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The lectures summarized in this publication were presented by their authors at a workshop held on the 13th through the 15th of June, 1991, at the Universidad Internacional Menéndez Pelayo in Cuenca.

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265 Workshop on Adhesion Receptors in the Immune System

SERIE UNIVERSITARIA

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Workshop on Adhesion Receptors in the Immune System

Organized by

T. A. Springer and F. Sánchez-Madrid

S. J. Burakoff
A. L. Corbi-López
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I N D E X

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PROGRAMME

1. Thursday, June 13th:

IMMUNOGLOBULIN SUPERFAMILY
Chair: A.F. Williams
Co-chair: S.J. Burakoff

A.F. Williams	- The structure of the CD2 T lymphocyte antigen and its function in signal transduction.
P. Moingeon	- Structural and functional characterization of human CD2.
P. Aparicio	- Short presentation.
H. He	- Short presentation.
S.J. Burakoff	- The role of CD4 and CD2 in T cell activation.
A.de Fougerolles	- Short presentation.
B. Malissen	- The T cell receptor-CD3 complex is composed of at least two autonomous transduction modules.
J.A. López de Castro	- Clonal heterogeneity in LFA-3 and ICAM-1 requirement for lysis by alloreactive T lymphocytes.
D. Jaraquemada	- Short presentation.
R.R. Lobb	- Vascular cell adhesion molecule 1: current studies.

2. Friday, June 14th:

INTEGRIN FAMILY
Chair: T.A. Springer
Co-chair: S. Shaw

T.A. Springer	- Cooperation between leukocyte rolling on a selection and adhesion strengthening through integrins.
C. Figdor	- Activation of LFA-1: Role of cations.
T.A. Yednock	- Short presentation.
J.A. Brieva	- Short presentation.
A.A. Postigo	- Short presentation.

N. Hogg	- Leukocyte integrin activation.
J.C. Gutiérrez-Ramos	- In vitro induction of embryonic stem cells to develop into T- and B-cell progenitors.
K. Okumura	- Murine lymphocyte adhesion molecules involved in cellular interactions and activation.
F.Sánchez-Madrid	- Regulation of VLA-4 adhesive functions
A. Ager	- Short presentation.
S. Shaw	- Adhesion molecules in post-thymic T cell diferentiation.
A.L. Corbi-López	- Preliminary studies on integrin gene structure and regulation of expression
M. Letarte	- Short presentation.
C. Bernabeu	- Short presentation.
F. Takei	- Short presentation.

3. Saturday, June 15th:

SELECTIN FAMILY
Chair: B. Furie
Co-chair: T.F. Tedder

T.F. Tedder	- Regulation of leukocyte interactions with endothelium through the leukocyte adhesion molecule-1, LAM-1.
L. Lasky	- Functional aspects of LECAM-1.
A. Hamann	- Adhesion molecules involved in lymphocyte traffic.
P. Ruiz	- Short presentation.
B. Furie	- Padgem: A leukocyte receptor on platelets and endothelial cells-role in inflammation and thrombosis.
J.C. Paulson	- The sugar coating and leukocyte trafficking.

INTRODUCTION

F. Sánchez-Madrid
Hospital de la Princesa. Madrid. Spain

INTRODUCTION

The last years have seen dramatic advances in our understanding of cellular adhesion. The mechanisms of leukocyte interactions with other cells and with the extracellular matrix can now be explained in terms of molecular recognition mediated by cell membrane glycoproteins of three supergene families: IMMUNOGLOBULINS, INTEGRINS AND SELECTINS. Processes such as immune recognition, leukocyte migration into inflammatory sites and lymphoid organs, and tumor metastasis have been found to be dependent on the function of these adhesion molecules.

The identification and characterization of new adhesion molecules and their ligand specificity, the molecular mechanisms of their interaction with counter-receptors and cytoskeleton, and their effect on the regulation of gene expression are areas of very active research. The determination of the relevant functional sites on the leukocyte receptors and their three-dimensional structure are of great interest to allow the definition of highly potent therapeutic tools to prevent adverse immunological and inflammatory reactions in different critical pathological stiuations.

The Workshop held in Cuenca was designed to bring together researchers actively working on the molecular mechanisms of leukocyte interactions. The following aspects were covered and discussed: adhesion receptors from the Ig superfamily, structure-function of integrins, functional regulation of integrins, structure function of homing receptors, adhesion receptors and signal transduction and role of adhesion receptors in inflammation.

FIRST SESSION
IMMUNOGLOBULIN SUPERFAMILY

A. F. WILLIAMS
P. MOINGEON
S. J. BURAKOFF
B. MALISSEN
J. A. LÓPEZ DE CASTRO
R. R. LOBB

The Structure of the CD2 T Lymphocyte Antigen and Its Function in Signal Transduction

Alan F. Williams+, Paul Driscoll*, Iain Campbell*, Jason Cyster+, Louise Spruyt+, Albert Beyers+ and Martin Glennie^

+MRC Cellular Immunology Unit, Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE; *Department of Biochemistry, University of Oxford, Oxford OX1 3QU; ^ Tenovus Research laboratory, Tremona Rd, Southampton SO9 4XY.

Domain 1 of the rat CD2 antigen was expressed as a fusion protein with glutathione S-transferase in the pGEX expression system and the product was purified on a glutathione agarose affinity column. The CD2 domain was released from the fusion protein by cleavage with thrombin and purified by antibody affinity chromatography and gel filtration. The CD2 domain was expressed in a form labelled with ^{15}N as well as in the ^{14}N form. The structure of the CD2 domain was solved using NMR techniques to reveal an Ig V-domain fold. In the core of the fold the disposition of β-strands was very similar to that of strands in Ig V_L and V_H domains and CD4 domain 1. Residues previously determined to be involved in the binding of LFA-3 by Peterson & Seed were found to map to the face of one of the β-sheets making up the Ig-like fold.

