Instituto Juan March
de Estudios e Investigaciones

53 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Vascular Endothelium and Regulation of Leukocyte Traffic

Organized by

T. A. Springer and M. O. de Landázuri

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INTRODUCTION

T.A. Springer and M.O. de Landázuri

The circulatory and migratory properties of white blood cells have evolved to allow efficient surveillance of tissues for infectious pathogens and rapid accumulation at sites of injury and infection. Lymphocytes continually patrol the body for foreign antigen by recirculating from blood, through tissue, into lymph, and back to blood. Lymphocytes acquire a predilection, based on the environment in which they first encounter foreign antigen, to home to or to recirculate through that same environment.

The first critical step in lymphocyte migration from circulation into tissue is the adhesion of lymphocytes to vascular endothelium. In lymphoid organs, lymphocyte adherence and transendothelial migration occur at specialized postcapillary vascular sites called high endothelial venules (HEVs). Although HEVs are particularly abundant in the T-cell areas surrounding the B-cell follicles, they serve as the sites of entry both for T and B lymphocytes. In humans, HEVs are found in all secondary lymphoid organs (with the exception of spleen, where lymphocyte emigration occurs via the blood sinusoids in the marginal zone), including hundreds of lymph nodes dispersed in the body, tonsils and adenoids in the pharynx, Peyer's patches in the small intestine, appendix, and small aggregates of lymphoid tissue in the stomach and large intestine. Moreover, HEV-like vessels are observed in chronically inflamed nonlymphoid tissues and are believed to support lymphocyte recruitment in these sites. In contrast to the endothelial cells from other vessels, the high endothelial cells of HEVs have a distinctive appearance, express specialized ligands for lymphocytes and are able to support high levels of lymphocyte extravasation.

The different homing and recirculation behaviours of lymphocytes depend on expression of specific adhesion receptors by lymphocytes, endothelial cells, and tissue cells and on interactions with the extracellular matrix. Expression of these receptors is finely regulated according to cell type, functional state, and anatomical localization, and builds up a complex network in interactions that simultaneously involve several of these receptors

working as "traffic signals" or "postcodes" for lymphocyte migration and homing. There are five main families of adhesion molecules: immunoglobulin superfamily, integrins, selectins, cadherins, and mucin-like molecules.

Many different adhesion systems are known to be subject to regulation, but the most-studied and best-understood class is the integrins. Integrins are known to be regulated at several levels. Modulation of the affinity of the adhesion receptor for ligand (called affinity modulation) is a well-documented mechanism for the activation of platelet aggregation and is thought to underlie activation of leukocyte adhesion. Adhesive strengthening by the clustering of adhesion receptors or by cytoskeletal-dependent processes such as cell spreading is known to be crucial for strong cell attachment, the control of cell growth and cell motility. These regulatory changes occur either in response to intracellular event (hence, sometimes called inside-out signalling), as a result of EC ligand binding (often called postreceptor occupancy events), or in many instances from both.

Regulation of integrin-mediated adhesion may involve conversions among several different states. For example, leukocytes exhibit several different adhesive behaviours as they interact with endothelial cells of vessel wall during homing or extravasation at sites of inflammation. In the now classic three-step model, under the high shear forces present in flowing blood, leukocytes first become tethered and then roll along the vessel surface. When a local signal (for example, a cytokine) is released in their vicinity, they arrest, develop firm adhesion, and then migrate across the endothelium. Until recently, it has been thought that the rolling phase was mediated solely by the selectins, a family of carbohydrate-binding adhesion molecules implicated in leukocyte homing. Arrest and tightening of adhesion are known to result from the activation of leukocyte integrins. However, it has been recently shown that a single type of integrin can mediate all adhesive phases, including the initial tethering and rolling. For example, $\alpha 4\beta 1$ (VLA-4) mediates tethering and rolling on vascular cell adhesion molecule 1 (VCAM-1), an endothelial integrin ligand belonging to the immunoglobulin superfamily. As expected, this integrin can also become activated to bring about arrest and tight adhesion. Thus, prior to activation, the integrin exhibits binding properties that support tethering and rolling.

The HEV endothelium is unique amongst vascular endothelium by virtue of its capacity to recruit large numbers of lymphocytes. In the human body it is estimated that as many as $5x10^6$ lymphocytes extravasate from the blood through HEVs every second. The specificity

and efficiency of this process is explained by specialized features of the HEV endothelium, such as the expression of mucin-like glycoproteins decorated with HEV-specific oligosaccharide. In the future, it will be important to define precisely the molecular mechanisms involved in the induction and maintenance of the specialized HEV phenotype. HEV-like vessels induced by chronic inflammation in extra lymphoid sites appear to be phenotypically and functional similar to HEVs from lymphoid tissues. Thus, a better understanding of the mechanisms controlling development and maintenance of HEVs could provide the basis of a novel therapeutic approaches for the treatment of human chronic inflammatory diseases, including rheumatoid arthritis and inflammatory bowel diseases, in which HEV-like vessels facilitate large-scale influx of lymphocytes, leading to ampliation and maintenance of chronic inflammation.

I. Endothelial cell development and heterogeneity

Chairman: Irving L. Weissman

Vascular Endothelial Growth Factors and Receptors Involved in Angiogenesis

Kari Alitalo, Vladimir Joukov, Katri Pajusola, Arja Kaipainen, Eija Korpelainen, Birgitta Olofsson*, Ralf Pettersson*, Ulf Eriksson*, Eola Kukk, Erika Hatva and Karri Paavonen Molecular/Cancer Biology Laboratory, Haartman Institute, University of Helsinki, POB 21, 00014 Helsinki, FINLAND, and Ludwig Institute for Cancer Research*, Stockholm, Sweden, e-mail: Kari.Alitalo@Helsinki.FI

Angiogenesis, the sprouting of new blood vessels from preexisting ones, and the permeability of blood vessels are regulated by vascular endothelial growth factor (VEGF) via its two known receptors Flt1 (VEGFR-1) and KDR/Flk-1 (VEGFR-2). The Flt4 receptor tyrosine kinase is related to the VEGF receptors, but does not bind VEGF and its expression becomes restricted mainly to lymphatic endothelia during development. We have purified the Flt4 ligand, **VEGF-C**, and cloned its cDNA from human prostatic carcinoma cells. While VEGF-C is homologous with other members of the VEGF/platelet derived growth factor family, it is made as a precursor protein having an extended N-terminus and a C-terminal half containing extra cysteine-rich motifs characteristic of a protein component of silk. VEGF-C is proteolytically processed, and binds and activates Flt4, which we rename as **VEGFR-3**. In addition, VEGF-C stimulated the migration of bovine capillary endothelial cells in collagen gel. VEGF-C is thus a novel regulator of endothelia, and its effects may extend beyond the lymphatic system, where VEGFR-3 is expressed. Another related novel growth factor, **VEGF-B** was also cloned and found to be co-expressed with VEGF and VEGF-C in heart, muscles and less in other tissues. VEGF-B formed cell surface-associated, disulfide-linked homodimers and heterodimerized with VEGF when coexpressed. Our results suggest that VEGF-B has a role in endothelial cell growth and angiogenesis, particularly in muscle.

Tie, one of the receptor tyrosine kinases we have cloned, is expressed in mouse hematopoietic stem cell fractions and in all studied fetal endothelial cells. In transgenic mice the Tie gene promoter directs endothelial specific expression of heterologous genes. Mouse embryos homozygous for a disrupted Tie allele developed severe edema, their microvasculature was ruptured and they died between days 13.5 and 14.5 of gestation. Thus Tie is required during embryonic development for the integrity and survival of vascular endothelial cells, particularly in the regions undergoing angiogenic growth of capillaries, but it is not essential for vasculogenesis. Our results thus demonstrate an increased complexity of signalling for endothelial cell proliferation, migration, differentiation and survival.

Proc. Natl. Acad. Sci. 87: 8913, 1990; 92: 3566, 1995; 93: 2576-2581, 1996, ***EMBO J.*** 10: 1347, 1991; 11: 4273, 1992; 14: 5884, 1995; 15, 290, 1996, ***Blood*** 80: 2548, 1992; 86: 1828, 1995; ***Oncogene*** 8: 2931, 9: 395, 3545, 3683, 1994; ***Circulation***, 93: 1079, 1996; ***Cancer Res.*** 52: 2004, 5738, 1992, 54: 6571, 1994; ***J. Exp. Med.*** 178: 2077, 1993; ***Am. J. Pathol.*** 146: 368, 1995; 148: 763, 1996, ***J. Biol. Chem.***, 268: 6271, 18320, 1994; 271: 1270, 1996, ***Mol. Cell Biol.*** 15: 711, 1995, ***J. Cell Biol.*** 129: 895, 1995.

The Blood-Brain Barrier: Development, Differentiation and Inflammation

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The vascular system of the central nervous system (CNS) is derived from endothelial cells, which have invaded the early embryonic neuroectoderm by a process which is called angiogenesis and which is probably regulated by brain derived factors. Vascular endothelial cell growth factor (VEGF) seems to be a key regulator in the development of the vascular system in the CNS. As VEGF is not uniquely expressed in the CNS, factors other than VEGF must be responsible for the induction of the blood-brain barrier (BBB) specific endothelial characteristics, such as complex tight junctions, low pinocytotic vesicles and the expression of specialized transport systems. These factors are probably provided by the interaction with astrocytes and other cells, which are closely associated with cerebral vessels. The permanent presence of these neuroectodermal signals is a prerequisite for the proper maintenance of the BBB characteristics of cerebral endothelial cells, as these characteristics readily change, when brain endothelial cells are put into culture or during inflammation in the CNS.

Several approaches to study the molecular mechanisms underlying BBB differentiation and maintenance are used in our laboratory and will be discussed. Morphological characterization of the complex tight junctions using freeze fracture analysis revealed that tight junction particles associate with the P-face during BBB differentiation. The molecular characterization of BBB specific molecules revealed a new member of the Ig-superfamily. Furthermore, we investigate the immunological and functional characteristics of the BBB endothelium, focusing on the question whether the specialization of BBB endothelium extends to distinct characteristics in the interaction with circulating leukocytes and the recruitment of leukocytes during inflammation.

We would like to put forward a "2-phase-model" of BBB differentiation, where in a first step of endothelial-neuroectodermal interaction endothelial cells become irreversibly committed to a BBB lineage. A second subsequent step of endothelial-neuroectodermal interaction leads to a fully differentiated BBB phenotype in endothelial cells. This phenotype is reversible, as disturbances of the endothelial-neuroectodermal environment result in loss of BBB characteristics in cerebral endothelial cells.

TITLE: Redox regulation of the expression of ICAM-1 in endothelial cells
by Manuel O. de Landázuri.

The redox status of the cell plays an essential role in regulating signal transduction, transcription factor activity and expression of cell surface molecules. Pyrrolidine dithiocarbamate (PDTC) is a potent antioxidant agent that regulates the expression of transcription factors such as NF-κB and AP-1. In this study, we show that PDTC up-regulated the cell surface expression of intercellular adhesion molecule-1 (ICAM-1) in human endothelial cells (EC), whereas the low baseline expression of vascular adhesion molecule-1 (VCAM-1) or E-selectin was not affected. On the other hand, PDTC delayed initially the up-regulation of ICAM-1 by inflammatory mediators such as TNF-α or LPS but this partial inhibition was progressively reverted and not detectable by 12-16 h of treatment. Under the same conditions, the induction of E-selectin and VCAM-1 by TNF-α or LPS remained inhibited. Further analysis of PDTC-mediated ICAM-1 up-regulation revealed that PDTC increased ICAM-1 mRNA levels and augmented its gene promoter activity. Transfection experiments in EC with reporter constructs harboring nested deletion fragments of the ICAM-1 promoter, indicated the presence of a functional PDTC responsive region located between positions -136 to -353 of the promoter. Gel retardation assays together with supershift analysis revealed that PDTC induced the binding of c-fos and c-jun to a consensus AP-1 binding site located at position -284. PDTC alone or in combination with TNF-α enhanced AP-1-dependent transactivation in HUVEC as determined by DNA-binding assays. The functional implication of AP-1 in the transcription of ICAM-1 gene was further demonstrated by cotransfection experiments in which a c-jun expression vector induced the promoter activity of the PDTC responsive element of the ICAM-1 promoter. Taken together, these results indicate that the antioxidant PDTC induces transcriptional activation of ICAM-1 and that this induction is mediated at least in part by the transcription factor AP-1. This mechanism might be operative in pathological conditions in which a redox imbalance plays

DEVELOPMENT OF T AND B CELLS IN THE BONE MARROW REQUIRES α4 INTEGRINS.

