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MOLECULAR, BIOLOGIC, IMMUNOHISTOCHEMICAL, AND ULTRASTRUCTURAL ASPECTS OF LYMPHATIC SPREAD OF THE HUMAN IMMUNODEFICIENCY VIRUS

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A major unsolved question concerns the mode of spread of the human immunodeficiency virus type-1 (HIV-1) in the body. Many emphasize the importance of viremia but viremia is not a constant finding during the entire course of infection. Infected cells in the blood circulation probably also contribute to the spread, but the number of these cells detected is extremely low (1). Many individuals with HIV-1 infection develop persistent generalized lymphadenopathy (PGL). Morphologic study has provided evidence that germinal centers (GC) in these lymph nodes harbor many cell-free retrovirus particles (2-8). Examining repeated biopsy specimens, we could demonstrate that the infection of GC persists for long periods of time (8,9). We assume that GC represent an important virus reservoir where permissive cells become infected (8-12). Knowledge of the route of entry of infectious particles into the node would contribute to our understanding of the pathogenesis of the disease.

One route through which infected cells may reach the lymph node is via the high endothelial venules. It is known that circulating lymphocytes enter the lymph nodes via these venules (13). Lymph-borne cells could also carry infection to the node from a prenodal area. Recent studies indicate that macrophages (14-18) and Langerhans cells (19,20) are also permissive for HIV-1. These cells can enter the lymph node via lymphatics. Nothing is known however about the spread of HIV-1 through lymphatics. Therefore, applying complex morphologic methods we looked for the presence of infected cells in the sinuses, afferent and efferent lymphatics of the lymph nodes with follicular hyperplasia caused by HIV-1.

The presence of infected cells in the lymphatics and lymph node sinuses

Immunostaining with monoclonal antibodies against *gag* proteins, p18 and p24, of HIV-1 shows positively reacting cells in the sinuses as well as the afferent and efferent lymphatics. These monoclonal antibodies detect positivity in the GC of patients with PGL and react with some cells scattered throughout the extrafollicular parenchyma (10-12). As long as the positivity in the GC is highly specific (i.e., never seen in control nodes), this feature distinguishes HIV-1-induced lymphadenopathy from other viral lymphadenitis and follicular hyperplasia of unknown origin (6,10-12,21). Positive cells in the pulp however have no diagnostic value, because they are seen also in control nodes. These cells probably cross-react with HIV antigens (21). Although

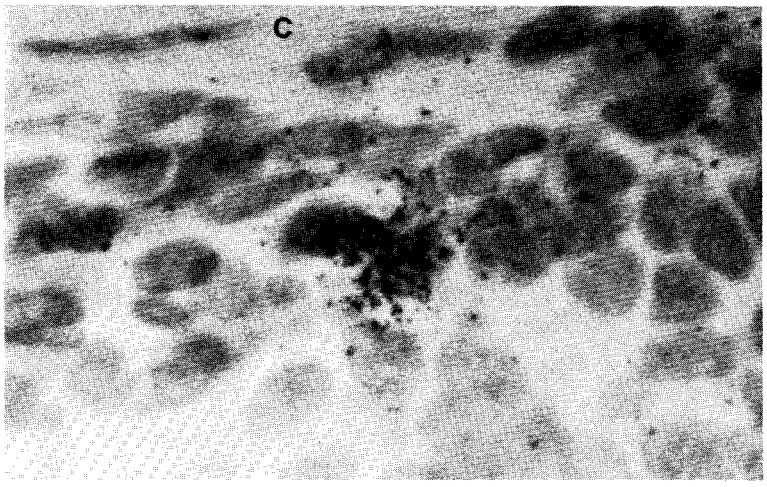

Fig. 1. A cell expressing RNA of HIV-1 in the marginal sinus. In situ *hybridization with ^{35}S-labeled RNA probe. PGL. C=capsule. Original magnification: x800.*

we could not find positive cells in the lymphatics and sinuses of control nodes, we applied *in situ* hybridization to gain evidence for the presence of infected cells in this location.

A ^{35}S-labeled RNA probe prepared by Harper et al. (1,22) was used, and the *in situ* hybridization was carried out on 15 lymph nodes with PGL and 15 controls according to published protocol (1,22,23). The results provided strong evidence that in the sinuses and lymphatics of lymph nodes with PGL, infected cells are circulating *(Fig. 1)*. The number of cells expressing viral RNA was low in this location. One to four cells can be found per section. In 4 patients with PGL, we evaluated serial sections at 10 cutting levels. Each level contained approximately the same amount of labeled cells in this location. Therefore, if we extrapolate the number of positive cells to the whole marginal sinus system of the lymph node we may conclude that a significant number of cells containing HIV-1 RNA at levels consistent with viral replication arrive in the lymph nodes through the lymphatics. Very likely, the number of infected cells carried to the lymph nodes via lymphatics is still higher than can be estimated on the basis of results with the applied RNA probe because this probe does not detect latently infected cells containing only provirus not undergoing viral replication.