The requirements for signal transduction via CD2 were investigated in terms of which other molecules were necessary to be expressed at the cell surface for CD2 to function. Previous studies had showed that CD2 functioned in T cells or NK cells and that signalling was poor in T cell lines that lacked a T cell receptor (TCR). Thus it was thought that in NK cells, which are TCR negative, there must be another molecule that was functionally equivalent to the TCR. One candidate was the CD16 Fc receptor which is expressed at the cell surface along with γ-chain which is related in sequence to ζ-chain of the TCR complex. On T cells CD16 can be expressed in a complex with ζ-chain and it seemed possible that signalling might occur on a TCR negative cell line that expressed the CD16:ζ complex. Jurkat cells of this phenotype were thus constructed and at first signalling in terms of a rise in cytoplasmic free $[Ca^{2+}]$ was observed using $F(ab')_2$ anti-CD2 MAb crosslinked with $F(ab')_2$ rabbit anti-mouse IgG second antibody. However IgG was found to be a contaminant in the second antibody at a level of 1% and when this was removed the signal transduction effect was lost. Signal transduction on NK cell lines was then reinvestigated and it was found that crosslinking of CD2 gave a Ca^{2+} signal only when there was some IgG contaminating the $F(ab')_2$ preparations. It is thus concluded that the presence of ζ-chain at the cell surface is not sufficient for signalling via CD2 and that efficient signalling does not occur on NK cells unless the Fc receptor is bound to the crosslinked CD2 complex.

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF HUMAN CD2:

Philippe MOINGEON and Ellis L.REINHERZ, Laboratory of Immunobiology, Dana Farber Cancer Institute, Harvard Medical School, Boston, USA.

CD2 facilitates T cell responses by mediating adhesion between T lymphocytes and their cognate partners and by transducing activation signals.

We have demonstrated that in T lymphocytes signal transduction through the CD2 molecule requires surface expression of the CD3-Ti antigen/MHC receptor complex(TCR). Functional characterization of various forms of CD2 with serial truncations within the cytoplasmic domain revealed that the cytoplasmic domain of CD2 plays an essential role in transduction of activation signals and that the segment between residues 253 and 287 is necessary for activation. To analyse the apparent TCR independence of CD2 signaling in NK cells, we transfected a cDNA encoding the transmembrane form of CD16 (the low affinity receptor for the Fc fragment of IgG) into TCR- variants of the Jurkat cell line. This results in surface expression of CD16 in association with endogenous CD3 zeta homodimers, and reconstitutes CD2 signaling as seen by calcium flux and IL2 production. To test the role of CD3 zeta in CD2 triggering, we then transfected human CD2 into each of two murine T-T hybridomas which express TCR containing either a full-length CD3 zeta subunit or a truncated CD3 zeta subunit. Given that CD2-mediated signaling is observed only in the T cells containing wild type CD3 zeta these findings demonstrate that CD2 signal transduction is dependent upon the CD3 zeta subunit.

The CD2 adhesion domain is encoded by a single exon (exon 2),and binds to LFA-3 with a micromolar Kd.The affinity of the CD2 extracellular segment for LFA-3 is not affected by truncations in the CD2 cytoplasmic domain which abrogates signaling.In addition the adhesion function of such truncated versions of human CD2 expressed into murine T cells was sufficient in itself to facilitate T lymphocyte responses to antigenic stimulation. In contrast to the LFA-1 pathway of adhesion, protein kinase C activation, TCR stimulation or increasing intracellular levels of cAMP have no detectable effects on CD2 mediated adhesion as seen using cell-cell binding assays. The TCR independence of CD2 adhesion function implies a critical role of the CD2 pathway of adhesion in initiating cell-cell interactions prior to TCR engagement and LFA1-ICAM-1 binding and suggests a sequential rather than parallel involvement of adhesion pathways during T cell responses.

The role of CD4 and CD2 in T cell activation

Steve J. Burakoff[o], Tassie Collins[o], William Hahn[o], Robert Mittler[•] and Barbara E. Bierer[o+]

We have studied the role of several T cell co-receptors in T cell activation. Recently, we have observed several novel functions associated with the CD4 and CD2 molecules. By fluorescence resonance energy transfer (FRET), we have previously demonstrated that upon anti-CD3 monoclonal antibody mediated activation of a $CD4^+$ T cell hybridoma, CD4 moves into close association with the T cell receptor (TCR)/CD3 complex. It was shown that this association between CD4 and the TCR/CD3 complex was dependent upon the presence of an intact CD4 cytoplasmic domain. We have now expressed, in a murine T cell hybridoma, mutated forms of CD4 containing cysteine to serine point mutations at positions 420, 422, or 430. The mutations at positions 420 and 422, but not 430, abolish association with $p56^{lck}$. Using FRET, we demonstrated that mutations of CD4 which fail to interact with $p56^{lck}$ are unable to associate with the TCR/CD3 complex under conditions in which wild-type CD4 and the 430 mutant CD4 do associate with the TCR/CD3 complex. We propose that the association between CD4 and the TCR/CD3 complex during T cell activation is dependent on the ability of CD4 to interact with $p56^{lck}$, suggesting that a substrate for $p56^{lck}$ may be expressed in the TCR/CD3 complex.

While the TCR/CD3 complex confers the ability to recognize specific antigen, the T cell-specific proteins CD2 and lymphocyte function-associated molecule-1 (LFA-1, CD11a/CD18), by binding to their ligands on target cells, provide the T cell with two major mechanisms for regulating cellular adhesion. The T cell glycoprotein CD2 not only increases intercellular adhesion but also plays a direct role in T cell activation. We have now demonstrated that the interaction of CD2 with LFA 3 is regulated by TCR/CD3 signaling. Using human peripheral blood T cells and murine T cell hybridomas expressing human CD2, we showed that TCR/CD3 crosslinking by specific antigen or monoclonal antibodies rapidly increases the avidity of adhesion between cell surface CD2 and immunoaffinity purified LFA-3. Upregulation of CD2 avidity was dependent on both protein kinase C (PKC) and tyrosine kinase activity. Using murine T cell hybridomas expressing human CD2 cytoplasmic domain deletion mutants, we showed that the five carboxyterminal amino acids of the CD2 cytoplasmic domain are essential for TCR/CD3-induced increases in CD2 avidity but not for CD2-mediated T cell activation. These observations establish that the cytoplasmic portion of CD2 consists of distinct functional domains.

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The T cell receptor-CD3 complex is composed of at least two autonomous transduction modules.

Anne-Marie Wegener, François Letourneur, and Bernard Malissen.