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To elucidate the roles played by the integrins α4β1 and α4β7 during *in vivo* hematopoiesis, chimeric mice carrying $\alpha4^{-/-}$ cells were generated. T lymphocytes were present in normal percentages in the blood and spleen from $\alpha4^{-/-}$ chimeric animals, and were α4 null phenotypically and genotypically. $\alpha4^{-/-}$ T lymphocytes were absent from the Peyer's patches of the chimeric animals. Interestingly, thymi from $\alpha4^{-/-}$ chimeric mice became depleted of α4 null thymocytes and atrophic one month after birth suggesting a problem at the T cell precursor level. However, the bone marrow from $\alpha4^{-/-}$ chimeras was able to short-term reconstitute the T cell compartment in sublethally irradiated recipients ruling out an inability of $\alpha4^{-/-}$ T cell precursors to migrate or differentiate in the thymus and suggesting a defect at the bone marrow level. Only a few $\alpha4^{-/-}$ B lymphocytes could be detected in the blood, lymphoid organs, and peritoneal cavity of $\alpha4^{-/-}$ chimeric mice. The bone marrow from these $\alpha4^{-/-}$ chimeras showed a greatly decreased percentage of CD45R(B220)+ cells mostly immature B cells, demonstrating an early defect at the B cell precursor level. Populations of monocytes and natural killer cells (NK) lacking α4 integrins were also present in the blood of the $\alpha4^{-/-}$ chimeric mice. These data show that there is a switch in the requirements for α4 integrins during lymphopoiesis; α4 is essential for normal development of lymphoid (T and B) precursor cells in the bone marrow during adult life but not during embryonic lymphopoiesis, and α4 is also essential for T cell homing to gut-mucosal associated lymph tissue. In contrast, normal myelopoiesis and NK development can occur in the absence of α4 integrins regardless of the age of the animal.

THE CRYSTAL STRUCTURE OF SOLUBLE ICAM-2

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Intercellular adhesion molecule-2 (ICAM-2) (CD102) is a cell surface glycoprotein expressed on hematopoietic and endothelial cells that binds to lymphocyte function-associated antigen-1 (LFA-1). ICAM-2 is the major LFA-1 ligand on unstimulated lymphocytes or endothelium, suggesting that ICAM-2 may be important for normal lymphocyte recirculation and initial T cell adhesion with antigen presenting cells.

The extracellular portion of human ICAM-2 (soluble ICAM-2, sICAM-2) has two immunoglobulin-like domains and six potential N-linked glycosylation sites. Domains 1 and 2 of human ICAM-2 are highly homologous with domains 1 and 2 of human ICAM-1 (34% identity) or ICAM-3 (37% identity). Domain 1 of ICAM-2 contains an extra disulfide bond characteristic of a subclass of the Ig-superfamily, which also comprises ICAM-1, -3, MadCAM-1, and VCAM-1. Therefore, it is expected that domain 1 of all these molecules will be structurally similar. There is also functional similarities between the members of this Ig-superfamily subclass because all these receptors bind to leukocyte integrins and mediate cell adhesion interactions essentials in immunity.

We expressed and crystallized a sICAM-2 fragment containing the extracellular portion of the molecule. The three-dimensional structure of the protein was determined by X-Ray crystallogrphy using the Multiple Isomorfous Refinament (MIR) method. The crystal structure of sICAM-2 will be presented and discused.

II. Leukocyte traffic and selectins
Chairman: Timothy A. Springer

Glycoprotein ligands of the endothelial selectins

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The Selectins are carbohydrate-binding cell adhesion molecules acting in the vascular system. They mediate the docking of leukocytes to the blood vessel wall and the rolling of these cells along the endothelial cell surface. These adhesion phenomena initiate the entry of leukocytes into sites of inflammation. Blocking selectin function with antibodies or oligosaccharides has proven to be beneficial in various animal models of inflammation and models of ischemia/reperfusion damage. This has raised much interest in the identification of the physiological ligands of the selectins.

Analysing mouse myeloid cells, we have searched for glycoprotein ligands for the two endothelial selectins - E- and P-selectin - using Selectin-Ig fusion proteins as affinity probes. We found a 230/130 kD pair of proteins, which bound to both selectins and most likely is the homolog to human PSGL-1 (1). In addition, we found an E-selectin-specific ligand of 150 kd which did not bind to P-selectin and which we named ESL-1 (2,3). Cloning of ESL-1 allowed to generate an ESL-IgG fusion protein, which - if fucosylated - supported adhesion of E-selectin-transfected CHO cells. Polyclonal antibodies against the ESL-IgG fusion protein blocked binding of the myeloid cell line 32Dcl3 and of mouse PMNs to E-selectin.

The complete amino-acid sequence of the mature ESL-1 protein is 94% identical to a recently identified chicken cysteine-rich FGF receptor (CFR), except for a 70 amino acid domain at the N-terminus, replacing the first 35 amino acids of the N-terminus of CFR. These data suggested that mouse ESL-1 might be a splicing variant of the mouse homolog of chicken CFR. Searching for such a variant, we have found a spliced form of mouse ESL-1 by PCR in embryonal mouse brain (day 13), which lacks the entire Pro-Gln rich region. The function of the spliced domain is still under investigation. Although antibodies against ESL-1 recognize a 150 kD protein in many different, non-myeloid cell types, in none of these cells could the protein be affinity isolated with E-selectin-IgG. Thus, while the glycoform of ESL-1, which is able to bind to E-selectin, is very specifically expressed, the protein scaffold shows a broad distribution and serves yet another function.

Fucosylation of ESL-1 seems to be the essential glycosylation step which makes ESL-1 an E-selectin ligand. Endogenous "ESL-1" in CHO cells, as well as transfected mouse ESL-1, only bind to E-selectin if co-expressed with a fucosyl transferase (FucT). We have expressed different human FucTs (obtained from Dr. Mike Bird, Glaxo; and from Dr. John Lowe, Univ. of Michigan) in CHO cells and have analysed cell lysates of these cells in

affinity isolation experiments with E-selectin-IgG. Expression of each of FucT III, IV, V, VI and VII allowed to generate an E-selectin binding form of ESL-1. However, not only ESL-1, but a rather large panel of glycoproteins could be affinity isolated with E-selectin-IgG from Fuc T III-expressing cells. In contrast, ESL-1 was the only ligand which could be affinity isolated from FucT VII-expressing cells. In cell extracts of FucT IV, V and VI transfected CHO cells, ESL-1 was the major but not the exclusive ligand for E-selectin. Thus, human FucT III can artificially transform many glycoproteins in CHO cells into "ligands" for E-selectin, while FucT VII exclusively generates one E-selectin ligand and that is ESL-1. It will be important to elucidate the structural determinants on ESL-1 and the molecular mechanisms which specify this exclusiveness.

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Title: Selectin-mediated neutrophil and lymphocyte adhesive interactions under flow.

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Selectins are a family of leukocyte, endothelial cell, and platelet adhesion molecules that regulate adhesive interactions during blood flow which are important in effective host inflammatory events and in regulating constitutive lymphocyte trafficking. Two selectins (E- and P-selectin) are expressed by inflamed endothelial cells, with P-selectin also being expressed by thrombin-activated platelets. A third selectin, L-selectin, is expressed by most circulating leukocytes. Specific carbohydrate moieties that decorate limited numbers of glycoprotein (or possibly glycolipid) scaffolding structures serve as selectin ligands on target cells. E- and P-selectin have been shown to mediate adhesive interactions of myeloid cells and certain lymphocyte subsets under physiological flow which results in a rolling interaction of the cell along the vessel wall. L-selectin on myeloid cells and lymphocytes mediates a similar type of rolling interaction of these cells along inflamed vessels and vessels found in secondary lymphoid tissues, such as peripheral lymph nodes. Selectin-dependent rolling represents the first step of the extravasation process, which is followed by specific signaling events, stopping of the cell, and eventual transendothelial migration. Lymphocytes, which can roll on E- or P-selectin, represent specific subsets, defined by a memory phenotype and/or expression of CLA. At birth, virtually all bovine γ/δ T cells have the capacity to roll on E-selectin; whereas, 40-60% of the cells can roll on P-selectin. In contrast, virtually none of the α/β T cells in newborns interact with either selectin. O-sialoglycoprotease, an enzyme which selectively cleaves mucin-like glycoproteins, abrogates γ/δ T cell rolling on P-selectin, but has minimal effect on rolling on E-selectin. Preliminary data suggest that the γ/δ T cell ligand for P-selectin is similar to P-selectin glycoprotein ligand-1 (PSGL-1), previously described on human neutrophils. The E-selectin ligand is molecularly distinct from PSGL-1. Affinity isolation using recombinant human E-selectin immobilized on Sepharose 4B results in the purification of a predominant 240-250 kD glycoprotein from bovine γ/δ T cell detergent lysates. An E-selectin/Ig chimera selectively stains γ/δ T cells in flow cytometric analysis and reacts with 4 glycoproteins in Western Blots of γ/δ T cell detergent lysates, including the 240-250 kD molecule. Cell surface biotin labeling experiments show that the 240-250 kD molecule as well as a second molecule at 180 kD are expressed on the cell surface of the γ/δ T cell. Monoclonal antibodies raised against a gel purified 240-250 E-selectin ligand, preferentially stain γ/δ T cells. These large molecular weight glycoproteins may represent the first defined E-selectin ligands unique to lymphocytes.

During our analysis of leukocyte-selectin interactions under flow, we found that once leukocytes become immobilized along endothelial cells, they themselves can support continued rolling of newly arriving leukocytes. This leukocyte on leukocyte rolling interaction occurs with both neutrophils and lymphocytes and may represent an amplification phase of the extravasation process. Anti-L-selectin mAbs completely block leukocyte on leukocyte rolling. L-selectin mediates these interactions by binding O-sialoglycoprotease-sensitive molecules, perhaps PSGL-1, which present carbohydrates moieties requiring sialic acid and fucose. Data supporting these findings will be summarized.

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Interactions of PSGL-1 with Selectins

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Under shear stress, the initial rolling of leukocytes on endothelial cells, platelets, or other leukocytes requires interactions of selectins with cell-surface glycoconjugates. The expression of the selectins is controlled by a variety of mechanisms. During acute inflammation, P-selectin is rapidly redistributed from secretory storage vesicles to the endothelial or platelet cell surface and then rapidly internalized. During allergic or chronic inflammation, P-selectin has been observed on the apical surface of endothelial cells for prolonged periods. TNF-α or IL-1β increase transcription of P-selectin mRNA in murine but not human endothelial cells. In contrast, IL-4 or oncostatin M increases transcription of P-selectin mRNA in human endothelial cells, suggesting that these cytokines may facilitate sustained expression of P-selectin during allergic or chronic inflammation. P-selectin mediates tethering and rolling of leukocytes through interactions with PSGL-1, a mucin-like glycoprotein expressed on the microvilli of leukocytes. P-selectin appears to bind preferentially to an N-terminal region of PSGL-1 that includes at least one tyrosine sulfate and at least one sialylated and fucosylated, core-2 O-glycan. L-selectin also appears to bind to this site, although with different kinetics. In contrast, E-selectin binds to one or more additional sites on PSGL-1. Remarkably, only a minority of the O-glycans on PSGL-1 are fucosylated, and some of these have novel structures.

CONVERSION OF CD4$^+$ T CELLS FROM NAIVE TO MEMORY CONFERS ROLLING ON E- AND P-SELECTINS UNDER FLOW.

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Compared to naive T cells, memory T cells preferentially interact with peripheral activated endothelium. On 6 hr TNF-α activated human umbilical vein endothelial monolayers, adhesion of CD4$^+$ CD45RO$^+$ (memory) was significantly higher than CD45RA$^+$ (naive) cells. The current study examines the molecular mechanisms which underlie the observed preferential interactions of memory CD4$^+$ T cells with activated HEC monolayers, focusing on the role of both E- and P-selectin using an *in vitro* flow chamber (J. Exp. Med. 181:1179, 1995) and CHO cell monolayers expressing human E- or P-selectin (CHO-E and CHO-P, respectively). Under flow, memory T cells rolled extensively at low velocity on both CHO-E and CHO-P monolayers, whereas very few if any naive T cells interacted with these monolayers. Purified CD4$^+$ CD45RA$^+$ could be converted to RO$^+$ cells by mitogen stimulation and 7 day culture *in vitro*, and this correlated with the acquisition of E- and P-selectin dependent rolling under flow. RT-PCR analysis was performed to determine the presence of mRNA encoding two glycosyltransferase enzymes, Core 2 and α1,3-fucosyltransferases (FUC-T), implicated in the biosynthesis of sialylated Lewis ^{x} determinants (sLe^x). The results revealed no difference between CD4+ naive and memory T cells for expression of genes encoding Fuc-T III, IV, VI, VII expression or Core II transferase. In conclusion, memory CD4$^+$ T cells interact with E- and P-selectin much more effectively than do naive CD4$^+$ T cells under flow conditions, and this may contribute to the preferential recruitment of memory T cells to peripheral sites of inflammation. The ability of T cells to interact with vascular selectins under flow appears to be a characteristic induced by T cell receptor stimulation.