Histologic changes in the sinuses which may affect lymphatic flow

Paraffin histology of lymph nodes with PGL shows dilatation of the afferent and efferent lymphatics as well as the sinus system *(Fig. 2)*. These changes could develop as a consequence of increased lymphatic flow from the prenodal area or because of a postnodal efferent block. The observed hypercellularity and the presence of inflammatory cells in the submarginal sinus are in favor of increased lymphatic flow.

Although not specific for HIV-1 induced lymphadenopathy, B-cell sinus reaction often develops in PGL (6,12,24-31). This reaction represents uni- or multifocal aggregates of distinctive mononuclear cells lying densely packed in the sinuses *(Fig. 3)* and hindering lymph flow. The characteristics of this unique cell type were first described by Lennert in Toxoplasma lymphadenitis (32). They are medium-sized cells with ovoid or indented nuclei, small basophilic nucleoli, mild basophilic nuclear sap, distinct nuclear membrane

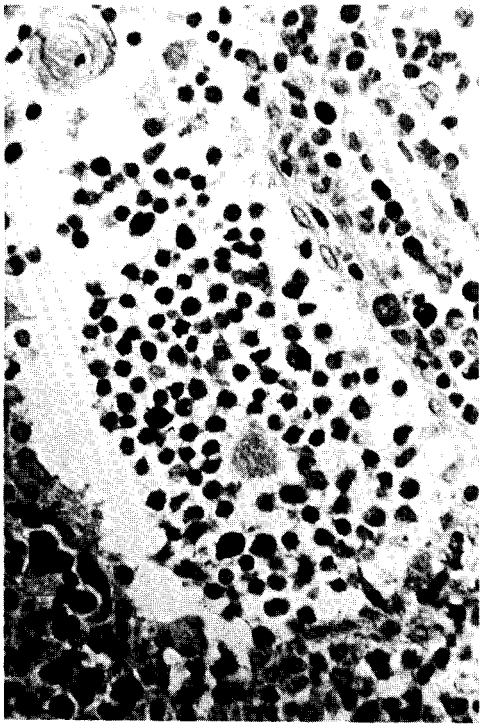

Fig. 2. Enhanced cellularity and dilatation of an intermediate sinus. PGL. Giemsa stain. Original magnification: x160.

and pale cytoplasm. They constantly express B-cell-restricted antigens and polyclonal surface immunoglobulins (6,28,32). In contrast to B-cell sinus reaction seen in conditions other than HIV-1 infection, we found that in PGL aggregates of this reaction also contain markedly increased number of $CD8^+$ lymphocytes instead of $CD4^+$ T cells.

Another sinus reaction which can influence lymphatic flow is vascular sinus transformation. This condition has been described by Haferkamp et al. (34) and can be reproduced experimentally by incomplete occlusion of the veins combined with complete blockage of the lymphatics or by complete occlusion of the lymphatics alone (35). A framework of channels lined by endothelium and anastomosis between them and the para- and intranodal blood vessels develop. There is proliferation of fibroblasts. Although infrequent, vascular sinus transformation may occur in PGL and this benign condition should not be confused with Kaposi's sarcoma. Vascular sinus transformation, however, provides an open route for HIV-1 infected cells and virus particles of the sinus to enter the blood circulation directly.

These changes may restrict lymphatic flow through the sinuses, direct it toward the lymph node parenchyma and facilitate the migration of cells through the sinus wall into the deeper parts of the lymph node.

Spread of HIV-1 infected cells from the marginal sinus into the lymph node parenchyma

We and others have reported (36-38) that the parenchymal side of the submarginal sinus is lined by discontinuous endothelium *(Fig. 4)* making it especially suitable for transporting antigens and micropathogens into the underlying lymphoid parenchyma.

Our electronmicroscopic examinations demonstrated many gaps between the sinus endothelial cells of the parenchymal sides of the marginal sinus in HIV-1 infected lymph nodes. These gaps contained migrating lymphocytes and macrophages *(Fig. 4)*. We assume that HIV-1-infected cells enter through these gaps into the lymph node. In addition, we found in PGL gaps containing long slender cytoplasmic processes resembling those of follicular dendritic cells *(Fig. 5)*. Immunohistochemical examinations on frozen sections stained with monoclonal antibodies detecting FDC shows that dendritic processes can, indeed, reach the marginal sinus *(Fig. 6)*. This phenomenon is probably due to the exuberant follicular hyperplasia and attenuation or lack of a mantle zone.