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Antigen recognition by most T cells is mediated through a clonally expressed $\alpha\beta$ heterodimer know as the antigens-specific T cell receptor (TcR). This TcR is noncovalently associated with a set of five polypeptides denoted CD3-γ, -δ, -ϵ, -ζ and -η and thought to be involved in signal transduction. CD3-γ, -δ, and -ϵ chains are structurally and genetically related. The CD3-ζ subunit exist predominantly as a 32 kD homodimer within the TcR-CD3 complex and is structurally and genetically distinct from the other CD3 polypeptides.

To analyze the functional role of the CD3-ζ chain, we have created partial or almost complete deletions of the CD3-ζ gene and transfected it into a CD3-ζ negative thymoma variant. In parallel, a set of CD8α/CD3ζ chimeric molecules was constructed and expressed by gene transfer into the BW5147 $\alpha^-\beta^-$ variant of the BW5147 thymoma.

Deletion of the cytoplasmic tail of the CD3-ζ does not dramatically affect the surface expression of the reconstituted TCR-CD3 complex. Moreover the surface expressed TcR-CD3 complex expressing the tailless CD3-ζ polypeptide are still able to stimulate IL-2 production in response to antigen, superantigen or the anti-CD3-ϵ monoclonal antibody 2C11. However, in contrast to wild-type TcR-CD3 complexes, the above mutated complexes do not transduce signals initiated via other surface molecules such as Thy-1 or Ly-6.

When considered together with the analysis of the functional ability of the CD8α/CD3-chimeras, these results suggest that the TcR/CD3 complex is composed of at least two autonomous transduction modules (i.e. ζ-ζ and $\gamma\epsilon\delta\epsilon$). Furthermore, each of these modules may be differentially connected to accessory molecules such as Thy-1, Ly-6 or CD2.

CLONAL HETEROGENEITY IN LFA-3 AND ICAM-1 REQUIREMENT FOR LYSIS BY ALLOREACTIVE T LYMPHOCYTES. **Begoña Galocha, Daniel López, Susana Rojo and José A. López de Castro. Fundación Jiménez Díaz y C.S.I.C. Avda. Reyes Católicos, 2 28040 Madrid (Spain).**

HLA-B27-specific cytotoxic T Lymphocytes are heterogeneous in their capacity to lyse the murine mastocytoma cell line P815 transfected with HLA-B27. Failure to kill mouse transfectants may be due to insufficient avidity of the human effector cells towards the murine targets, as these lack some of the ligands for human T cell adhesion molecules. Alternatively, it might imply alteration of HLA-B27-specific T cell epitopes upon expression of HLA-B27 on mouse cells. To discern between these alternatives two approaches were undertaken. First, various anti-B27 CTL clones were selected with highly similar fine specificity, as measured with a series of HLA-B27 structural variants, but differing in avidity on the basis of blocking experiments with an anti-CD8 monoclonal antibody. For these particular clones, their capacity to lyse mouse transfectants correlated directly with avidity (1). Second, P815 cells were cotransfected with HLA-B27, human ICAM-1 and LFA-3, and these transfectants were used as targets against a series of HLA-B27 specific CTL clones. Some clones with low lytic activity towards HLA-B27$^+$ P815 cells showed markedly increased lysis of the transfectants co-expressing human adhesion molecules. However, a significant fraction of the CTL examined were unable to kill mouse targets regardless of the presence of human ICAM-1 and LFA-3. These results strongly suggest that many HLA-B27-specific epitopes are altered upon expression on mouse cells. Presumably this is due to involvement of tissue and/or species-specific peptides in recognition by the corresponding CTL clones. Subtle alterations of these epitopes, probably reflecting recognition of distinct but structurally related peptides, can also be detected for clones recognizing mouse transfectants. This was shown by demonstrating changes in the fine specificity patterns of one such CTL clone in the killing of a series of HLA-B27 mutants expressed on either human or mouse target cells (2).

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2.- Rojo, S., López, D., Calvo, V., and López de Castro, J.A. (1991). J. Immunol. 146: 634-642.

VASCULAR CELL ADHESION MOLECULE 1: CURRENT STUDIES

ROY R. LOBB, BIOGEN INC., CAMBRIDGE, MA, U.S.A.

Vascular cell adhesion molecule 1 (VCAM1) is a member of the immunoglobulin (Ig) superfamily expressed on endothelium at inflammatory sites in vivo or stimulated by cytokines in vitro. VCAM1 is also expressed on macrophage-like and dendritic cells in both normal and inflammed tissues. VCAM1 binds to the integrin VLA4, present on all leukocytes except neutrophils, and helps recruit these cells to inflammatory sites in vivo. Two forms of VCAM1 are generated in vitro by alternative splicing, encoding proteins with six and seven Ig-like domains, respectively. No tissue- or cell-type specific expression of either form of VCAM1 has been detected to date, and both forms support VLA4-dependent cell adhesion. A truncated cDNA for VCAM1 has been constructed, stably expressed in CHO cells, and the secreted recombinant soluble form of VCAM1 (rsVCAM1) purified by immunoaffinity chromatography. Immobilized rsVCAM1 is a functional adhesion protein which selectively binds only VLA4-expressing cells. T cell subset analyses indicate preferential binding of memory T cells, and of CD8+ versus CD4+ T cells. A cDNA encoding murine VCAM1 has been cloned, contains seven Ig-like domains, and shows 76% identity with human VCAM1. Transient expression in COS cells generates a functional adhesion molecule which binds both human and murine lymphoblastoid cell lines in a VLA4-dependent manner.

SECOND SESSION

INTEGRIN FAMILY

T. A. SPRINGER
C. FIGDOR
N. HOGG
J. C. GUTIÉRREZ-RAMOS
K. OKUMURA
F. SÁNCHEZ-MADRID
S. SHAW
A. L. CORBI-LÓPEZ

COOPERACTION BETWEEN LEUKOCYTE ROLLING ON A SELECTION AND ADHESION STRENGTHENING THROUGH INTEGRINS

Timothy A. Springer. The Center for Blood Research, INC. BOSTON, MA. (USA).

Rolling of leukocytes on vascular endothelial cells, an early event in inflammation, can be reproduced in vitro on artificial lipid bilayers containing purified CD62, a selectin also named PADGEM and GMP-140 that is inducible on endothelial cells. Neutrophils roll on this selectin under flow conditions similar to those found in postcapillary venules. Adhesion of resting or activated neutrophils through the integrins LFA-1 and Mac-1 to ICAM-1 in a lipid bilayer does not occur at physiologic shear stresses; however, static incubation of activated neutrophils allows development of adhesion that is greater than 100-fold more shear resistant than found on CD62. Addition of a chemoattractant to activate LFA-1 and Mac-1 results in the arrest of neutrophils rolling on bilayers containing both CD62 and ICAM-1. Thus at physiologic shear stress, rolling on a selectin is a prerequisite for activation-induced adhesion strengthening through integrins.