Mobilization of stem cells, developmental switches in homing receptor and addressin expression, and a role for homing receptors in the invasive and extravasation stages of solid tumor metastases.

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In adult mice lymphocytes expressing integrin $\alpha4\beta7$ home to Peyer's Patches (PP) by binding to and rolling on PP HEV MAd CAM-I, while lymphocytes bearing L-selectin bind to and roll on LN HEV PNAd. During late fetal and perinatal development the only LN addressin is MadCAM-1, and $\alpha4\beta7^+$, but not $\alpha4\beta7^-$ (L-selectin$^+$) cells are admitted, despite the rarity of $\alpha4\beta7^+$ and the great abundance of L-selectin$^+$ cells in fetal and neonatal blood. Begining at day 1 after birth concomitantly in all LN's PNAd expression is initiated; postnatal day 7-14 MadCAM-1 disappears from LN HEV. Two rare "fetal program" lymphocytes populations populate the fetal/neonatal LN's: $\alpha4\beta7^+$ TCRVγ2 cells and $\alpha4\beta7^+$ CD4$^+$3$^-$ cells, CD4$^+$3$^-$ cells lack Ig or TCR rearrangements, are mainly IA$^+$ ICAM-1$^+$ and express surface LTβ. We propose that CD4$^+$3$^-$ cells are involved in the formation of lymph node vasculature and architecture, while TCR Vγ 2 cells play other regulatory roles.

Cytoreductive agents and cytokines such as G-CSF, GM-CSF,etc. can induce the appearance of hematopoietic stem cells (HSC) and other hematopoietic progenitors in the blood of mice, dogs, and all primates tested. With Miyake and Kincade we had shown previously that hematolymphoid progenitors bind to bone marrow (BM) and thymic stroma via hematopoietic cell integrin $\alpha4\beta1$ and stromal cell VCAM-1. Here we show that cyclophosphamide /G-CSF treatment results in a rapid mobilization of HSCs from BM→blood→ spleen, followed by massive expansion of HSC numbers in BM and spleen. The "mobilized" HSCs either lose expression of integrin $\alpha4$ or express surface $\alpha4$ that has lowered affinity for plate -bound VCAM-1; pre-mobilization BM HSC's all express high levels of integrin $\alpha4$ (but not $\beta7$), and bind avidly to VCAM-1. Most late mobilized HSCs begin to express high levels of L-selectin.We propose that downregulation of $\alpha4(\beta1)$ leads to de-adherence from stromal cells, and perhaps that upregulation of L-selectin leads mobilized HSCs to bind to vascular sulfoglycoproteins such as CD34 preparatory to reestablishing HSC niches for post-treatment hematopoiesis.

The B16 melanomas in mice have been passaged selectively to identity clonal variants that have high or low pulmonary metastatic efficiency. We have shown that a class of high metastatic variants do not express integrin $\alpha4$, but express $\beta1$ mRNA, while other variants that grow at the same rate subcutaneously are far less metastatic. Transfection of $\alpha4^-$ high metastatic variants with $\alpha4$ transgenes reproducibly generates neoplasms that grow locally but metastasize poorly. Both $\alpha4+$ and $\alpha4^-$ B16 variants give equivalent pulmonary metastases when injected i.v., and so the likely difference between variants is pre-hematogenous, or involves local events that allow invasion. We showed that $\alpha4\beta1^+$ melanoma cells aggregate <u>in vitro</u> in liquid culture and on matrigel surfaces at high cell density. Invasion

of matrigel occurs with both $\alpha 4^+$ and $\alpha 4^-$ melanomas at low cell density, but only by $\alpha 4^-$ melanomas at high cell density. MAbs that are directed to $\alpha 4$ and block cell-cell adhesion allow $\alpha 4^+$ melanomas to invade matrigel in vitro and to generate pulmonary metastases in vivo. As $\alpha 4^+$ melanomas are fibronectin and VCAM-1 negative, homotypic / homophilic interactions that lead to in situ tumor cell adhesion seems most likely to be responsible for the reduced invasive property. In this view, spontaneous $\alpha 4^-$ (or $\beta 1^-$) variants would be free to detach, a condition that allows (but does not wholly determine) invasion.

Can lymphocyte homing receptors play a role in site-specific metastasis following hematogenous spread ? With Douglas Hanahan (UCSF) we crossed transgenic mice from his lab expressing SV40 T antigen driven by the insulin promoter with mice prepared by us that express lac Z and L-selectin also using the rat-insulin promoter. L-selectin negative transgenic SV40 T^+ mice regularly develop pancreatic islet cell adenocarcinomas that grow locally, metastasize to liver, but not distant lymph nodes. Insulin promoter L-selectin positive / SV40 T positive mice virtually all additionally have distant LN metastases. Since L-selectin expression is unlikely to affect invasive capacity, we believe these results indicate: a) these tumors do spread to the bloodstream; and b) L-selectin expression may be sufficient for at least some stages of LN localization by nonlymphoid cells. These results are obviously from a highly manipulated model, but should alert investigators to the posibility that even nonlymphoid tumors might inappropriately activate lymphocyte homing receptors and thereby gain new metastatic sites.

Abbreviaton's (in order used)

PP	Peyer's patches
HEV	High endothelial venule
MAd CAM-1	Mucosal addressin cell adhesion molecule 1
LN	Lymph node
HSC	Hematopoietic stem cells
BM	Bone marrow
VCAM-1	Vascular cell adhesion molecule 1

III. Endothelial cell junctions and transmigration.

Chairman: Charles R. Mackay

REGULATION OF ENDOTHELIAL CELL JUNCTION ORGANIZATION BY POLYMORPHONUCLEAR CELL ADHESION

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The endothelium forms the main barrier to the passage of macromolecules and circulating cells from blood to tissues. Endothelial permeability is in large part regulated by intercellular junctions. These are complex structures formed by transmembrane adhesive molecules linked to a network of cytoplasmic/cytoskeletal proteins. At least four different types of endothelial junctions have been described (*tight junctions, gap junctions, adherence junctions and complexus adherentes*). These organelles have some features and components in common with epithelial cells but also some which are specific for the endothelium. Interendothelial junctions present a different degree of complexity along the vascular tree responding to different functional requirements. They are well organized and numerous in large arteries or in the blood vessels of the brain where the control of permeability must be strict, whereas they are very primitive and almost disappear in the post capillary venules, where cell extravasation and exchange of plasma constituents need to be particularly efficient. The mechanisms that regulate the opening and closing of endothelial junctions are still obscure. It is conceivable that inflammatory agents increase permeabilty by binding to specific receptors generating intracellular signals which in turn cause cytoskeletal reorganization and opening of interendothelial cell gaps. Endothelial junctions also control leukocyte extravasation. Once leukocytes have adhered to the endothelium, a coordinated opening of interendothelial cell junctions occurs. The mechanism by which this takes place is unknown but it might present characteristics similar to that triggered by soluble mediators.

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TRANSENDOTHELIAL MIGRATION OF MONONUCLEAR PHAGOCYTES IN VITRO: Regulation of their accumulation within and clearance from connective tissue. Martha B. Furie, JoAnn Meerschaert, and Gwendalyn J. Randolph. Department of Pathology, School of Medicine, State University of New York at Stony Brook, Stony Brook, New York, 11794-8691, USA.

Mechanisms that control the migration of mononuclear phagocytes (MP) across vascular endothelium were explored using an *in vitro* model of the vessel wall. This model consisted of human umbilical vein endothelial cells (HUVEC) cultured to confluence on connective tissue substrates prepared from human amniotic membrane. Purified human peripheral blood monocytes placed above the apical surface of HUVEC-amnion cultures began to adhere to and migrate across the endothelium within minutes. However, 2 hours were required for maximum numbers of MP (~50% of those added) to traverse the HUVEC monolayer. Stimulation of the HUVEC with interleukin-1β (IL-1β) prior to addition of the leukocytes increased the rate at which MP adhered to the endothelium, but had no effect on the kinetics of their migration. To determine the fate of these emigrated leukocytes, MP were allowed to enter HUVEC-amnion cultures for 2 hours, nonadherent leukocytes were washed away, and incubation was continued. With time, the number of MP within the amniotic tissue decreased, and the numbers adherent to the apical surface of the endothelium or suspended in the apical medium increased. These data indicated that MP that had emigrated into HUVEC-amnion cultures later exited by re-traversing the endothelium in the basal to apical direction. Indeed, over a 4 day period, as many as 80% of MP initially within cultures subsequently exited, with a $t_{1/2}$ of approximately 24 hours. To determine whether exiting occurred in a diluting environment, as would be afforded by flowing blood, HUVEC-amnion cultures containing MP in the connective tissue were suspended in a large volume of stirred medium. Monocytes still exited from the cultures under these conditions, supporting the possible physiological relevance of this phenomenon. However, the rate of exiting was slowed, suggesting that a soluble factor may be involved. In contrast, the initial rate of exiting was accelerated when HUVEC were first treated with IL-1β.

Blocking monoclonal antibodies (MAbs) were used to identify adhesion molecules that participated in the trafficking of MP into and out of HUVEC-amnion cultures. MAbs to CD18 or CD11a greatly inhibited entrance of MP into HUVEC-amnion cultures when the monocytes were incubated with unstimulated endothelium for 20 minutes. Much less inhibition was observed when the incubation period was extended to 2 hours or when HUVEC were pretreated with IL-1β. A MAb to VLA-4 had little or no effect under any of these conditions. However, a combination of MAbs to CD18 and VLA-4 greatly suppressed (from 70% to 98%) entrance of MP into HUVEC-amnion cultures, regardless of the time of incubation or whether the endothelium was first stimulated with IL-1β. Thus, MP are able to traverse endothelium in the apical to basal direction via either CD11/CD18- or VLA-4-dependent pathways. Although CD11a/CD18 and CD11b/CD18 are known to bind to ICAM-1, results of antibody-mixing experiments indicated that alternative ligands may also be used for entrance of MP into endothelial-amnion cultures. In contrast, exiting of MP from HUVEC-amnion cultures was highly dependent on ICAM-1. To analyze use of adhesion molecules during exiting, MP were allowed to migrate into IL-1-pretreated HUVEC-amnion cultures for 2 hours. Nonadherent leukocytes were washed away, MAbs were added, and incubation was continued for an additional 3 hours. MAbs to ICAM-1 or CD18 inhibited exiting of MP from such cultures by 100% and

71%, respectively, whereas MAbs to VCAM-1 or E-selectin were without effect. Immunostaining experiments verified that ICAM-1 was present on the basal surfaces of HUVEC both *in vitro* and *in situ*. Inhibition by the MAb to ICAM-1 decreased to only 53% when the period of incubation was extended to 10 hours, suggesting that alternative adhesion pathways for exiting are upregulated with time.

The role of chemoattractants in trafficking of MP in this model system was also assessed. Medium conditioned by unstimulated or IL-1β-stimulated HUVEC contained a soluble chemotactic activity for MP, and this activity was completely ablated by a neutralizing MAb to monocyte chemoattractant protein-1 (MCP-1). Moreover, MAb to MCP-1 prevented apical-to-basal migration of MP across unstimulated or IL-1β-stimulated HUVEC grown on amnion by 74% and 45%, respectively. Addition of MCP-1 itself to the apical compartment of unstimulated HUVEC-amnion cultures also reduced entrance of MP, but only when it was present at a concentration equal to or greater than that endogenously deposited beneath the endothelium. As much as 90% of the MCP-1 deposited by the HUVEC into the underlying basement membrane and connective tissue could be washed away, and migration of MP into these washed cultures was reduced by 69% as compared to unwashed controls. These data indicate that MCP-1 promotes the entrance of MP into HUVEC-amnion cultures by establishing a predominantly soluble, rather than haptotactic, concentration gradient. Surprisingly, MAb to MCP-1 had no effect on exiting of MP from the endothelial-amnion cultures.

It is clear, then, that different mechanisms govern the initial migration of MP across endothelium in the apical to basal direction and their eventual re-traversal in the opposite direction. Entrance of MP into HUVEC-amnion cultures utilizes a variety of adhesion mechanisms and is, at least in part, mediated by MCP-1. Exiting, in contrast, is highly dependent on ICAM-1 and does not involve MCP-1 at all. Whether these same mechanisms operate *in vivo* remains to be determined.

