ENDOCYTIC STRIPPING OF LIGANDS FROM MIGRANT LYMPHOCYTES IN HIGH ENDOTHELIAL VENULES (HEV): IMPLICATIONS FOR IMMUNOMODULATION vs VIRAL PATHOGENESIS

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INTRODUCTION

"Grooming" of membrane detritus from lymphocyte surfaces by mononuclear phagocytes during migration through lymphatic tissues was proposed as a cause of differences in the potency of comparable populations of lymphocytes to produce the normal lymphocyte transfer reaction (LTR) when equivalent numbers of T cells from blood, afferent lymph, and efferent lymph were injected SC\(^1\). Three- to five-fold greater LTR activity was found with efferent lymph cells compared to blood or afferent lymph cells. It is possible that these differences in reactivity were due to anti-idiotypic antibodies or other "blocking factors" occupying lymphocyte receptors. Isotypic, allotypic and idiotypic immunoregulation occurs in mice, rats, rabbits, and man\(^2-5\). The present studies employ light and electron microscopy, cytochemistry and immunocytochemistry to analyze the endocytic potential of Lewis Rat HEV endothelium toward both particulates and toward autologous lymphocytes whose surfaces have been modified by liganding. Results indicate that HEV endothelial cells nonspecifically remove receptor-blocking proteins during blood to tissue transit.

MATERIALS AND METHODS

Male, Lewis Rats of 180-200g body weight were used in these studies. Outbred Rabbits were used for antibody production. Colloidal carbon, inactivated Pneumococci type 25, zymosan, glutaraldehyde-fixed sheep erythrocytes (SRBC), rabbit anti-thymocyte serum (ATS)-treated thoracic duct lymphocytes and chicken erythrocytes were suspended in tissue culture media and injected retrograde into the surgically-exposed brachial arteries in order to perfuse the axillary lymph node vasculature. The axillary...

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the author(s) do not purport to reflect the positions of the Department of the Army or the Department of Defense.
Table 1. RETICULOENDOTHELIAL FUNCTION OF HEV FOLLOWING I.V. INFUSION

<table>
<thead>
<tr>
<th>Agent</th>
<th>Lymph node HEV</th>
<th>Blood Monocytes</th>
<th>Liver and Spleen Phagocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colloidal Carbon</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Type 25 Pneumococci</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Zymosan Particles</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Saccharomyces Cerevisiae</td>
<td>±</td>
<td>±</td>
<td>+++</td>
</tr>
<tr>
<td>Sheep Red Blood Cells</td>
<td>+</td>
<td>±</td>
<td>ND</td>
</tr>
<tr>
<td>Chicken Red Blood Cells</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Autologous Lymphocytes</td>
<td>-</td>
<td>±</td>
<td>ND</td>
</tr>
<tr>
<td>+ Anti-Immunglobulin</td>
<td>-</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>Autologous Lymphocytes + Anti Thymocyte Serum</td>
<td>++</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
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Lymph nodes were removed at 30 min, 3 hours and 18 hours after perfusion and either snap frozen for histochemistry or fixed in phosphate buffered glutaraldehyde for transmission electron microscopy. Lymph nodes from HIV virus-infected patients (from ref. 6) were also examined by immunohistochemistry for viral antigen and lymphocyte surface receptors. Thoracic duct lymph (TDL) was obtained via surgically prepared Bollman fistulae as described elsewhere. Aliquots of 3 x 10⁸ Fresh TDL were washed and resuspended in media containing 3H-Uridine 10 mcI/ml and incubated for 30 minutes at 37°C, chased with cold Uridine, washed and resuspended in aliquots of Alcian blue dye 2-200 µg/ml, Concanavalin A 1-5 µg/ml, Rabbit anti-rat Immunoglobulins (IgG fraction) FAB₁, or Rabbit anti-rat thymocyte serum (IgG fraction) FAB₁, and then infused intravenously (i.v.). Lymphatic tissues were removed, dissolved in scintillant and the percent of injected CPM calculated. TDL treated with Alcian blue dye or ferritin or horseradish peroxidase (HRP) conjugated FAB₁ antibody fragments of anti-immunoglobulins and anti-thymocyte serum. Autologous Lewis rat peripheral blood lymphocytes were collected on Percoll gradients after lysing erythrocytes and surface ¹²⁵I iodinated using lactoperoxidase (11). Axillary lymph nodes of these rats were excised, fixed and processed for EM peroxidase cytochemistry or autoradiography.