Activation of LFA-1 : Role of cations.

Carl Figdor and Yvette van Kooyk.
Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

Despite the fact that lymphocytes express both LFA-1 and ICAM-1 they do not spontaneously bind to each other, indicating that additional signals are required to induce cell adhesion. Recent data showed that LFA-1 can be activated through different cell surface receptors expressed by lymphocytes, such as CD2, CD3 or MHC class II molecules. Activation of LFA-1 by these receptors involves signals transduced via PKC, which may lead to phosphorylation of the LFA-1β chain. However, also intracellular Ca^{2+} levels are generated upon triggering of these receptors, with kinetics that correlate directly with LFA-1 dependent adhesion. We have made a unique anti-LFA-1 antibody (NKI-L16) which activates the LFA-1 molecule, resulting in enhanced adhesion to its ligand ICAM-1. The antibody induces homotypic aggregation of lymphocytes and monocytes and stimulates adhesion of these cells to endothelium and L cells transfected with ICAM-1 (L-ICAM-1). Monoclonal antibody NKI-L16 recognizes a unique Ca^{2+} dependent epitope on LFA-1. We showed that the surface expression of the epitope recognized by NKI-L16 correlates with the activation/maturation state of the cells. Expression is low on resting lymphocytes and monocytes, whereas it is induced upon in vitro activation of these cells by cytokines. Furthermore expression of the L16 epitope correlates well aendothelial cells, and can thereby determine participation of LFA-1 in cell adhesion. From these data it is concluded that LFA-1 can be expressed on the cell membrane in an inactive, intermediate aand active state, in which both Ca^{2+} and Mg^{2+} cations play an important role.

LEUKOCYTE INTEGRIN ACTIVATION

Nancy Hogg, Carlos Cabañas and Ian Dransfield

Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX

LFA-1 is a β_2 integrin which mediates adhesive interactions when leukocytes are activated, for example during an immune response. It requires the divalent cation Mg^{2+} to function and has three putative "EF-hand"-like cation binding domains. The binding of LFA-1 to its ligand ICAM-1 is carefully regulated in order to prevent random cellular clustering or aggregation. There are presently thought to be two ways by which an increase in receptor avidity might occur. Receptor clustering would give an increase in avidity of binding to ligand, simply by increasing the opportunity for the interaction to take place and changes in conformation of the receptor could potentially cause an increase in affinity of binding of individual receptor/ligand pairs. The use of monoclonal antibodies defining activation states of the integrins such as the β3 integrin GPIIbIIIa, have indicated that conformational changes occur extracellularly which correlate with observed induction of functional activity.

We have characterised a monoclonal antibody (mAb) named 24 which has been shown to bind to a novel epitope present on the α subunits of the three leukocyte integrins, LFA-1, CR3 and p150,95 (Dransfield *et al*, EMBO J 12, 3759, 1989; Dransfield *et al*, Imm. Reviews 114, 29, 1990). The effect of mAb 24 on leukocyte integrin function has been analysed using systems which are leukocyte integrin-dependent, for example antigen presentation by monocytes to T cells (Dransfield *et al*, submitted). This LFA-1-dependent function was found to be inhibited by the presence of mAb 24, implicating the recognised epitope as a functionally important region of the leukocyte integrins. Recent work has centred around activation of leukocyte integrins, using the 24 epitope as a "reporter" of such activation with the hope that an understanding might be gained as to how mAb 24 blocks integrin mediated events.

A second novel feature of the 24 epitope is that its recognition in the intact heterodimer is Mg^{2+}-dependent and for intact cells, expression parallels receptor activity. We have therefore suggested that control of the affinity of Mg^{2+} binding represents a potential mechanism for regulation of receptor function (Dransfield, EMBO J 12, 3759, 1989). Cross-linking studies have shown that the RGD sequence recognised in matrix proteins by the β3 receptors binds within the cation binding domains, suggesting an association between presence of divalent cation and ligand binding to integrin. In spite of the fact that neither ICAM-1 nor ICAM-2, possesses such an RGD sequence, we wondered whether mAb 24 would directly block binding of LFA-1 to these ligands. The result has been that binding of LFA-1 to ICAM-1 or ICAM-2 is unaffected by the presence of mAb 24 (Dransfield *et al*, submitted). One suggestion would be that the mode of binding of ligand by integrins may depend on the type of integrin and/or target protein.

A plausible explanation for the ability of mAb 24 to inhibit β_2 integrin functions has come from another set of experiments, in which the crosslinking of the CD3/TcR complex causes an increase in the ability of LFA-1 to bind to ICAM-1. These experiments have indicated that mAb 24 may be locking LFA-1 in the high avidity or adhesive mode of ICAM-1 binding preventing receptor/ligand deadhesion (Dransfield *et al*, submitted). Why transient adhesion between LFA-1 and ICAM-1 is important for progress in an immune response is not yet certain. It may be simply a logistical problem, allowing access of large numbers of T cells to antigen presenting cells for a brief period of time. Alternatively, there is present interest in the possibility that LFA-1 mediates crucial intracellular signalling required for the T cell programming leading to proliferation. These issues are currently under investigation. Whether this type of transient activation is a general feature of the functioning of all integrins has still to be determined.