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CHEMOKINES REGULATE CELLULAR POLARIZATION AND ADHESION RECEPTOR REDISTRIBUTION DURING LYMPHOCYTE INTERACTION WITH EC AND ECM

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Leukocyte recruitment is a key step in the inflammatory reaction. During lymphocyte migration T cells become polarized, developing a well defined cytoplasmic projection designated as cellular uropod. We found that the chemokines RANTES, MCP-1, MIP-1α, MIP-1β and IL-8, were able to induce uropod formation and ICAM-3 redistribution in T lymphoblasts adhered to ICAM-1 or VCAM-1. A similar effect was observed during T cells binding to the fibronectin fragments of 38 and 80-kD, that contain the binding sites for the integrins VLA-4 and VLA-5, respectively. The uropod structure concentrated the ICAM-3 adhesion molecule (a ligand for LFA-1), and emerged to the outer milieu from the area of contact between lymphocyte and protein ligands. Other adhesion molecules such as ICAM-1, CD43, and CD44, also redistributed to the lymphocyte uropod upon RANTES stimulation, whereas other cell surface receptors did not redistribute. Chemokines displayed a selective effect among different T cell subsets; MIP-1β had more potent action on $CD8^+$ T cells and tumor infiltrating lymphocytes (TIL), whereas RANTES and MIP-1α targeted selectively $CD4^+$ T cells. Several cAMP agonists were able to induce uropod formation and ICAM-3 redistribution, whereas H-89, a specific inhibitor of the cAMP-dependent protein kinase, abrogated the chemokine-mediated uropod formation, thus pointing out a role for cAMP-dependent signaling in this process. Since the lymphocyte uropod induced by chemokines was completely abrogated by *Bordetella pertussis* toxin, the formation of this membrane projection appears to be dependent on G proteins signaling pathways. Uropod formation and ICAM-3 redistribution in response to chemokines was prevented by the myosin-disrupting agent butanedione monoxime. Altogether, these results demonstrate that uropod formation and adhesion receptor redistribution is a novel function mediated by chemokines (1); this phenomenon may represent a mechanism that significantly contributes to the recruitment of circulating leukocytes to inflammatory foci (2).

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IV. Leukocyte-endothelial interactions (I)

Chairman: John M. Harlan

Apoptotic Endothelial Cells are Proadhesive for Leukocytes

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The balance between endothelial survival and death is critical to vascular development and homeostasis. Pertubations of this balance may contribute to pathogenesis of diverse inflammatory and immune processes. Recently, we have examined the molecular basis of endothelial cell survival and death in inflammation, seeking to identify endogenous proteins that "protect" or "execute" (1). Additional studies have addressed the potential consequences of endothelial cell apoptotic death. Necrotic cell death is generally associated with a loss of cell membrane integrity, resulting in the release of cytoplasmic constituents and induction of an inflammatory response. In contrast, during apoptosis there are alterations in the plasma membrane that provoke phagocytosis by neighboring cells; cells not engulfed degenerate into membrane-bound apoptotic bodies. Consequently, apoptosis of most cells is not generally associated with a significant inflammatory response. Vascular endothelium, however, is situated at the interface of blood and tissue and is continuously exposed to circulating leukocytes. We hypothesize that proadhesive changes in endothelial cell membrane during apoptosis may represent a novel pathway of leukocyte recruitment in inflammation.

Several recognition-adhesion pathways involved in this process have been reported in other cell types (2, 3), including: 1) the $\alpha_v\beta_3$ vitronectin receptor on the macrophage surface interacts with apoptotic neutrophils via thrombospondin, which appears to act as a molecular bridge between the two cell types (4, 5); 2) exposure of phosphatidylserine on apoptotic leukocytes leads to an interaction between phosphatidylserine on the apoptotic cell and a putative phosphatidylserine receptor / ligand on the macrophage (6); 3) interaction of murine thymocytes with macrophages is inhibited by monosaccharides, suggesting a lectin-carbohydrate ligand interaction (7); and 4) recognition of apoptotic leukocytes by human macrophages is inhibited by a macrophage-specific mAb which recognizes an antigen distinct from $\alpha_v\beta_3$ (8).

We examined the process of leukocyte recognition/adherence of apoptotic HUVEC and bovine aortic EC. Apoptosis was induced by growth factor deprivation or staurosporine, a non-specific kinase inhibitor that rapidly triggers apoptosis in a wide variety of cell lines. Findings of these studies are summarized as follows:

- Growth-factor deprivation for 24 hours induced adhesion to HUVEC of human peripheral blood mononuclear several leukocytes (PBM) and several leukocyte cell lines.
- Staurosporine treatment of HUVEC or bovine aortic EC induced apoptosis as determined by morphology, TUNEL, and DNA laddering. Staurosporine-induced apoptosis was time- and dose- dependent. Other kinase inhibitors failed to induce apoptosis or adhesion.
- Staurosporine induced a marked increase in mononuclear leukocyte and U937 cell adhesion to HUVEC in a time- and dose-dependent manner, and was not associated with expression of ICAM-1, VCAM-1, or E-selectin.
- Staurosporine induced adhesion was not dependent on *de novo* protein synthesis, as it was observed in the presence of cycloheximide.
- Staurosporine also induced marked leukocyte adhesion to bovine aortic EC, HMEC-1, and human skin fibroblasts but minimal adhesion to human VSMC.

- By scanning electron micoscopy leukocytes bound to cell processes of apoptotic HUVEC, but not to exposed subendothelial matrix.
- Adhesion of PBM and most leukocyte cell lines to staurosporine-treated HUVEC was blocked by pre-treatment of the leukocytes, but not the EC, with an adhesion-blocking β_1 mAb. A blocking β_3 mAb was without effect. Neutrophils, a β_1-negative cell, failed to bind to apoptotic HUVEC.
- U937 cell adhesion to staurosporine-treated HUVEC was inhibited partially by blocking mAbs to α_4, α_5, or α_6 subunits, but not by blocking mAb to α_v or $\alpha_v\beta_3$. The combination of mAbs to α_4, α_5, and α_6, produced comparable inhibition of adhesion to that observed with the β_1 mAb.

These studies demonstrate that EC that are induced to undergo apoptosis by the global protein kinase inhibitor staurosporine or by growth factor deprivation become adhesive for leukocytes by a novel mechanism involving one or more β_1 integrin receptor(s) on the leukocyte and an undetermined ligand(s) on the apoptotic EC.

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Leukocyte migration to tissues. Examples of selective homing based on integrin expression, and chemokine/chemokine receptor expression

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Leukocyte migration to tissues is a highly selective process, mediated by adhesion molecules expressed on leukocytes and on endothelium. One of the best examples of tissue selective homing by a leukocyte subset is the migration of the a4b7 integrin-positive memory T cell subset through mucosal tissues. This is brought about by the expression of an endothelial ligand for a4b7, MAdCAM-1, which restricted to HEV of mucosal lymphoid tissue, as well as endothelium of the lamina propria. The differential expression of chemokines on endothelium, and their receptors on subsets of leukocytes, is another mechanism that has been suggested as a potential mechanism for selective leukocyte recruitment. This may be especially important for eosinophils, which accumulate in mucosal tissuesin a higly selective manner in response to parasitic infection. Eosinophils areOOO not known to express specific adhesion molecules. The chemokine eotaxin is a highly specific chemoattractant for eosinophils. Ligand binding studies with radiolabeled eotaxin demonstrated a receptor on eosinophils distinct from the known chemokine receptors CKR-1 and CKR-2 (1). We have now isolated a cDNA from eosinophils, termed CKR-3, with significant sequence similarity to other well characterized chemokine receptors (2). Cells transfected with CKR-3 cDNA boundradiolabeled eotaxin specifically and with high affinity, comparable to the binding affinity observed with eosinophils. This receptor also bound RANTES and MCP-3 with high affinity, but not other CC or CXC chemokines. Furthermore, receptor transfectants generated in a murine B cell lymphoma cell line migrated in transendothelial chemotaxis assays to eotaxin, RANTES, and MCP-3, but not to any other chemokines. A monoclonal antibody recognizing CKR-3 was used to show that eosinophils but not other leukocyte types expressed high levels of this receptor. This pattern of expression was confirmed by Northern blot with RNA from highly purified leukocyte subsets. An analysis of tissues with prominent eosinophil influx showed expression of eotaxin, particularly by leukocytes, and also by endothelial cells. The restricted expression of CKR-3 on eosinophils, and the fidelity of eotaxin binding to CKR-3, provide a mechanism for the selective recruitment and migration of eosinophils to certain tissues.

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TISSUE-SELECTIVE INTERACTIONS OF LEUKOCYTES WITH VASCULAR ENDOTHELIUM IN INFLAMMATION

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A critical feature of inflammation is the infiltration of the involved tissue by blood leukocytes which cause tissue injury by the production of cytokines, proteolytic enzymes and oxygen radicals. The steps controlling the migration of leukocytes out of blood vessels across the vascular endothelium have in recent years been elucidated. Our studies have focused on the contribution of the two major families of leukocyte integrins, the α_4 (CD49d) and β_2 (CD18) integrins in lymphocyte, monocyte, and neutrophil migration *in vivo*. Our investigations have demonstrated a tissue-specific pattern of integrin utilization by leukocytes for infiltration in inflammation. T lymphocyte migration from the blood to cutaneous inflammatory sites is mediated through the combined actions of $\alpha_L\beta_2$ (LFA-1) and $\alpha_4\beta_1$ (VLA-4). Migration to joint inflammation in rat adjuvant arthritis is also mediated by these two integrins, while migration to the inflamed CNS in experimental allergic encephalomyelitis (EAE) is completely inhibited by blocking α_4 integrins. In addition, T cell accumulation in intestinal inflammation appears to be primarily dependent upon $\alpha_4\beta_7$. Monocyte adhesion to cytokine activated endothelium is mediated by LFA-1, Mac-1 and VLA-4 *in vitro* and *in vivo* migration to both cutaneous inflammation and to inflamed joints in adjuvant arthritis can be partially inhibited by blocking VLA-4 and the CD18 integrins, and abolished by blocking all three receptors. Our studies of neutrophil migration have not only demonstrated that LFA-1 and Mac-1 mediate neutrophil accumulation in inflammatory sites in the skin induced by C5a, IL-1, TNF and LPS, but that these cells can also utilize additional integrins for migration out of the blood. We have shown that rat blood neutrophils express low levels of VLA-4 on their surface and this VLA-4 is functional. Blocking both LFA-1 and Mac-1 or CD18 inhibited 50-75% of the neutrophil accumulation in adjuvant arthritis and 90% of the migration to dermal inflammation. Treatment with anti-VLA-4 plus anti-LFA-1 but neither mAb alone, strongly (60-75%) inhibited neutrophil accumulation in arthritic joints. This mAb combination also inhibited neutrophil migration to dermal inflammatory reactions by 30-70%. Blocking VLA-4 together with the CD18 integrins inhibited neutrophil accumulation by 95-99%, virtually abolishing neutrophil accumulation in cutaneous inflammation. A similar blockade of VLA-4 and CD18 decreased neutrophil accumulation in inflamed joints by 70-83%, but a significant proportion of neutrophil migration to these joints still remained. Thus, neutrophils, as well as monocytes and lymphocytes, utilize VLA-4 and LFA-1 for migration out of the circulation in inflammation. The relative importance of these integrins appear to depend upon the inflamed tissue. In addition there is a CD18- and VLA-4-independent pathway on neutrophil migration to arthritis.

The Pathophysiologic Role of Leukocyte α4 Integrins

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The α4 integrins are heterodimeric cell surface molecules central to leukocyte-cell and leukocyte-matrix adhesive interactions. The integrin α4β1, expressed on all leukocytes except neutrophils, interacts with the immunoglobulin (Ig) superfamily member vascular cell adhesion molecule-1 (VCAM1), and with an alternately spliced form of fibronectin (Fn). The integrin α4β7 is also restricted to leukocytes, and can bind not only VCAM1 and Fn, but also to a homing receptor is selectively expressed on mucosal high endothelial venules called the mucosal addressin cell adhesion molecule (MAdCAM), and which contains Ig-like domains related to VCAM1.

Immobilization of functional purified integrins such as platelet αIIbβ3, leukocyte Mac1 and LFA1, and αVβ3, has proved useful as a means to probe integrin function, to investigate mechanisms of integrin activation, to define alternative ligands, and to define peptide binding sequences by phage methodology. Recently we have developed methods for the purification of intact VLA4 from detergent extracts of α4 integrin-transfected K562 cells. The VLA4 can be immobilized on plastic with retention of its adhesive function, as measured by its ability to bind a VCAM-Ig fusion protein in the presence of suitable activating stimuli, such as certain anti-β1 mAbs (e.g. mAb TS2/16) and manganese ions. Immobilized purified VLA4 should prove useful to further probe α4 integrin structure and function.

