning migration is tempting but unjustified. We will only consider those leukemic cells as traveling through an endothelium if somewhere near one third to one half of the cell is found on either side of the gap, and this we did not observe in the hepatic sinusoids. Neither could be develop satisfactory evidence that the numbers or types of gaps were really increased as the result of the leukemic infiltration, as compared to normal control animals. To rephrase this, are these gaps indeed the result of a specific interaction between the endothelium and the neoplastic cells?

In the second stage of infiltration leukemic cells are in the liver plates, but they are still separated from the sinusoid by an intact endothelium (Fig. 2). Occasional blunt processes do extend into the sinusoidal lumen, sometimes referred to as tails by other authors (Dingemans et al. 1978). It is tempting to link this stage of hepatic invasion to the first stage but it is quite disturbing that in no instance could we, or other investigators (Dingemans et al. 1978) document intermediate stages in which a cell crosses the endothelium, as defined by us previously as about one-third way through.

In the third grade of hepatic parenchymal invasion the sinusoids are markedly distended by leukemic cells, but the endothelium now is fragmented and may have completely disappeared. The process of endothelial fragmentation could not be followed well: the endothelia are either fairly intact, or have entirely disappeared. The origin of these large endothelial defects has been the subject of much speculation. According to some (Locker et al. 1970; Chew et al. 1976; Fonck-Cussac et al. 1969) there is attrition following the stretching of the sinusoid; according to others (Cotmore and Carter 1973) there is possible anoxic damage to the endothelial cells, still others (Dingemans et al. 1978) believe there is retraction followed perhaps by regeneration of the endothelium. Retraction, with exposure of the underlying basal membrane, has been seen in vitro in binding of malignant cells to monolayers of vascular endothelia (Kramer and Nicolson 1979). Movement of neoplastic cells to the basal membrane occurs because the basement membrane is closely adherent to the endothelium and thus becomes exposed once the endothelium retracts (Kramer et al. 1980; Nicolson et al. 1981). To what extent this is important in the liver where the basal lamina is so incomplete is still in question. A different mechanism of malignant extravasation, similar to that of leukocytes crossing the endothelium during inflammation (Marchesi and Florey 1960) has been proposed with the cells passing through the endothelial junctions (Ludatscher et al. 1967; Sindelar et al. 1975; Carr et al. 1976).

All the leukemic animals show extensive leukemic infiltrates in the portal triads, they are continuous with those in the periphery of the hepatic lobules. Whether the portal infiltrates develop first in the portal triads as has been suggested in human lymphocytic leukemia (Goldberg 1960) or are secondary to the parenchymal infiltration is unclear. The parenchymal infiltration shows no zonal preference and therefore cannot itself be derived from the portal infiltrates. The veins, but not the arteries or the bile ducts, do show extensive leukemic infiltration and it is clear that there is transendothelial passage of leukemic cells. The direction of the migration, whether from the lumen outwards or back into the lumen can of course not be established by static morphologic studies. The leukemic cells, either singly or in groups, cross through a pore in an otherwise intact endothelial cell. Such pores are generally close to the cell junction but the junctions themselves remain intact. The portal veins in the triads normally show fenestrations (Grisham et al. 1975), these are differentiated from pores in that the fenestrations remain covered by a thin layer of cytoplasmic membrane. In addition, fenestrations occur at random through the endothelial cell whereas the pores are always close to the cell junctions. Passage through pores, as described here, has previously been reported for leukemic myelogenous elements in the bone marrow (DeBruyn et al. 1977), of erythroblasts in rats with murine erythroblastosis in the spleen (Cho and DeBruyn 1975), in subcutaneously implanted myelogenous tumors (DeBruyn and Cho 1979) and by us in the leptomeningeal veins of L2C leukemic guinea pigs (Azzarelli et al. 1984). This crossing of the blood vessel wall, so characteristic of malignant cells, is of course not limited to such cells but is the usual mechanism of egress into the circulation of normal, myelogenous elements in the bone marrow (DeBruyn et al. 1971; Campbell 1972). Only mature cells will cross and crossing is through transient pores, characteristically located close to the cell junction. This selectivity for mature elements is lost when leukemic cells enter the bone marrow sinusoid following the same route (DeBruyn et al. 1977).

It is interesting to observe that in no instance could we demonstrate the presence of fibrin or platelets around or near tumour cells, either in the hepatic sinusoids or in the portal vessels. This fits in with observations by other authors (Cotmore and Carter 1973; Dingemans 1973). This negative finding may be significant, since it has been postulated that the attachment of embolized malignant cells to the vascular walls is largely dependent upon entrapment of tumor cells within a platelet thrombus and/or a fibrin mesh-work (Wood 1958; Wood et al. 1961; Chew and Wallace 1976; Sindelar et al. 1975; Jones et al. 1971).

In summary, we believe that the invasion of the hepatic parenchyma proper follows intrasinusoidal leukemic cell proliferation. This proliferation leads to endothelial disruption or perhaps retraction. The possibility that occasional leukemic cells migrate through gaps of a previously intact endothelium cannot be ruled out.

We also have documented that there is extensive passage of cells through the endothelium in all the veins in the portal triads, which then must be

true for both the portal and the hepatic system. But whether this represents intra or extravasation could not be determined.

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