IN VITRO INDUCTION OF EMBRYONIC STEM CELSS TO DEVELOP INTO T- AND B- CELL PROGENITORS

José C. Gutiérrez-Ramos. Basel Institute for Immunology.
Basel (Switzerland)

We have defined culture conditions that support in vitro the differentiation of the embryonic stem cell line D3 into T-cell progenitors, B-cell progenitors and myeloid cells as assesed, at various times of culture, by FACS with a panel of monoclonal antibodies against several surface markers (Thy1.2, Pgp1, B220, Joro37.5, IgM, F4/80 and Mac-1). We found that different bone marrow stromal and fetal liver stromal cell lines have distinct capacities of inducing differentiation of D3 stem cells into T- and B-cell progenitors ($Thy1^+Pgp1^+Joro37.5^+$ or $B220^+IgM^-$) and myeloid cells ($F4/80^+$ $Mac1^+$). Such functional properties of the different stromal cell lines can be distinctly modified by recombinant interleukins (IL3,IL4, IL5,IL6 and IL7) when added at different times of culture. Thus, we have been able to define a precise sequence of signals required to induce in vitro embryonic stem cells into T- and B-cell progenitors or myeloid cells Under certain culture conditions, the D3 stem cells develop preferentially along a particular pathway of haemopoietic differentiation.Also , if the sequence of signals provided (by stromal cell lines and interleukins) is altered, the final products as well as the efficiency of the system dramatically change. We shall also present data concerning the functional potential of the lymphoid progenitors generated in vitro by the D3 ES cells.

Murine lymphocyte adhesion molecules involved in cellular interactions and activation

Ko Okumura, M.D. and Hideo Yagita, Ph.D.

Department of Immunology
Juntendo University School
of Medicine

We recently found that some T cell surface molecules other than CD2 and LFA-1 might be involved in the LAK cell cytotoxicity against certain target cells. In order to characterized such unknown molecules, we established a mAb (RMV-7) which could inhibit CD2/LFA-1-independent LAK cell cytotoxicity and binding to target cells at the effector site. The antigen identified by RMV-7 was predominantly expressed on myeloid cells in vivo but appeared on splenic T cells late after mitogenic stimulation, which is a character of very late activation antigens (VLA). The RMV-7 antigen was a noncovalently linked heterodimer composed of a 140-kDa α-chain and 95-kDa β-chain, which is reminiscent of integrin superfamily molecules. RMV-7 blocked LAK cell binding to fibronection (FN), fibrinogen (FB), and vitronectin (VN) but not that to laminin or type IV collagen, indicating that the RMV-7-defined molecule (gp140/95) is a unique extracellular matrix (ECM) receptor. Purified gp140/95 by RMV-7 affinity chromatography was cross-reactive with rabbit polyclonal antibodies to human VN receptor but not with those to human FN receptor, suggesting that the gp140/95 may be the murine VN receptor ($\alpha_v\beta_\epsilon$) or its relating molecule. One of its ligand, FN, was found on the surface of several target cells, against which LAK cell cytotoxicity was inhibited by RMV-7, and LAK cell cytotoxicity againt them was blocked by anti-FN antibody at the target site. Similarly, cytotoxicity of a H-2^d-specific CTL clone was inhibited by RMV-7 and anti-FN antibody as well. These results indicate that a VLA-like ECM receptor on murine CTL and LAK cells contributes to target cell biniding and cytotoxicity.

We next addressed a possible involvement of the gp140/95 in T cell activation, and if was revealed that FN, VN, and, to a lesser extent, FB as well as RMV-7 synergized to induce the serine esterase (SE) release from a CTL clone (OE4) and the IL-2 production by a helper T cell hybridoma (2B4) when co-immobilized with a suboptimal dose of anti-CD3 mAb. In addition, immobilized FN or VN alone could induce both responses. All these co-stimulatory and stimulatory effects of ECM proteins were blocked by soluble RMV-7, indicating that the gp140/95 mediates all these signals.

These findings indicate that the gp140/95 not only mediates cell-ECM and cell-cell adhesion but also delivers a signal that augments T cell antigen responses and that triggers effector functions of pre-activated T cells in an antigen-independent manner. This implies an important role of T cell surface integrins in regulating T cell responses.

REGULATION OF VLA-4 ADHESIVE FUNCTIONS

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The human integrin VLA (very late activation antigens)-4 (CD49d/CD29), the leukocyte receptor for both the CS-1 region of plasma fibronectin (Fn) and the vascular cell surface adhesion molecule-1 (VCAM-1) also mediates homotypic aggregation upon triggering with specific anti-VLA-4 monoclonal antibody (mAb) (1). Epitope mapping of this integrin on the human B-cell line Ramos, performed with a wide panel of anti-VLA-4 mAb by both cross-competitive cell binding and protease sensitivity assays, revealed the existence of three topographically distinct epitopes on the α4 chain, referred to as epitopes A-C. By testing this panel of anti VLA-4 mAb for inhibition of cell binding to both a 38 kDa Fn fragment containing CS-1 and to VCAM-1, as well as for induction and inhibition of VLA-4 mediated homotypic cell adhesion, we have found overlapping but different functional properties associated with each epitope. Anti-α4 mAb recognizing epitope B inhibited cell attachment to both Fn and VCAM-1, whereas mAb against epitope A did not block VCAM-1 binding and only partially inhibited binding to Fn. In contrast, mAb directed to epitope C did not affect cell adhesion to either of the two VLA-4 ligands. All mAb directed to site A, as well as a subgroup of mAb recognizing epitope B (called B2), were able to induce cell aggregation, but this effect was not exerted by mAb specific to site C and by a subgroup against epitope B (called B1). Moreover, although anti-epitope C and anti-epitope B1 mAb did not trigger aggregation, those mAb blocked aggregation induced by anti-epitope A or B2 mAb. In addition, anti-epitope A mAb blocked B2-induced aggregation, and conversely, anti-epitope B2 mAb blocked A-induced agggregation. Further evidence for multiple VLA-4 functions is that anti-Fn and anti-VCAM-1 antibodies inhibited binding to Fn or VCAM-1, respectively, but did not affect VLA-4-mediated aggregation. In summary, we have demonstrated that there are at least three different VLA-4-mediated adhesion functions, we have defined three distinct VLA-4 epitopes, and we have correlated these (2).

We have found that the VLA protein β1 subunit is also involved in the induction of homotypic cell aggregation. We have obtained three novel anti-β1 mAb with the ability to induce cell aggregation on different leukocyte cell types. These mAb recognize an antigenic site on the common β1 chain of VLA proteins which is either topographically or functionally different from those defined by previously described anti-β1 mAb is epitope specific, isotype and Fc independent, and displays cell requirements and kinetics similar to α4-mediated aggregation. For Ramos cells (which bear only the VLA α4β1 heterodimer), cell adhesion induced through the VLA-β1 chain could be selectively inhibited by other anti-β1 mAb or by anti-α4 mAb. Interestingly, anti-β1 mAb, which had minimal effect on the $\alpha2^-$ $\alpha4^-$ $\alpha5^+$ K-562 cell line, induced strong aggregation of VLA-α2 or VLA-α4 transfected K-562 cells. Furthermore, the β1-mediated induction of cell aggregation on α2-K562 and α4-K562 transfected cells was blocked by preincubation with either anti-α2 or anti-α4 mAb, respectively, as well as by other anti-β1 mAb. Together these results provide strong evidence supporting the involvement of either the α2/β1 or α4/β1 heterodimers in the induction of intercellular interactions and underline the pivotal role of the common β1 chain of VLA proteins in the integrin-mediated induction of cell aggregation (3).