Certain monoclonal antibodies (mAbs) to the α4 chain of α4β1 and α4β7 can block their in vitro adhesive function, and these mAbs are efficacious in numerous animal models of autoimmune and inflammatory pathologies. The data argue that α4 integrin-dependent adhesive interactions with VCAM1, MAdCAM, and Fn play a central role in the recruitment, priming, activation, and apoptosis of certain leukocyte subsets, and offer novel targets for drug intervention. To this end we and others have generated a4 integrin small molecule antagonists based on the sequence EILDVPST, which serves as the a4 integrin recognition motif in the CS1 Fn connecting segment. These small molecule antagonists show in vivo efficacy in several animal models, and may be suitable for development as aerosolized antagonists for the treatment of human asthma.

Eotaxin: a potent eosinophil-selective chemokine generated in allergic airways inflammation.

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Eosinophil accumulation is a characteristic feature of allergic asthma and the numbers of recruited cells correlate with lung dysfunction, implying a causal relationship. Thus, intervention preventing eosinophil accumulation is an attractive target for future therapy. The process of leukocyte accumulation is initiated and orchestrated by the generation of soluble chemical signals in the lung. The selective recruitment of a minority cell type from the blood suggests the existence of eosinophil-selective chemoattractants. Against this background we allergen-challenged sensitised guinea pigs and lavaged their lungs at intervals from 30min to 24h. The bronchoalveolar lavage (BAL) fluid was then assayed intradermally in naive guinea pigs previously injected intravenously with ^{111}In-eosinophils. The accumulation of these cells in the skin then provided an index of eosinophil chemoattractant activity in the BAL fluid. Activity was detected which was maximal between 3 and 6h post-challenge. This was then purified in a series of HPLC steps using the skin bioassay system throughout. Protein sequencing revealed a novel 73 amino acid C-C chemokine which we termed 'eotaxin'(1,2).

Eotaxin is a highly potent and eosinophil-selective chemoattractant. Its potent activity on human eosinophils suggested the existence of a human homologue and human eotaxin receptor. Using primers based on the guinea pig sequence a murine (3) and human eotaxin (4) have now been discovered which exhibit all the characteristics of the guinea pig protein. Guinea pig eotaxin was cloned (5) and eotaxin mRNA has been shown to be upregulated in sensitised lungs following allergen challenge (5,6). A similar expression has been demonstrated in allergen-challenged mice (7).

No chemoattractant activity was found in BAL fluid corresponding to IL-5 (indeed all the activity in BAL can be neutralised by antibody to eotaxin). However, it has been shown in several studies that antibodies to IL-5 suppress eosinophil accumulation in this model. This suggests that there may be synergism between eotaxin and IL-5. Accordingly, we have shown that local eosinophil accumulation induced by eotaxin is massively enhanced by giving low doses of IL-5 intravenously one hour previously (8). This effect correlates with the release of a rapidly-mobilisable pool of eosinophils from the bone marrow.

Radioimmunoassay of eotaxin has revealed the kinetics of its appearance in lung extracts and BAL fluid after allergen challenge in guinea pig. Interestingly, significant protein and mRNA (5,6) have also be detected in the lungs of naive guinea pigs. This may be responsible for maintaining the basal eosinophil population in the lung.

Application of eotaxin to the mesentery of anaesthetized guinea pigs induces eosinophil extravasation, as determined using intravital microscopy. Eotaxin is also potent in this respect when administered down the airways and into the skin.

We found that human RANTES, although active on human eosinophils (9) was inactive as an agonist on isolated guinea pig eosinophils (2). However, human

radiolabelled RANTES binds to guinea pig eosinophils and this can be displaced by cold RANTES and guinea pig eotaxin (2). This suggested that human RANTES may act as a receptor antagonist; an effect which we have now demonstrated *in vitro* and *in vivo* (10). This provides the first prototypic eotaxin receptor antagonist.

These observations illuminate possible mechanisms operating in the asthmatic lung and provide a possible target for future therapy.

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CELLULAR LOCALIZATION AND FUNCTIONAL RELEVANCE IN MIGRATION OF THE β 1 INTEGRIN LIBS EPITOPE HUTS-21

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We have generated recently a novel mAb, HUTS-21, that recognizes a specific epitope on the β1 integrin subunit (CD29) whose expression correlates with the ligand-binding activity of these heterodimeric glycoproteins. This correlation has been demonstrated for several integrin heterodimers in different cell systems using a variety of extracellular and intracellular stimuli for integrin activation. Thus, the presence of μM concentrations of extracellular Mn^{2+}, preincubation with the activating anti-β1 mAb TS2/16, and cell treatment with phorbol esters or calcium ionophores, induced the expression of the HUTS-21 β1 epitope on T lymphoblasts. Using a panel of human-mouse β1 chimeric molecules, we have mapped this epitope to the 355-425 sequence of the β1 polypeptide. This segment represents therefore a novel regulatory region of β1 that is exposed upon integrin activation.

The expression of the epitope recognized by mAb HUTS-21 is also induced on T lymphoblasts upon binding of soluble VCAM-1 and fibronectin to the β1 integrins VLA-4 and VLA-5 and this effect is dependent on ligand concentration. We have determined the specific localization of β1 integrins expressing this Ligand-Induced Binding Site on both adherent and non-adherent cells interacting with fibronectin. On fibroblastic cells, which deposit fibronectin fibrils on their surface, the expression of the epitope is restricted to the fraction of integrins interacting with these fibrils. On T lymphoblasts adhering to immobilized fibronectin, the HUTS-21 epitope is localized exclusively to sites of integrin binding to fibronectin. These results indicate that mAb HUTS-21 recognizes a Ligand-Induced Binding Site on the common β1 subunit of VLA proteins that is specifically expressed at sites where cellular integrins interact with ligands. Engagement of β1 integrins through this LIBS epitope inhibited T lymphoblast movement on fibronectin, as assessed by quantitative time-lapse video microscopy studies. Furthermore, the HUTS-21 mAb also prevents T lymphoblast-directed migration through gradients of β1 ligands such as fibronectin or VCAM-1 and reduces migration of T lymphoblasts across high endothelial cells. Our results demonstrate that HUTS-21 defines a novel LIBS epitope on β1 integrins and represents a good reporter of functional activation of β1 integrins during their interaction with ligands.

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Modulation of endothelial cell functions by Hevin, an acidic protein associated with high endothelial venules (HEV)

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In lymphoid organs, lymphocyte adherence and trans-endothelial migration occur at specialized post-capillary vascular sites called high endothelial venules (HEV). The high endothelial cells of HEV have a plump, almost cuboidal appearance, express specialized ligands for lymphocytes and are able to support high levels of lymphocyte extravasation.

To characterize further the specialization of the HEV endothelium at the molecular level, we designed a new strategy to isolate HEV-specific genes. We purified high endothelial cells from human tonsils by immunomagnetic selection with the monoclonal antibody MECA-79, which recognizes HEV-specific sulfated carbohydrate ligands for lymphocyte L-selectin and stains all HEV within lymphoid tissues but does not react with non-HEV cells of the human body. We constructed a cDNA library with mRNA isolated from the purified high endothelial cells. Then, we screened this library by differential hybridization with cDNA probes from HEV (plus) or flat-walled vessel (minus) endothelial cells. This strategy allowed us to characterize a novel human cDNA expressed to high levels in HEV, that encodes a secreted acidic calcium-binding glycoprotein of 664 aa residues, designated Hevin, exhibiting 62% identity with the antiadhesive extracellular matrix protein SPARC. Hevin, is associated with basal, lateral and apical surfaces of HEV cells and unlike MECA-79 antigen is not expressed on the underlying basement membrane.

In contrast to fibronectin or other adhesive extracellular matrix proteins, purified hevin does not support endothelial cell adhesion *in vitro*. Moreover, addition of soluble exogenous hevin inhibits attachment and spreading of endothelial cells on fibronectin substrates. Hevin treated cells do not form focal adhesions and exhibit a rounded morphology. Recently, we tested the capacity of hevin to modulate endothelial cell adhesion and migration during neovascularization. Preliminary results in the rabbit cornea assay suggest that hevin has angiogenic activity *in vivo* . The role of hevin in the regulation of angiogenesis and lymphocyte migration through HEV will be discussed.

V. Leukocyte-endothelial interactions (II)

Chairman: Manuel O. de Landázuri

MODULATION OF LEUKOCYTE ENDOTHELIAL ADHESION UNDER CONDITIONS OF FLOW.

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We have investigated primary intercellular adhesive mechanisms under hydrodynamic flow using two experimental models. The first uses cultured endothelial cells and isolated leukocytes or leukocytic cell lines for the study of leukocyte-endothelial interactions. Here we have analyzed the effects of cytokine stimulation of the endothelial cells over a 24 hour observation period. After 24 hours of IL-1 or IL-4 stimulation, neutrophils, T cells, and the cell lines THP-1 and Ramos were each captured from the flow stream when wall shear stresses were in the range of 2 dynes/cm^2. The adhesive mechanism responsible for this primary capture apparently differs from the known selectins in the case of neutrophils and T cells, since monoclonal antibodies against the selectins exhibited only minimal inhibition. In the case of the two cell lines, much of the primary capture could be attributed to VCAM-1/VLA-4 interactions, and with the remaining adhesion being refractory to anti-selectin antibodies. With each cell type the primary adhesion not accounted for by VCAM-1 could be completely inhibited by sialidase treatment of the leukocytes. Thus, in contrast to endothelial monolayers stimulated for 4-8 hours with IL-1, 24 hour stimulation resulted in a sialidase sensitive adhesive mechanism that could not be blocked with anti-selectin antibodies. An additional experimental condition prevented detection of this primary adhesion. That was, costimulation of the endothelial cells with IL-1 and IL-4 for 24 hours. Under that stimulus condition, primary adhesion at wall shear stresses of 2 dynes/cm^2 failed to occur. Not only the sialidase sensitive component failed, but that attributed to VCAM-1 as well. This latter observation was interesting in light of the fact that VCAM-1 levels on the monolayer were higher than with IL-1 or IL-4 stimulation alone. Endothelial cells appear to modulate the function of expressed tethering molecules following some combinations of cytokine stimulation.

The second experimental model uses shear mixing conditions to investigate the homotypic adhesion of neutrophils. In this model we have found that both L-selectin and Mac-1 (CD11b/CD18) are necessary. Two aspects of the transition from L-selectin dependent to Mac-1-dependent adhesion are under investigation. The first is the ability of L-selectin to function as a signaling molecule for changes in Mac-1 avidity. The second uses cone and plate viscometry to study the adhesion efficiency of each component over a wide range of shear rates. In these studies L-selectin was absolutely required at shear rates $\geq$800 sec^{-1} where contact durations were in the range of 4 msec, but failed to participate in adhesion at shear rates of 200 sec^{-1} where contact durations were in the range of 20 msec. It appears that primary adhesive (tethering) mechanisms are critical to homotypic adhesion of neutrophils as well as adhesion of neutrophils to endothelial monolayers.

THE DYNAMICS OF SELECTINS AND THE BIOLOGY OF ADHESION IN VASCULAR SHEAR FLOW

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Traffic signal molecules for leukocytes are displayed on endothelium that regulate emigration into inflammatory sites. The selectins mediate the first steps, tethering of a cell in flow to the vessel wall, and support labile adhesion to the wall that allows the cell to roll in response to hydrodynamic drag. This is a passive process that reflects the kinetics of receptor binding (k_{on}) and dissociation (k_{off}). We have studied the dynamics and biophysics of this process in flow chambers bearing purified selectins on the wall. When the density of selectins is lowered below a threshold of about 20 sites/mm^2, they do not support rolling, but support transient tethering. The duration of transient tethers can be used to calculate a cellular k_{off}, which appears to reflect the receptor k_{off}. The selectins have fast k_{off} of 0.95/s for P-selectin and 6.6/s for L-selectin, and are predicted to have fast k_{on}, allowing efficient tethering and rolling. The faster k_{off} of L-selectin correlates with faster rolling, even when strength of adhesion to the substrate through P-selectin and L-selectin is the same. Measurement of the distance a cell moves when it flips on its tether as flow is reversed, 6 mm, suggests tethering through a microvillus 1 mm long, and allows calculation of the force on the bond. Selectins can very effectively resist tensile force on the bond; i.e., the k_{off} only increases 3-fold when a 200 pN force is applied, allowing them to maintain adhesion to the vessel wall in spite of the considerable hydrodynamic drag forces exerted on a tethered cell. We have discovered a novel shear-dependent mechanism that regulates adhesion through L-selectin. Although transient tethers develop over a wide range of shears, they only progress to rolling adhesions above 0.4 dyn/cm^2. Lack of adhesion through L-selectin at low shear may counterbalance other adhesion pathways that are maximal at low shear, and prevent inappropriate adhesion in hypoperfusion. Recent reports show that there are L-selectin ligands on neutrophils. We show that once neutrophils adhere to a substrate, such as stimulated endothelial cell monolayers, L-selectin dependent tethering of neutrophils in flow to adherent neutrophils nucleates subsequent rolling on the endothelium. This results in synergy of L-selectin with E-selectin and P-selectin.