With regard to antigen trapping and retaining ability of FC (for review see 39,40), it is very likely that these processes of FDC extending into the marginal sinus can also capture HIV transported by the lymph from remote sites or produced intrasinusoidally by infected cells. Studying transport of

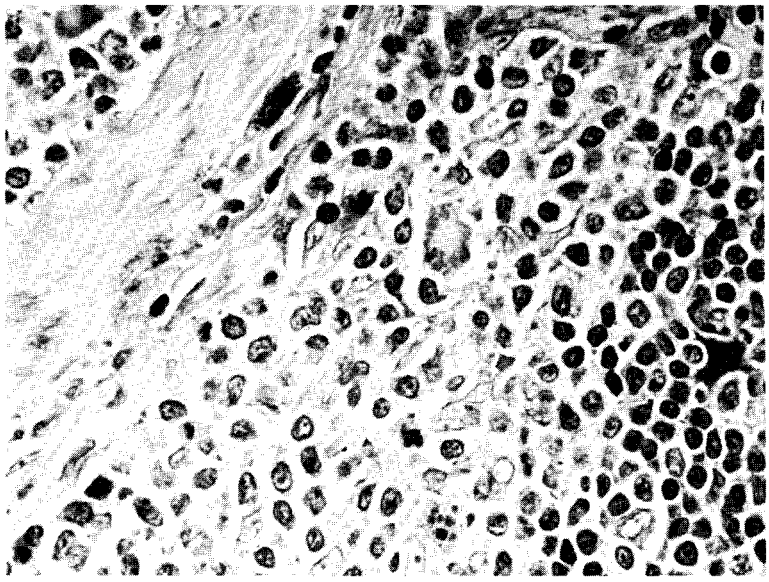

Fig. 3. B-cell sinus reaction in PGL. Giemsa stain. Original magnification: x160.

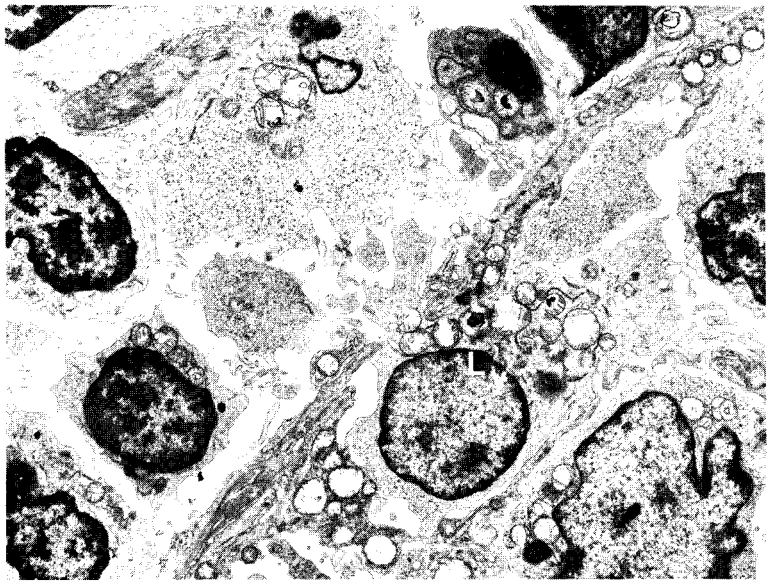

Fig. 4. Discontinuous lining of the parenchymal side of the marginal sinus. A gap with a migrating lymphocyte (Ly). PGL. Original magnification: x4,500.

immune complexes from the subcapsular sinus to lymph nodes of mice, Szakal et al. (41) found that an active cell-mediated mechanism of antigen transport existed. In this transport, special cells were involved with processes of various complexity that trapped antigen on their plasma membrane by an antibody-dependent mechanism. The antigen transporting cells located near the sinus resembled non-Birbeck granules containing Langerhans cell precursor or veiled cells, and those lying deeper in the cortex shared morphologic similarities with FDC. The authors suggested that antigen was transported from the marginal sinus into the