The functional activity of integrins is regulated during cellular activation. We investigated the role of different cell activation pathways in the regulation of T cell binding to both VLA-4 ligands. Either activation of protein kinase C (PKC) or increments in the iCa^{++} levels enhanced VLA-4-mediated binding to Fn and VCAM-1. The increased attachment to Fn and VCAM-1 upon cell activation was independent of both RNA and protein synthesis and did not imply any alteration in the phosphorylation and proteolysis patterns of VLA-4. These data altogether indicate that VLA-4 undergoes a qualitative change upon activation of PKC or rise of the iCa^{++} levels that increases adhesion to both ligands (4).

We have also addressed the question whether a regulated expression and function of VLA-4 receptors could exist in "in vivo" activated T cells in a pathological activation. Therefore, we examined the expression and function of VLA-4 fibronectin receptors on T cells localized in the inflammed synovium of patients with rheumatoid arthritis (RA). A high proportion of T cells in both synovial membrane (SM) and synovial fluid (SF) expressed the activation antigens AIM (CD69) and gp95/85 (Ea2) as well as an increased number of VLA-4α and β1 adhesion molecules, as compared with peripheral blood (PB) T cells from the same patients. Furthermore, the majority of these activated SF and SM T cells were able to adhere to both a 38 kD Fn proteolytic fragment containing the connecting segment-1 (CS-1) and to a recombinant soluble form of the endothelial cell ligand VCAM-1, specifically through VLA-4 receptors, whereas a significantly lower proportion of PB T cells displayed this capacity. Therefore, our results show that activated T cells selectively localize at sites of tissue injury in RA disease and provide evidence for the in vivo regulation of the expression and function of the VLA-4 integrin. This regulatory mechanism may enable T cells either to facilitate migration or to persist at sites of inflammation (5).

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ADHESION MOLECULES IN POST-THYMIC T CELL DIFFERENTIATION

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We have been systematically exploring changes in expression/function of adhesion molecules with T cell differentiation. The results confirm and extend the concepts that adhesion molecules are regulated with differentiation and that adhesion molecules often mark T cell subsets. At least four molecular pathways mediate binding of normal human T cells to endothelial cells, when studied in the in vitro model system of CD4+ T cell adhesion to cytokine-stimulated cultured umbilical vein endothelial cells (HUVEC): T cell LFA-1 binding to ICAM-1 and an alternative ligand (presumably ICAM-2), T cell VLA-4 binding to VCAM-1, and T cell binding to ELAM-1. Operationally we use CD45 isoform expression to distinguish two fundamentally different subsets of peripheral T cells: naive and memory. The receptors on T cells which mediate these adhesion pathways (and a number of others) are preferentially expressed on memory cells. All four of these pathways preferentially mediate binding of memory T cells. T cell binding to HUVEC is thought to model T cell interactions with flat endothelium in non-lymphoid tissue and therefore these findings are consistent with the predominance of memory cells in non-lymphoid sites; in addition they help explain the preferential responsiveness of memory cells in many cellular interactions. We have now sought molecular mechanisms to account for preferential entry of naive cells into lymphoid tissue. LECAM-1 (LAM-1, Leu-8, MEL-14) is preferentially expressed on naive cells and certainly contributes to this entry. We have explored two additional molecular mechanisms of preferential adhesion of naive T cells: CD31 and CD7.

Our work indicates that CD31 is likely to be an important participant in a T cell adhesion cascade. CD31/PECAM-1/endoCAM has been inferred to mediate adhesion between leukocytes/platelets and endothelial cells and therefore potentially function in immunity, inflammation and wound healing. We report four novel features of CD31 on T lymphocytes: 1) Unique subsets of CD4+ and CD8+ T cells, including all CD8 naive cells, express CD31; 2) Purified CD31 is sufficient to mediate T cell adhesion; 3) Engagement of CD31 powerfully induces the adhesive function of $\beta 1$ and $\beta 2$ integrins, particularly among naive T cells. 4) CD31 preferentially induces binding to VCAM-1, fibronectin and laminin which are particularly important in T cell capture by endothelium and subsequent migration; in contrast, crosslinking of the CD3/T cell receptor preferentially induces binding to ICAM-1, which is of paramount importance in antigen-specific recognition. Thus, CD31 functions in an "adhesion cascade" by inducing integrin-mediated adhesion of selected T cell subsets. CD31 may be particularly important in T cell interactions with endothelium, especially in high endothelial venules (HEV) of lymph nodes through which naive cells recirculate.

CD7, one of the earliest T-cell specific differentiation markers, is also capable of inducing adhesion mediated by integrins on resting T cells. CD7 is preferentially expressed on naive cells, and induces better integrin-mediated adhesion of naive cells than memory cells.

Thus, adhesion is regulated with differentiation in two distinct ways: change in level of expression of adhesion receptors (e.g. increased expression of adhesion receptors on memory cells); and change in expression/function of molecules which regulate adhesion (e.g. increased adhesion-induction by CD7 on naive cells).

PRELIMINARY STUDIES ON INTEGRIN GENE STRUCTURE AND REGULATION OF EXPRESSION

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Unlike most of the members of the VLA family, which are widely expressed, the expression of the adhesion glycoproteins LFA1, Mac-1 and p150,95 is absolutely restricted to leukocytes: LFA1 is found on all leukocytes while Mac-1 and p150,95 are mainly expressed by cells of the myeloid lineage and their expression is regulated during differentiation and activation. For this reason, we decided to study the regulation of the expression of the alpha subunits of these two integrins in order to characterize the mechanisms involved in their constitutive and regulated expression.