In Vivo Analysis of Cell Adhesion in Lymph Node Microvessels.
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Leukocyte adhesion to endothelial cells in physiologically perfused microvessels is thought to follow a multi-step cascade of adhesion and activation events (for reviews see Butcher, 1991, and Springer, 1994). Intravital microscopy studies have documented the critical importance of this process for the accumulation of neutrophils in acutely inflamed non-lymphoid tissues (von Andrian et al. 1991) and for homing of lymphocytes to Peyer's patches (Bargatze et al. 1995). *In vivo* and *in vitro* data also suggest that sequential engagement of distinct receptor-ligand interactions may be similarly involved in the trafficking of lymphocytes to peripheral lymph nodes (PLN). However, this has not been analyzed *in situ*.

We have recently developed a new model that allows us to observe single lymphocytes in subiliac LN microvessels of anesthetized mice by epifluorescence videomicroscopy. As reported for other vascular beds, lymphocytes initially rolled and subsequently became stuck in LN venules, but not arterioles or capillaries. Rolling was nearly completely dependent on L-selectin and correlated with the localization of intravenously injected MAb FITC-MECA-79. The MECA-79 antigen, a sulfated carbohydrate also known as the peripheral node addressin (PNAd), was prominently expressed on the adluminal surface of paracortical high endothelial venules (HEV), but was sparse or undetectable in medullary and hilus venules. MAb MECA-79 effectively inhibited rolling of L-selectin transfectants in the former, but had little effect on rolling in the latter suggesting the presence of at least two functional L-selectin ligands in PLN that differ both spatially and immunologically.

A fraction of rolling lymphocytes (~15 - 20%) became stuck in HEV. This transition from rolling to sticking was abolished by a blocking MAb to the activation-dependent integrin LFA-1 (CD11a/CD18) or by pretreatment of lymphocytes with pertussis toxin, whereas neither treatment affected rolling. In contrast, when rolling was blocked by systemic treatment of mice with the anti-L-selectin MAb Mel-14, sticking was abolished indicating that rolling via L-selectin is a prerequisite first step for subsequent LFA-1 dependent sticking.

Taken together, these studies have identified at least three distinct sequential interactions during lymphocyte homing to PLN: L-selectin mediates the initial attachment and rolling in HEV largely, but not exclusively, via interactions with MECA-79-reactive PNAd. Rolling cells become activated through an as yet unidentified stimulus that requires intracellular signalling events via pertussis toxin-sensitive g αi-linked proteins. Activation, in turn induces engagement of LFA-1 which mediates firm adhesion and thus allows adherent cells to emigrate into the nodal parenchyma.

In addition to this 'classic' homing cascade which largely confirms current concepts of lymphocyte trafficking, we have uncovered an alternative pathway that may allow lymphocyte delivery to sites of PNAd expression even in the absence of functional L-selectin. We found that activated, but not resting human platelets readily roll in LN HEV through an interaction that requires P-selectin on activated platelets and PNAd on HEV.

In vitro studies in a flow chamber using immobilized components of PNAd as a substrate indicate that P-selectin is similarly effective as L-selectin in mediating rolling of platelets and transfected lymphoma cells by binding to the

MECA-79 epitope. Importantly, a small number of rolling human platelets on PNAd dramatically enhanced the rolling frequency of co-infused human lymphocytes through interaction of platelet P-selectin with its lymphocyte ligand, PSGL-1. Moreover, while the blockade of L-selectin lectin function completely abolished lymphocyte rolling on PNAd in the absence of platelets, rolling was preserved when activated platelets were first allowed to adhere to the PNAd-coated substrate and anti-L-selectin treated lymphocytes were infused subsequently. Similarly, rolling of endogenous mouse leukocytes in HEV *in vivo* was effectively blocked by intravenous administration of anti-L-selectin MAb Mel-14. Rolling leukocytes rapidly reappeared, however, when activated human platelets were infused. This rolling interaction was no longer L-selectin-dependent, but was mediated by P-selectin on human platelets that were attached to the surface of the rolling leukocytes.

These experiments suggest that activated platelets can amplify the recruitment of lymphocytes in HEV. Moreover, activated platelets can deliver blood-borne cells to vascular beds that express PNAd, even if the circulating cells possess only P-selectin ligands, but not L-selectin. Both mechanisms may contribute to the pathogenesis and/or maintenance of several chronic inflammatory diseases in which elevated numbers of activated platelets have been found in the circulation and PNAd expression has been detected in venules of the chronically inflamed lesion.

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Functional implications of the actin filament cytoskeleton in endothelial cells

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The vascular endothelium forms a cellular barrier that separates the blood compartment from the extravasal space and controls the passage of blood cells, macromolecules and solutes across the vascular wall. In fulfilling these functions, the endothelium has to cope with various types of mechanical stresses resulting from blood flow (shear stress), blood pressure (wall distension), and osmotic gradients. There is increasing evidence that paracellular permeability and mechanical resistance of the endothelium is controlled by a contractile cytoskeleton consisting of actin, myosin, and several associated proteins that comprise about 20 % of total cellular protein of endothelial cells. The ratio between myosin, the force-generating motor protein, and actin is about 1:15 in endothelial cells and lies between the myosin to actin ratio of smooth muscle (~1:30) and striated muscle (~1:6). Various actin-associated proteins have been identified in endothelial cells that are involved in the regulation of actin polymerisation (e.g. gelsolin, profilin), filament cross-linking (α-actinin, filamin), stabilisation of filaments (tropomyosin), or attachment of actin filaments to the plasma membrane (α-actinin, talin, vinculin, catenins). Three main sets of actin filaments occur in endothelial cells in situ and in vitro: (i) a subplasmalemmal layer underneath the lumenal plasma membrane, (ii) a junctional (marginal) filament system that is associated with the cytoplasmic aspect of the intercellular junctions and (iii) straight bundles, denoted as stress fibres, that emanate from focal adhesion sites of the basal cell surface to the substratum.

The subplasmalemmal actin layer seems to be attached to the plasma membrane via a membrane-bound spectrin meshwork that is well developed underneath the lumenal plasma membrane. This membrane-associated cytoskeleton appears not only to be involved in mechanical support of the lipid bilayer but also to participate in various other events, such as endo- and exocytosis and leucocyte attachment.

The junctional belt of actin, myosin, α-actinin, and other actin-associated proteins is associated via certain bridging molecules (catenins, plakoglobin) to the cytoplasmic domains of Ca^{2+}-dependent adhesion molecules, cadherins. Changes of the contractile tonus of this junction-associated cytoskeleton affect the width of the intercellular clefts and modify paracellular permeability. Increase of paracellular permeability induced by thrombin, histamine and other inflammatory agents depends on intracellular rise in Ca^{2+} and activation of the myosin ATPase by phosphorylation of the regulatory myosin light chain.

Endothelial stress fibres are contractile filament bundles that resemble in several structural and functional aspects myofibrilles of muscle. Stress fibres are particularly abundant in endothelial cells exposed to high levels of fluid shear stress. Their formation in endothelial cells in situ and in vitro can be induced by experimental elevation of shear stress levels. The main function of stress fibres appears to help the endothelium to withstand the shear forces of blood flow and to protect the cells from hydrodynamic injury and detachment.

POSTERS

Specific regulation of VLA-4 and alpha4 beta7 integrin expression on human activated T lymphocytes

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Modulation of VLA integrins was studied on several human T cell clones upon specific and non-specific cellular activation. Human activated T lymphocytes down-regulated both $\alpha4\beta1$ and $\alpha4\beta7$ integrins upon specific recognition of alloantigens (cytotoxic T cells) or in the presence of Staphylococcus enterotoxin B (SEB)[2] (superantigen recognizing non-cytotoxic T cells). In contrast, the expression of other membrane integrins, such as VLA-1 and VLA-5 integrins, was not modified. Down-regulation of $\alpha4\beta1$ and $\alpha4\beta7$ integrins was observed as early as 3 hr after stimulation, lasted later than 72 hr and was partially inhibited by cytochalasin D. Interestingly, neither target cells nor NK cells modulated CD49d expression after interaction with T cells or K562 respectively, suggesting that CD49d expression was linked to specific T cell activation. The down-regulation of CD49d chain in T cell clones stimulated with immobilized anti-CD3 mAbs confirmed the role of TCR-mediated activation in CD49d regulation. However, the CD3-independent cellular aggregation induced by soluble anti-CD43 mAb was also able to strongly down-regulate $\alpha4\beta1$ and $\alpha4\beta7$. The present work shows the first evidence that CD49d-integrin expression is distinctly regulated from other integrins after antigen or superantigen recognition by human activated T cells. CD49d modulation may be relevant for the traffic and tissue localization of locally activated T cells during immune responses.

Migration of T lymphocytes modelled *in vitro*: Enhanced migratory capacity of T cells with a "recently activated/ memory" phenotype

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We established an *in vitro* transmigration assay in order to investigate the phenotype of T lymphocytes spontaneously migrating across a monolayer of unstimulated endothelial cells. During a 4 hour time period 3-4% of lymphocytes from either peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN) or periphheral blood (PBL) spontaneously migrated across a monolayer of the endotheliomal cell line bEnd.5. Further investigations focusing on the T cell population in these assays showed an enrichment for T lymphocytes expressing a "recently activated/memory" phenotype: LFA-1/CD44/ICAM-1^{high} in the transmigrated T cell population. The transmigrated T cell population was also enriched for L-selectin$^{low/negative}$ T lymphocytes. Besides that, the migratory T cell populations could be distinguished from their original population and from each other by their distinct expression of α4- and β7-integrin and partially also by their PECAM-1 expression, pointing to the presence of different migratory T cell populations in MLN, PLN and PBL. Additionally, PMA-activated lymph node cells show a phenotype that can easily be distinguished from that of transmigrated T cells. This provides evidence that the distinct phenotype of spontaneously migrating T lymphocytes is not merely due to activation. Besides that T cell lines did not show a difference in phenotype when compared before and after transmigration. Interestingly, the ratio of $CD4^+$ versus $CD8^+$ T lymphocytes was reversed towards an enrichment of $CD8^+$ T cells after transmigration, which seems to be dependent on preferential migration of $CD8^+$ T cells.

In our *in vitro* assay system recently activated and/or memory T cells preferentially migrate across monolayers of unstimulated endothelial cells. The T cells migrating across the endothelial monolayer differ in their expression of cell adhesion molecules depending on their origin. Our *in vitro* transmigration assay mimicks closely observations from *in vivo* studies and thus provides a versatile tool to study the molecular mechanism involved in T lymphocytes migration across the endothelial cell wall during immunosurveillance.

SUSCEPTIBILITY TO INFECTION AND ALTERED HEMATOPOIESIS IN MICE DEFICIENT IN BOTH P-AND E-SELECTINS.

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The genes for P-and E-selectin are closely linked on chromosome 1. To study their role *in vivo*, we generated mice lacking both endothelial selectins by two rounds of homologous recombination in embryonic stem cells. The animals are viable and fertile therefore endothelial selectins are not necessary for embryonic angiogenesis. In contrast to the rather mild phenotypes observed in mice deficient in a single selectin gene, the doubly deficient mice present extreme leukocytosis, elevated cytokine levels and alterations in hematopoiesis. Granulocytopoiesis is increased both in bone marrow and spleen while erythropoiesis is partially translocated to the spleen. Virtual lack of leukocyte rolling and low extravasation at sites of inflammation make these animals susceptible to opportunistic bacterial infections to which they succumb. Our results show that the absence of endothelial selectins severely affects leukocyte homeostasis and indicate that these two selectins are as important for normal leukocyte function as the leukocyte β2 integrins.

B cell intracellular events induced by interaction of the α4β1 integrin with different sites in fibronectin or with VCAM-1. Carmen Domínguez-Jiménez, Paloma Sánchez-Aparicio, and Angeles Garcia-Pardo, Centro de Investigaciones Biológicas, CSIC, Madrid, Spain.