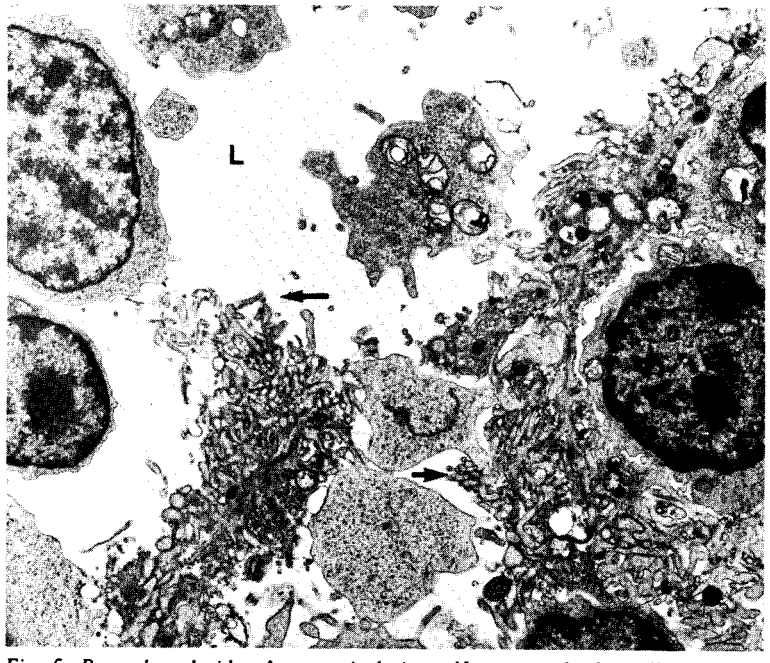

Fig. 5. Parenchymal side of a marginal sinus. Numerous slender cell processes (arrows) resembling those of follicular dendritic cells beneath the sinus wall or protruding in the lumen (L) of the sinus. PGL. Original magnification: x4,500.

follicles by a group of nonphagocytic non-lymphoid cells. Macrophages are probably also involved in the transport of HIV-1. We found macrophages with HIV enclosed in vacuoles in the submarginal region *(Fig. 7)* and in the GC (9). The ultrastructure of these cells was well preserved without signs of cellular damage.

We detected infected cells in the submarginal area also by *in situ* hybridization. The majority of cells expressing viral RNA were, however, localized in the GC. We found no labeled cells in or around the high endothelial venules, although morphologic evidence of an enhanced lymphocytic traffic through these vessels was regularly seen in PGL. This finding does not exclude the possibility that a small number of infected cells corresponding to that in blood circulation (1) may migrate through high endothelial venules. Overall results suggest, however, strongly that most of the cells undergoing viral replication come to the lymph node via afferent lymphatics.

Fig. 6. Follicular dendritic cells reaching the marginal sinus (arrows). PGL. Immunoperoxidase reaction with monoclonal antibody Ki-M4. Original magnification: x100.

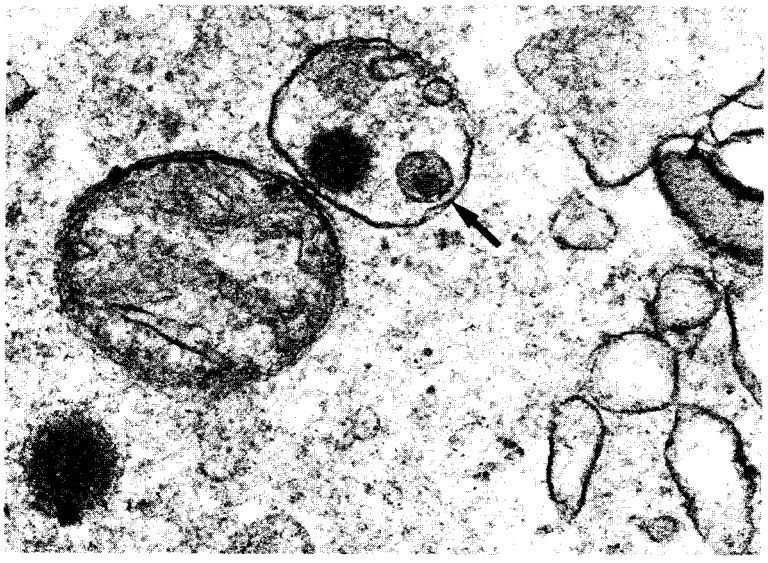

Fig. 7. Detail of a submarginal macrophage with a virion (arrow) located in an intracytoplasmic vacuole. PGL. Original magnification: x30,000.

CONCLUSIONS

Combined morphologic methods provide evidence that lymphatics are an important pathway in the spread of HIV-1 infected cells and may be involved in transporting virus at the initial infection. Unless HIV-1 gains direct access to blood by inoculation or through wounds, it may enter the body through lymphoepithelium overlying the mucous membrane associated lymphoid tissue (MALT), which is especially well developed in the gut. There is a large body of data supporting that lymphoepithelium of the MALT transports antigens and micropathogens to the underlying lymphoid tissue (42-48).

Because the transported material remains in vacuoles and is delivered to the lymphoid tissue without entering the cytosol of lymphoepithelial cells, it is possible that these cells are able to take up HIV-1 even if they do not have the CD4 receptor. Once delivered in lymphoid tissue, HIV-1 could interact with permissive target cells and disseminate through efferent lymphatics of MALT which drain to regional lymph nodes. Suitable experimental models would be necessary to substantiate this hypothesis.

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