RESULTS

Endothelial cell "coated pits" were frequently found associated with sites of membrane contact with untreated lymphocytes emigrating along interendothelial spaces in HEV (Fig. 1a). Lymphocyte contacts were in the form of microvillus-pit interactions or adhesion plaques. The endothelial cells of HEV exhibited modest endocytic activity for colloidal carbon, CRBC and SRBC, whereas intra-arterial infusion of opsonized microorganisms did not reveal significant reticuloendothelial function (Table 1). Carbon was found in vacuoles within most of HEV endothelial cells after intra-arterial injection. Much less carbon and none of the other particles were found in HEV following intravenous infusion of equivalent doses. Carbon particles, Pneumococci, zymosan granules and fixed SRBC were sequestered in Kupffer cells in the liver and macrophages in the red pulp and marginal zone of the spleen after intravenous infusion. Autologous ¹¹¹I-Uridine labeled TDL treated with FAB₁ fragments of anti-immunoglobulin, ATS and alloantisera, Con A and Alcian blue exhibited reduced homing to lymph nodes and spleen without concomitant sequestration in the liver (Table 2). Intact Rabbit anti-rat thymocyte serum resulted in significant reticuloendothelial sequestration in the liver, spleen and in the endothelium of lymph node HEV. Infusions of lymphocytes coated with
monovalent ligands resulted in endocytic but not phagocytic activity by HEV endothelial cells. In vitro treatment of TDL with doses of HRP-labeled FAB fragments prepared from anti-lymphocyte serum and selected ligands, (that permitted reversible suppression of entry into lymph nodes) resulted in stripping of the labeled ligand from the surface of lymphocytes as they emigrated from the blood across HEV (Fig. 1b). The HRP complexes were "zipped" from the lymphocyte surface by coated pits in the endothelial cell membrane while sparing the lymphocyte. In vitro treatment of TDL with higher doses of ATS resulted in endocytosis and destruction of the labeled cells by HEV after intra-arterial infusion (Fig. 2). Fragments of migrating lymphocytes appeared to be in the process of being pinched off by the adjacent endothelial cells (Fig. 1c). In other preparations, active pinocytosis and membrane ruffling was associated with endothelial membranes that were in contact with anti-immunoglobulin-treated lymphocytes. Smooth membrane arrays of tubules were rarely seen emptying into the space between
endothelial cell and lymphocytes that had been treated with ATS (Fig 1d). Lymphocyte grooming was further studied using non-specific lactoperoxidase $^{125}\text{I}$ iodination of peripheral blood lymphocyte surface proteins followed by autoradiography of lymph node sections from rats who received i.v. infusions of surface-labeled lymphocytes. Lactoperoxidase iodination labels endogenous anti-lymphocyte antibodies as well as membrane glycoproteins because freshly isolated peripheral blood lymphocytes label 80-85% with FITC-anti-immunoglobulins before pronase treatment and 15-20% after treatment and reexpression in culture (unpublished data, Anderson and Anderson). Silver grains were found in the autoradiographs above inter-endothelial clefts and lysosomal granules of HEV and perivascular lymphocytes (Fig. 3). There were fewer labeled cells by this method than was seen for peroxidase labeled antibodies. This can be attributed to the
<table>
<thead>
<tr>
<th>Dose (μg/ml)</th>
<th>Alcian Blue Treatment</th>
<th>CON A Treatment</th>
<th>Antibody Treatment</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Percent Viability by Trypan Blue</td>
<td>Lymph Node Immigration</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>20</td>
<td>2</td>
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<tr>
<td></td>
<td>10.4</td>
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<td>4.40</td>
</tr>
<tr>
<td>Control</td>
<td>96.8</td>
<td>17.90</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>96.0</td>
<td>0.30</td>
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<tr>
<td></td>
<td>82.0</td>
<td>0.12</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>96.0</td>
<td>17.70</td>
<td>14.90</td>
</tr>
<tr>
<td></td>
<td>96.4</td>
<td>17.82</td>
<td>6.37</td>
</tr>
</tbody>
</table>