The lymphocyte integrin α4β1 (CD49d/CD29) is the receptor for the Hep II (H1) and CS-1 sites in fibronectin (Fn) as well as for VCAM-1. We have recently shown that upon activation with anti-β1 mAb TS2/16, α4β1 also recognizes the RGD sequence in Fn. To determine the physiological role of these multiple interactions, we have now studied some intracellular events induced by "resting" and activated α4β1 binding to its different ligands. Analyses of actin and tubulin reorganization upon adhesion of B lymphoid cells to Fn fragments of 38 kDa (Hep II+CS-1), 58 kDa (Hep II), and 80 kDa (RGD), or to VCAM-1, showed that VCAM-1, the 38 kDa fragment and the CS-1 synthetic peptide induced formation of long cytoplasmic projections; however, cells attached to other fragments or the synthetic peptide H1 remained rounded. Because the cytoplasmic protrusions were transient, we tested whether they represented cell movement. Using trans-filter assays, we showed that VCAM-1, 38 kDa and CS-1 induced dose-dependent B cell migration mediated by α4β1, thus establishing a correlation between the two responses. Furthermore, these three ligands, but not the 80 kDa fragment or H1 peptide, induced tyrosine phosphorylation of a 110 kDa protein. Activation of α4β1 with TS2/16 mAb inhibited the cytoplasmic protrusions and cell migration, probably because of adhesion strengthening, but did not seem to affect the pattern of phosphorylation. Our results indicate that the various α4β1 ligands induce different cellular responses thus suggesting a regulation by the activation form of the receptor as well as by the ligand in events involving lymphocyte adhesion and migration.

IDENTIFICATION OF AMINO ACIDS IN THE LFA-1 I DOMAIN THAT MEDIATE ICAM-3 BINDING AND IMMUNE FUNCTION

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The leukocyte integrin LFA-1 (CD11a/CD18) is a cell surface receptor which mediates adhesive interactions and signal transduction in the immune system by binding its ligands ICAM-1, -2 or -3. Several studies indicate that the I domain located in the α chain (CD11a) of LFA-1 (CD11a/CD18), plays an essential role in ligand recognition. We have identified amino acid residues, within the evolutionarily conserved I domain of the α-chain (CD11a) of the leukocyte integrin LFA-1, that are critical for ICAM-3 binding. ICAM-3, a ligand of LFA-1, is thought to mediate intercellular adhesion essential for the initiation of immune responses. Using a panel of human/murine I domain chimeras and point mutants, we observed that the Ile-Lys-Gly-Asn motif, located in the N-terminal part of the CD11a I domain, is required for ICAM-3 binding. An aspartic acid located at position 137, that is essential to ICAM-1/LFA-1 interactions, was also critical for ICAM-3 binding. Anti-LFA-1 antibodies that selectively block LFA-1 binding to ICAM-3 bind the I domain in close proximity to the residues implicated in binding of ICAM-3. A synthetic peptide containing the Ile-Lys-Gly-Asn inhibited ICAM-3 dependent adhesion and proliferation of resting T cells, suggesting that this peptide interferes with immune recognition. These observations underscore the importance of ICAM-3 in leukocyte function, and may lead to development of a new category of immunosuppressive agents.

In a separate study we investigated the role of the cytoplasmic domain of the β subunit of LFA-1 in regulating integrin fucntion. Our finding that LFA-1, expressed in the non-leukocyte K562, can not be activated by the phorbol ester PMA to bind its ligand ICAM-1, demonstrates that K562 cells lack the correct signaling pathway for LFA-1 activation. Using chimeric LFA-1 receptors transfected in K562 cells, we observed that replacement of the β2 cytoplasmic tail for the β1 cytoplasmic tail but not the β7 cytoplasmic tail restore PMA responsiveness of LFA-1 in K562 cells. We observed that only the cytoplasmic tail of the β1 integrin is able to localize LFA-1 into clusters on K562 cells, thereby facilitating PMA induced ligand binding. Also the extracellular conformation was altered as evidenced by enhanced L16 epitope expression when the β2 cytoplasmic domain was replaced for the β1 cytoplasmic domain. This may be attributed to distinct interactions with cytoplasmic proteins affecting the extracellular conformations of the integrin molecule. The importance of interactions of integrins with kinases or cytoskeletal elements for its function is not yet known.

FACTOR J MEDIATES CELL MEMBRANE INTERACTION BY AN ADHESION MECHANISM.

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Factor J (FJ) is a complement regulator with inhibitory activity on the classical and the alternative pathway. Furthermore, FJ is a highly cationic glycosylated protein. FJ was found as a membrane protein in human peripheral blood cells (PBL) , mainly on B-lymphocytes. FJ is present on several cell lines, too. Some peculiarities of this molecule resemble those of the vitronectin as: high glycosylation, stickiness to many surfaces and molecules, the ability to regulate complement and the tendency to aggregate.

These characteristics could relate FJ with cell-cell or cell-matrix interactions. The eventual implications of FJ on cell adhesion mechanism was tested by using FJ coated on microtiter plates, in a similar way as fibronectin or vitronectin are regularly tested. For that, 1 μg of FJ was coated to the plates, blocked for nonspecific interactions and after that, PBL or different cell lines were added to the plates, and incubated for 30 min at 37 ^{o}C. Thereafter, the plates were washed and examined by light microscopy. The percent of bound cells was quantified by dyeing with violet crystal. Binding experiments were repeated on a fluid-phase assay by incubation of the cells with purified FJ, followed by the addition of mab anti-FJ and the appropriated second ab. Stained cells were analyzed by flow cytometry on a FACSscan.

Assays on microtiter plates evidenced that FJ was able to bind cells in a similar way that fibronectin. One μg of each protein induced a similar adhesion percentage. FJ was capable of binding PBL and cell lines as: K562, Jurkat or JY. By opposite, U937 cells were negative in these assays. Moreover, the interaction FJ-cells is perceptible by flow cytometry experiments. The adhesion was blocked by incubation of the FJ-coated plates with mabs anti-FJ or heparin, and it is not mediated by β1, β2, or β3 integrins.

When experimental requirements for FJ-cells interaction were established, we found that it was dependent of temperature, required active metabolism, cytoskeleton integrity and a functional Na^{+}/H^{+} antiporter.

A developmental switch in lymphocyte homing receptor and endothelial vascular addressin expression regulates lymphocyte homing and permits CD4+3- cells to colonize lymph nodes

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In adult mice, the dominant adhesion molecules involved in homing to lymph nodes are L-selectin homing receptors on lymphocytes and the peripheral lymph node addressins (PNAds) on specialized high endothelial venules. Here we show that from fetal life through the first 24 hrs of life the dominant functional adhesion molecules are the mucosal addressin MAdCAM-1 on lymph node high endothelial venules and its counterreceptor, the Peyer's patch homing receptor, integrin $\alpha4\beta7$ on circulating cells. Before birth 40-70% of peripheral blood leucocytes are L-selectin positive, while only 1-2% expresses $\alpha4\beta7$. However, the fetal lymph nodes preferentially attract $\alpha4\beta7$ expressing cells, and this can be blocked by fetal administration of anti MAdCAM-1 antibodies. During fetal and early neonatal life, when only MAdCAM-1 is expressed on HEV, an unusual subset of CD4+CD3- cells, as well as $\gamma\delta$ TCR+ cells, exclusively expressing $\alpha4\beta7$ as homing receptors, enter the lymph nodes. Beginning 24 hours after birth a developmental switch occurs, and the peripheral node addressins (PNAd) are upregulated on high endothelial venules in peripheral and mesenteric lymph nodes. This switch in addressin expression facilitates tissue-selective lymphocyte migration, and mediates a sequential entry of different cell populations into the lymph nodes.

DITHIOCARBAMATE-INDUCED ANTIOXIDANT STRESS PROMOTES FIRM ADHESION OF POLYMORPHONUCLEAR CELLS TO HUVEC UNDER PHYSIOLOGICAL FLOW.

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Interaction of polymorphonuclear cells (PMN) with the vascular wall is a key proccess in the extravasation of these cells to the inflammed tissues. In order to evaluate the involvement of the cellular redox status in the adhesion of PMN to endothelium we carried out adhesion assays under both static and flow conditions. We first demonstrated the antioxidant effect of Pirrolidine Dithiocarbamate (PDTC) by showing a decrease in the H_2O_2 intracellular levels upon treatment of PMN. Adhesion assays were then performed with PMN treated with or without the antioxidant agent to HUVEC monolayers stimulated with TNF-α. Under static conditions, PDTC treatment greatly enhanced adhesion of PMN to HUVEC stimulated with suboptimal doses of TNF-α. Adhesion studies under defined laminar flow conditions revealed that the antioxidant treatment produced a considerable enhancement of PMN stable adhesion to 20 h-stimulated HUVEC monolayers, and decreased dramatically the percentage of rolling PMN. The rolling velocity was significatively slower for the PMN treated with the antioxidant agent. Flow cytometry studies revealed that expresion of the integrin Mac-1, but not LFA-1 or p150, was upregulated upon treatment with PDTC. PDTC treatment also promoted expression of activation neo-epitopes on the surface of β_2 integrins. Surprisingly and diferentially from other PMN-activation agents such as phorbol esters or fMLP, this agent did not induce the shedding of L-selectin. The use of blocking mAbs directed to the different adhesion molecules on the surface of PMN on static adhesion assays revealed that uniquely Mac-1 was responsible for the proadhesive effect of PDTC, although this binding also implicated LFA-1.

Altogether these results demonstrate that induction of an antioxidant stress produces activation of polymorphonuclear cells, thus increasing their adhesion to stimulated endothelium under conditions of physiological flow. This effect is produced by the upregulation and activation of Mac-1 on the surface of PMN which is not accompanied by a proteolytic cleavage of L-selectin.

VE-cadherin competes with N-cadherin for cell to cell junction localization in endothelial cells.

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Cadherins are a superfamily of calcium-dependent transmembrane glycoproteins which are the major components of adherens junctions. These structures play an important role in the control of vascular permeability and in the organization of new vascular structures during angiogenesis. Human umbilical vein endothelial cells (HUVEC) express two major cadherins: VE- (which is specific for endothelium) and N-cadherin. These two proteins are expressed in a comparable amount in the endothelium but only VE-cadherin is localized at junctions while N-cadherin remains diffuse on the cell membrane. We attempted to define at a structural level the reasons for this dominant activity of VE-cadherin on N-cadherin. This dominant activity of VE-cadherin was not specific for cultured HUVEC since it was apparent also in vivo by immunohistochemical analysis of endothelial cells of different type of vessels. In addition, this phenomenon was not specific for endothelial cells in general since it could be reproduced in CHO cells cotransfected with the two types of cadherins. The inability of N-cadherin to go to junctions in presence of VE-cadherin was not due to a loss of its functional activity since it was still able to promote homotypic cell adhesion both in HUVEC and CHO cells . In addition, when N-cadherin was transfected in absence of VE-cadherin, it could regularly organize at cell to cell junctions. The dominant effect of VE- on N-cadherin was not linked to a differential association to catenins since immunoprecipitation analysis showed that comparable amounts of α-, β-, plakoglobin and p120 were associated to the two types of cadherins. Finally, a mutant VE-cadherin lacking the cytoplasmic domain responsible for catenin binding was still able to compete for N-cadherin localization at junctions. This strongly suggests that the dominant effect of VE- on N-cadherin is related to the non-catenin binding domain of the molecule. Overall these data show that different types of cadherins can compete for junction localization in the same cell type. The extrajunctional N-cadherin might play a role in localizing N-cadherin expressing cells (such as tumor cells) or N-cadherin binding substances (such as "the fibroblastic growth factor receptor") on the endothelial surface.

Chemokines regulate cellular polarization and adhesion receptor redistribution during lymphocyte interaction with endothelium and extracellular matrix. A possible novel regulatory mechanism for leukocyte recruitment.

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Leukocyte recruitment is a key step in the inflammatory reaction. Several changes in the cell morphology take place during lymphocyte activation and migration: spheric-shaped resting T cells become polarized during activation, developing a well defined cytoplasmic projection designated as cellular uropod. We found that the chemotactic and pro-inflammatory chemokines RANTES, MCP-1, and, to a lower extent, MIP-1α, MIP-1ß and IL-8, were able to induce uropod formation and ICAM-3 redistribution in T lymphoblasts adhered to ICAM-1 or VCAM-1. A similar chemokine-mediated effect was observed during T cells binding to the fibronectin fragments of 38 and 80-kD, that contain the binding sites for the integrins VLA-4 and VLA-5, respectively. The uropod structure concentrated the ICAM-3 adhesion molecule (a ligand for LFA-1), and emerged to the outer milieu from the area of contact between lymphocyte and protein ligands. In addition, we found that other adhesion molecules such as ICAM-1, CD43, and CD44, also redistributed to the lymphocyte uropod upon RANTES stimulation, whereas a wide number of other cell surface receptors did not redistribute. Chemokines displayed a selective effect among different T cell subsets; MIP-1ß had more potent action on $CD8^+$ T cells and tumor infiltrating lymphocytes (TIL), whereas RANTES and MIP-1α targeted selectively $CD4^+$ T cells. We have also examined the involvement of cAMP signaling pathway in uropod formation. Interestingly, several cAMP agonists were able to induce uropod formation and ICAM-3 redistribution, whereas H-89, a specific inhibitor of the cAMP-dependent protein kinase, abrogated the chemokine-mediated uropod formation, thus pointing out a role for cAMP-dependent signaling in the development of this cytoplasmic projection. Since the lymphocyte uropod induced by chemokines was completely abrogated by *Bordetella pertussis* toxin, the formation of this membrane projection appears to be dependent on G proteins signaling pathways. In addition, the involvement of myosin-based cytoskeleton in uropod formation and ICAM-3 redistribution in response to chemokines was suggested by the prevention of this phenomenon with the myosin-disrupting agent butanedione monoxime. Interestingly, this agent also inhibited the ICAM-3-mediated cell aggregation, but not the cell adhesion to substrata. Altogether, these results demonstrate that uropod formation and adhesion receptor redistribution is a novel function mediated by chemokines (M.A. del Pozo *et al.*, 1995, *J. Cell Biol.*, 131: 495-508); this phenomenon may represent a mechanism that significantly contributes to the recruitment of circulating leukocytes to inflammatory foci (M.A. del Pozo *et al.*, 1996, *Immunol. today*, 17: 127-131).