Initially we have used the U937 promonocytic cell line, whose treatment with phorbol esters drives them along the monocyte/macrophage differentiation pathway, and leads to their acquisition of functional and phenotypic properties of macrophages. The induction of the expression of p150,95 and Mac-1 is one of the changes that takes place during such a proccess. U937 cells do not express mRNA for the alpha subunits of Mac-1 and p150,95, while LFA1 and CD18 are constitutively expressed. After PMA treatment, mRNA for CD11b and CD11c appears and reaches a maximum 48-72 hours later. The CD11c mRNA induction was found to be dependent on PKC activation, since a PKC inhibitor (H7) completely abolished the induction of CD11c mRNA. Moreover, addition of cycloheximide to U937 cells caused the appearance of CD11c mRNA, suggesting that mRNA stability may be an important factor controlling the induction of CD11c mRNA. Preliminary results using cicloheximide and actinomicin D, and nuclear run-on experiments suggest that the induction of CD11c MRNA is also controlled at the transcriptional level. The half-life of the p150,95 alpha subunit transcripts after induction appears to be around 5-8 hours.

An additional demonstration that the induction of the CD11c expression has a physiological significance has been obtained through the study of the pattern of leukocyte integrin expression during B cell activation. Like U937 cells, tonsilar and peripheral blood B lymphocytes constitutively express LFA1, but not Mac-1 or p150,95. Activation of B cells with a number of agents like PMA, or anti-IgM or SAC plus cytokines, induces a change in the pattern of expression by causing the appearance of CD11c on the cell membrane. The induction of cell surface expression of CD11c was preceded by the appearance of CD11c mRNA, as demonstrated by Northern blot. The CD11c mRNA could be detected as early as 24 hours after induction, and appeared to reached a maximum after 72 hours. These results demonstrate that the induction of CD11c expression is not restricted to cells of the myeloid lineage, and that p150,95 may play an important role in the adhesive properties of both myeloid and activated B cells.

To analyze the molecular basis for the constitutive and regulated expression of the leukocyte integrin p150,95, we decided to identify the regulatory regions involved in these proccesses, and located its promoter region. Primer extension analysis and nuclease S1 assays allowed us to identify several transcription initiation sites for CD11c mRNA, although one of them seems to be the predominant. Sequence analysis around this major transcription initiation site revealed the absence of TATA box, consistent with the existence of several transcription initiation sites, the presence of a sequence resembling a CAAT box at around -48 bp., and the existence of a consensus site for binding of the ubiquitous Sp1 factor. Interestingly, the sequence in the major transcription initiation site is almost coincident with the INITIATOR sequence identified in the B-cell-specific gene coding for terminal deoxinucleotidil transferase, and such a similarity has also been found in the case of the transcription initiation site of the integrin gpIIb. A number of potencially interesting sequences have been also located within the CD11c promoter. First, a perfect consensus AP1 sequence, which could explain the inducibility of the CD11c gene after PMA treatment in both myeloid and B cells. The CD11c promoter also contains two sequences matching the IL-6 responsive element, and located 13 bp. away from each other, as described for some IL6 inducible genes, like the alpha2-macroglobulin gene. In this sense it is interesting to note that CD11c has been recently reported to be induced on IL-6 treated U937 cells, although to a lower extent than CD11b. Finally, a consensus octamer sequence is found at 170 bp 5' from the major initiation site, and is followed by a sequence resembling the heptamer which is usually located adjacent to the octamer, both of them known to contribute to the activity of the immunoglobulin heavy-chain promoters. Additional sequences resembling the E3 motiff of the heavy and light chain enhancers are also present within the promoter. Although the functional significance of these sequences remains to be demonstrated, it is tempting to speculate about their role on the CD11c induction on activated B cells.

To fully demonstrate the promoter activity of this region, we have performed CAT assays with a region from -960 to +45 ligated to the CAT gene. Transfection of this construct into HeLa cells revealed the promoter activity of this fragment, and strongly suggested that the tissue-specificity of the CD11c expression is not contained within this region. In any event, the promoter activity is weak both in myeloid (U937) and non-myeloid cells.

Since the pattern of expression of Mac-1 is almost coincident with that of p150,95, and together with LFA1 they are restricted to leukocytes, we are actually pursuing the isolation of the regulatory regions for the other two alpha subunits of the leukocyte integrins. Recently we have obtained the promoter regions of both Mac-1 and LFA-1. In the case of the alpha subunit of LFA1, several transcription initiation sites have also been mapped by primer extension, all of them lacking consensus TATA or CAAT boxes.

In addition to LFA1, Mac-1 and p150,95, VLA4 is the other integrin whose expression is almost absolutely restricted to leukocytes. Unlike the leukocyte integrins, whose expression is induced during U937 differentiation, flow cytometry revealed that the alpha subunit of VLA4 almost completely dissapears from the cell membrane after the PMA treatment. Northern blot analysis confirmed that the decrease in the cell surface expression is caused by a decrease in the mRNA steady state level for VLA4 alpha, which was barely detectable 10 hours after treatment. A similar phenomenon has also been observed after in vitro T cell activation, and suggests that during cell activation or differentiation, the expression of leukocyte integrins is differentially regulated, providing a mechanism for the selective regulation of the interaction that occur at inflammatory sites.

Apart from its pattern of expression, VLA4 also differs from the other members of the VLA subfamily by the presence of a totally unique proteclytic cleavage site. For this reason, we have been involved in the procces of isolating genomic clones for the alpha 4 gene, to determine the sequences controlling its pattern of expression. So far, we have obtained a number of clones which contain the region where the proteolytic cleavage of VLA4 occurs. The availability of such clones has allowed us to analyzed the genomic structure of the VLA4 alpha subunit around the proteolytic site. Restriction mapping and sequencing of one of these clones has shown that the proteolytic cleavage site, which has been determined by Martin Hemler's group, is encoded in an exon whose flanking sequences are homologous to corresponding exons from the genes for CD11c and gpIIb. Reverse PCR on cell lines with both forms of VLA4 indicates that all mRNA for VLA4 contain this exon, and therefore demonstrate that the distinct proteolytic forms of VLA4 arise by postranscriptional modifications, either inside the cell or on the cell membrane.

THIRD SESSION

SELECTIN FAMILY

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REGULATION OF LEUKOCYTE INTERACTIONS WITH ENDOTHELIUM THROUGH THE LEUKOCYTE ADHESION MOLECULE-1, LAM-1.