**Table 2. EFFECT OF LIGAND SURFACE BINDING ON LYMPHOCYTE FUNCTION**

**Antibody Treatment**
- FAB-α-IgG; 50
- FAB-α-IgG; 5
- Control Rat Serum
- FAB-ATS; 50
- FAB-ATS; 5

**Immigration**
- Lymph Node
- Spleen

**Notes:**
- %=% injected CPM at 24 hr after 3 x 10⁶ ³H-U lymphocytes.
- ATS = anti-thymocyte serum.

**Fig. 4(a & b).** a. HEV (outlined by small arrows) in a human lymph node from a patient with AIDS® contain immunoperoxidase labeled P2A antigen from HIV. b. Analogous sites in HEV from a deeper section also label with peroxidase tagged OKT-4 monoclonal antibody.
relative instability of the iodine label over the time course of the study. The ferritin labeled Con-A was endocytosed by the lymphocytes. Few of the Con-A treated TDL could be found to be migrating at HEV in EM preparations.

In lymph nodes of selected AIDS cases (Fig. 4), the P24 antigen of HIV virus was found in endocytic vacuoles near Golgi regions of HEV endothelium. Antibodies to the CD4 molecule, expressed on helper T-lymphocytes, labeled identical vacuoles only in HEV from HIV (+) lymph nodes. This suggests that endocytic stripping of lymphocyte CD4 molecules which are receptors for HIV may be a pathogenic mechanism for penetration of virus into HEV endothelium.

DISCUSSION

These data indicate that HEV endothelial cells exhibit active endocytic interactions with recirculating lymphocytes enroute from blood to lymphatic tissue which results in removal of ligands from the lymphocyte surface. In the extreme case, xenogeneic and heavily opsonized cells are destroyed as has been described in earlier studies of the effects of ATS in vivo. Griffin et al. have shown that mononuclear cells participate in removing capped immunoglobulin from B cells incubated with anti-immunoglobulin in vitro. Studies described here indicate that lymphocytes might also be "groomed" during migration into lymph nodes. PAB fragments were used to eliminate Fc receptor- or complement receptor-mediated endocytosis as an explanation for this phenomenon. Endocytic grooming of lymphocyte surfaces by HEV endothelium may be an essential mechanism for restoring responsiveness to idiotype-suppressed circulating cells as they enter lymphatic tissues. Idiotype suppression reverses in time and continuous exposure to anti-idiotype is necessary to maintain suppression. If grooming is occurring, then the intranodal lymphocytes should be more responsive than peripheral blood or spleen cells. Indeed, Charpentier et al. described spontaneous recovery of lymphocytes from idiotype suppression of responsiveness to major histocompatibility antigens quantitated by mixed lymphocyte responses, as might have occurred if lymphocytes were groomed during transit from blood to lymph as proposed by Scollay. In the case of endocytosis of HIV virus, pathogenesis of Acquired Immunodeficiency may follow if HIV destroys HEV's or if antigen-receptor bearing T-cells were denuded of CD4 molecules which are necessary for proper interaction with antigen presenting cells. There are no data presently available about whether HIV replicates in HEV or not.

Endocytosis of receptor complexes at sites of adhesion between migrating lymphocytes and HEV endothelial cells (Fig. 1a) may also represent a mechanism by which the HEV endothelium breaks the chemical bond between the "homing" receptor and its ligand so the lymphocyte can leave the interendothelial space. It is plausible that cleavage of the homing receptor is what we are observing because heterologous antibodies and lactoperoxidase 125I iodination were used to nonspecifically label glycoproteins on lymphocyte surfaces. Liganding receptors may alter or denature the surface glycoprotein so that accessory proteins that preserve native receptor structure are eliminated. The lymphocyte "homing" receptor recognized by MEL-14 may, in fact, be one of the molecular chaperones that have been associated with ubiquitous heat shock proteins. Finally, it is intriguing that the HEV presumably endocytosed HIV virus and its receptor from transmigrating helper T-cells. Could part of the loss of immunity in AIDS patients be due to loss of lymphocyte recirculation?
REFERENCES