CD24 is a ligand for human P-selectin destinct from PSGL-1

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P-selectin (CD62P) is a Ca^{2+} dependent lectin that participates in leukocyte adhesion to vascular endothelium and platelets. Counter receptors are carbohydrates expressed at the cell surface of myeloid cells, subsets of T lymphocytes and certain tumor cells. The nature of these ligands is not well understood. P-selectin glycoprotein ligand-1 (PSGL-1) has been identified as a major ligand for P-selectin on neutrophils and HL60 cells. We report here that CD24, a mucine-type glycosylphosphatidylinositol-linked cell surface molecule on human neutrophils, pre B lymphocytes and certain tumors can also be a ligand for P-selectin. Latex beads coated with purified CD24 from neutrophils or the human small cell lung carcinoma cells SW-2 were stained with P-selectin-IgG. The staining was dependent on divalent cations and was abolished by treatment with O-sialoglycoprotein endopeptidase but not Endoglycosidase F or sialidase. The beads were stained with a mab to CD57 (HNK-1 carbohydrate epitope) but did not react with mabs HECA 452 or CSLEX against the sialyLex/a epitope. Mab PL-1 directed against PSGL-1 which has been reported to block neutrophil rolling on P-selectin (Moore et al, J.Cell Biol. 128:661-671, 1995) could also stain the CD24-beads whereas mab PL-2 recognizing a different epitope on PSGL-1 did not. The CD24 beads were able to bind to activated platelets in a P-selectin-dependent manner and this binding was blocked by mab PL-1. These results establish a functional similarity between CD24 and PSGL-1. However, CD24 is different in its carbohydrate composition as well as in its signalling potential which could be important for distinct functions.

IL-8 mediates transendothelial migration of human skin homing T cells

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The cutaneous lymphocyte-associated antigen (CLA) is a carbohydrate epitope present on most skin infiltrating T cells but only on 7-20 % of circulating T cells and absent from T cells infiltrating other organs. CLA is a ligand for E-selectin expressed by endothelial cells (EC) in inflammation. We have recently shown that CLA defines a subset of circulating memory T cells that preferentially respond to skin-associated allergens and migrate across EC monolayers by CLA/E-selectin, VLA-4/VCAM-1 and LFA-1/ICAM-1 interaction. Here, we investigated the role of chemokines in transendothelial migration of CLA^+ and CLA^- memory T cells. Blood isolated CLA^+ and CLA^- $CD45RO^+$ T cells were incubated on IL-1/TNF-α activated EC monolayers. Pertussis toxin selectively inhibited the migration of CLA^+ T cells indicating that G-protein signals were involved.

Blocking mab to IL-8 selectively inhibited CLA^+ T cell transmigration, whereas mAbs to MCP-1, RANTES and ENA-78 did not have an effect. Preincubation of CLA^+ T cells with 20nM IL-8 for 30 min. also inhibited transmigration of CLA^+ T cells, indicating that the IL-8 receptor can be desensitized for this function. Furthermore, CLA^+ but not CLA^- sublines generated from a cutaneous T cell lymphoma (mycosis fungoïdes) cell line exhibited IL-8-dependent migration. In order to characterize which of the two forms of IL-8 receptor (IL-8R) mediated CLA migration we used blocking mAbs to IL-8RA and IL-8RB. Although only the IL-8RB was involved in CLA-dependent migration, both CLA^+ and CLA^- memory T cells and HUT-78 cells contained mRNA coding for IL-8RA and IL-8RB.

Similar results were obtained by using microvascular skin endothelial cells.

Our results support a role for IL-8 in transendothelial migration of a human skin-homing memory T cells to cutaneous sites.

HUMAN EOTAXIN INDUCES EOSINOPHIL ACCUMULATION IN RAT SKIN WHICH IS DEPENDENT ON BOTH VCAM-1 AND ICAM-1

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The novel C-C chemokine eotaxin, originally purified from bronchoalveolar lavage fluid of sensitized guinea-pigs following allergen challenge, is a potent eosinophil-selective chemoattractant (1,2). The human homologue exhibits the same potency and selectivity for human eosinophils and has been demonstrated to induce eosinophil accumulation in monkey skin (3). In the present study we have used synthetic human eotaxin to characterize the profile of eotaxin-induced eosinophil accumulation *in vivo*. For this purpose, we have used a previously described method to measure ^{111}In-eosinophil accumulation and oedema formation in rat skin (4). Using a 4h *in vivo* test period, intradermal-injected human eotaxin caused a dose-dependent accumulation of ^{111}In-eosinophils, significant responses being induced by 10 and 100 pmol/site. In contrast, the chemokine did not induce oedema formation or ^{111}In-neutrophil accumulation. Time-course studies indicated that the ^{111}In-eosinophil accumulation induced by human eotaxin was rapid and that all the accumulation occured within the first hour after i.d. administration of the chemokine. Co-injection of a rabbit anti-human eotaxin IgG with the chemokine inhibited the ^{111}In-eosinophil accumulation induced by human eotaxin but not the cell accumulation induced by PAF or LTB_4. The intravenous administration of an anti-rat VCAM-1 mAb (5F10, 5mg/kg) or an anti-α4 integrin mAb (HP2/1, 3.5mg/kg) significantly inhibited the eosinophil accumulation induced by 100 pmol human eotaxin by 40% and 67%, respectively. Further, an anti-rat ICAM-1 mAb (1A29, 5mg/kg i.v.) significantly reduced the ^{111}In-eosinophil accumulation induced by human eotaxin (78% inhibition). Our findings show that human eotaxin is a potent inducer of eosinophil, but not neutrophil, accumulation *in vivo*, and that this response is dependent on the expression of adhesion molecules VCAM-1, α4 integrins and ICAM-1.

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STRUCTURE-FUNCTION STUDIES OF HUMAN EOTAXIN.

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Asthma is an inflammatory disorder with a preponderance of eosinophils, and to a lesser extent, T cells and mast cells infiltrating the airways. Eosinophils may play a significant role in the pathogenesis of asthma and thus inhibition of eosinophil migration into the airways may alleviate the disease.
Eotaxin is a recently discovered CC chemokine (Jose P.J. et al, 1994; Ponath et al, 1996a.) which is a potent and selective chemoattractant for eosinophils. It binds and signals through the chemokine receptor CKR3 which is selectively expressed in eosinophils (Ponath et al.1996b). Eotaxin and CKR3 may be important mediators of asthma by participating in the recruitment of eosinophils to the airways. Therefore, CKR3 is a target for therapeutic intervention in asthma.
To study the molecular basis for the eotaxin-CKR3 interaction and thereby facilitate the development of a competitive antagonist, we have analyzed eight synthetic N-terminal deletion mutants of eotaxin in binding and functional assays. To complement the deletion studies we have prepared a panel of eotaxin mutants and produced these in a baculovirus expression system.
We will present data which supports the identification of distinct functional domains within eotaxin and suggest mechanisms by which these interact with the CKR3 receptor.

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STRUCTURE-FUNCTION RELATIONSHIP IN THE INTEGRIN VLA-4.

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The VLA-4 ($\alpha 4\beta 1$) integrin is involved in the adhesion of cells to fibronectin and VCAM-1. In order to study $\alpha 4$ structure-function relationship, we have expressed mutated $\alpha 4$ subunit by transfection into VLA-4-negative K562 cells. Substitutions at $\alpha 4$ residues ^{89}Arg^{90}Asp resulted in a reduction in the reactivity of all anti-$\alpha 4$ epitope A mAb tested, compared with the reactivity with anti-$\alpha 4$ epitopes B1, B2 and C mAb, both by transfectant flow cytometry, and by immunoprecipitation and SDS-PAGE analysis of transfectant surface iodinated proteins. In contrast, substitutions at nearby residues, ^{101}Q, ^{102}P and ^{108}I did not affect the reactivity of any anti-$\alpha 4$ mAb representing the known $\alpha 4$ epitopes. Homotypic cell aggregation triggered by anti-$\alpha 4$ epitope A mAb was prevented in the transfectants expressing mutated $\alpha 4$ ^{89}Arg^{90}Asp residues, while cell aggregation was fully achieved with either anti-$\alpha 4$ epitope B2 or anti-$\beta 1$ mAb. In addition, the adhesion of mutant ^{89}Arg^{90}Asp $\alpha 4$ transfectants to the CS-1-containing FN-40 fragment of fibronectin was diminished compared to wild type $\alpha 4$ transfectants, as well as to other mutant $\alpha 4$ transfectants. This adhesion to FN-40 was restored when the activating anti-$\beta 1$ TS2/16 mAb was present in the adhesion assays. In contrast, adhesion to VCAM-1 was not affected by mutations at ^{89}Arg^{90}Asp, nor at ^{101}Q, ^{102}P and ^{108}I $\alpha 4$ residues. These results indicate that $\alpha 4$ residues ^{89}Arg and ^{90}Asp are included in a region involved in homotypic cell aggregation, as well as in adhesion to FN-40, but not to VCAM-1. We have also found that substitutions at $\alpha 4$ residues ^{152}Q and ^{153}D resulted in a nearly complete abolishment in the reactivity of three diferent anti-$\alpha 4$ epitope B2 mAb, while substitution of residue ^{154}Y resulted only in a partial loss of reactivity towards the same antibodies. By contrast, the $\alpha 4$ subunit expressed by the transfectants carrying substitutions at these residues showed similar reactivity towards epitopes A, B1 and C anti-$\alpha 4$ mAb compared with wild type $\alpha 4$ transfectants. Substitution at residues ^{155}V, ^{156}K, ^{157}K and ^{158}F did not change the reactivity pattern towards any anti-$\alpha 4$ mAb so far tested. These results indicate that residues ^{152}Q, ^{153}D and ^{154}Y are included in the $\alpha 4$ epitope B2. Monoclonal antibodies which recognize this epitope completely inhibit VLA-4-mediated adhesion to fibronectin, partially inhibit adhesion to VCAM-1, and they are able to trigger homotypic cell aggregation. We are presently investigating the functional consequences of these mutations.

INCREASED ICAM-1 BUT NOT L-SELECTIN EXPRESSION DECELERATES B AND T LYMPHOCYTE SUBSET MIGRATION FROM BLOOD TO LYMPH. J. Westermann and R.Pabst. Centre of Anatomy, Hannover, Germany

To study the effects of different levels of ICAM-1 and L-selectin expression on the migration of B and T lymphocyte subsets from blood to lymph, rat thoracic duct lymphocytes were examined either immediately after collection or after additional culturing in normal medium. After overnight incubation ICAM-1 expression dramatically increased on B lymphocytes (mean fluorescence intensity (MFI) 580 vs. 660) but less so on T lymphocytes and their subsets (MFI: 330 vs. 350). In contrast, L-selectin showed a moderate increase on B cells (MFI: 620 vs. 630) and a dramatic increase on T cells (MFI: 550 vs. 690). The labelled (FITC) cells were injected into rats which had been subjected to thoracic duct cannulation. For 4 days the lymph was collected in 6 hourly portions. The number of $FITC^+$ lymphocytes was counted and, using appropriate antibodies and flow cytometry, the phenotype of these cells was determined.

Although the recovery of injected cells was similar for all subsets and both groups, culturing overnight lead to an increase in the mean transit time for B lymphocytes from 40 h to 60 h, whereas for T cells only an increase from 25 h to 30 h was seen. The migration of $CD4^+$ and $CD8^+$ lymphocytes was effected similarly.

Thus, the level of ICAM-1 but not L-selectin expression seems to determine the time needed to pass through lymphoid tissues in vivo.

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Workshop on

VASCULAR ENDOTHELIUM AND REGULATION OF LEUKOCYTE TRAFFIC

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