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LAM-1 (TQ1, Leu-8), is a member of the Selectin family of adhesion receptors that is expressed by most lymphocytes, NK cells, monocytes, neutrophils, thymocytes, and myeloid precursor cells. LAM-1, like its mouse homolog the MEL-14 antigen, mediates the adhesion of lymphocytes to specialized high endothelial venules (HEV) of peripheral lymph nodes. LAM-1 contains an amino terminal lectin domain followed by an epidermal growth factor (EGF)-like domain and two short consensus repeat units which precede transmembrane and short cytoplasmic domains. Studies using recombinant chimeric selectins and monoclonal antibodies that block LAM-1 function have revealed that adhesion mediated by LAM-1 may involve cooperativity between functionally and spatially distinct sites within the the lectin and EGF-like domains. In addition to HEV binding, we have recently shown that LAM-1 mediates the initial attachment of lymphocytes and neutrophils to cultured human umbilical vein endothelial cells. LAM-1 binds through a sialated cell-surface molecule that is expressed following stimulation of endothelium with inflammatory cytokines and LPS. Thus, LAM-1 function is not only regulated by selective expression of the receptor by leukocyte subpopulations, but by the restricted expression of the ligand(s) at sites of inflammation and by HEV. Additionally, LAM-1 function is influenced by a transient increase in receptor affinity for ligand immediately following leukocyte stimulation with lineage-specific stimuli. This event precedes the shedding of LAM-1 from the cell surface that is induced by leukocyte activation. However, the precise role of LAM-1 shedding in leukocyte attachment to endothelium is uncertain as many lymphocytes, monocytes and neutrophils that have recently transmigrated venules *in vivo* retain LAM-1 expression. Consistent with this observation, anti-LAM-1 monoclonal antibodies are able to significantly inhibit leukocyte transendothelial migration in an *in vitro* assay suggesting a role for LAM-1 beyond the initial attachment phase. Thus, LAM-1 and its inducible endothelial ligand constitute a new pair of adhesion molecules which regulate leukocyte/endothelial interactions at sites of inflammation.

FUNCTIONAL ASPECTS OF LECAM-1

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Recently, a new family of cell adhesion molecules have been discovered that mediate white cell-endothelial adherance during the inflammatory response. This family has been dubbed the LEC-CAM or selectin family, since it appears that they mediate cell adhesion through the offices of protein-carbohydrate interactions. One such molecule, the homing receptor, is found on all leukocytic cells and appears to mediate adhesion of lymphocytes during normal trafficking to lymphoid tissue as well as neutrophils during acute inflammatory responses. This molecule contains the requisite three protein domains found in all three members of the LEC-CAM family: an N-terminal lectin domain followed by an epidermal growth factor-like domain and two copies of a domain homologous to the short concensus repeats of complement binding proteins. We have recently found, using a recombinant homing receptor-immunoglobulin chimera (HR-IgG) that the ligand for this molecule is a ~50,000 dalton glycoprotein that is labeled by inorganic sulfate and whose expression is confined to the peripheral lymph node endothelium. In addition, the binding of this ligand to HR-IgG is calcium dependant and inhibited by the Mel 14 antibody, suggesting that it binds to the lectin domain of the homing receptor. This notion is further supported by the ability to block this interaction with polysacharrides as well as to inhibit binding by treating the ligand with neuraminadase. This latter result is consistent with the possibility that this ligand-receptor interaction is dependant upon sialic acid, a carbohydrate. We have also found that the binding of this ligand to the HR-IgG requires the other domains of the HR, most notably the complement binding-like motifs. This latter result suggests that the lectin domain may require the complement binding doomains for appropriate function. Finally, we have followed up on previous data demonstrating that the HR-IgG effectively blocks inflammation of neutrophils in vivo by examining the effect of this molecule on neutrophil adhesion in vivo by intravital microscopy. These results have shown that leukocyte rolling, a phenomenon first described over 100 years ago, appears to be mediated by the homing receptor. Thus, we have proposed a model whereby an activation event causes the neutrophil to roll along the endothelium by utilizing the homing receptor, after which a separate event triggers tight adhesion and ultimate extravasation. These results are consistent with the possibility that inhibiting adhesion mediated by the homing receptor, perhaps by using small carbohydrate-like compounds, may provide an effective anti-inflammatory drug.

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ADHESION MOLECULES INVOLVED IN LYMPHOCYTE TRAFFIC

The entry of lymphocytes into different tissues is thought to be mediated by interaction of endothelial ligands with "homing receptors" or other adhesion molecules on lymphocytes. In contrast to naive lymphocytes which more or less randomly recirculate through lymphoid tissues, activated lymphocytes, memory cells or effector cells display distinct patterns of traffic and an increased preference to home into non-lymphoid tissues. Mainly based on in vitro studies, a variety of lymphocyte molecules has been supposed to play a role in recirculation and homing, namely selectins as the leucocyte-endothelial cell adhesion molecule LECAM- 1(MEL-14 antigen), integrins as LFA-1 and ß1-integrins and the CD44 molecule.

However, the roles of these molecules seem to be more complex than previously thought and extrapolations from in vitro studies not always proove to be appropriate. Our in vivo-studies have shown that: -the selectin LECAM-1 has a key role as homing receptor for entry into lymph nodes, but is also involved in entry into mucosal lymphoid tissue, in contrast to earlier reports. It plays no significant role in entry into (normal) non-lymphoid tissues. However, studies with activated lymphocytes show that expression of functional LECAM-1 (and LFA-1) is not sufficient for entry into lymph nodes. The mechanisms regulating the traffic of such activated cells are still unknown.
A member of the integrin family, the murine α_4/β_1 and β_7-integrin called LPAM 1/2 has been proposed to play a role as homing receptor for mucosal lymphoid tissue. We could not confirm this proposal by in vivo experiments; functionally active concentrations of anti LPAM Fab failed to affect homing of normal lymphocytes into Peyer´s patches.
No role for lymphocyte recirculation through lymphoid tissues could likewise be detected for one endothelial ligand for LFA-1, the murine ICAM-1, indicating that the established function of LFA-1 in lymphocyte recirculation relies on a different ligand.

In vitro experiments are useful to identify mechanisms which may be involved in the one or other way in lymphocyte-endothelial interactions. Thus, studies on the molecules contributing to lymphocyte adhesion to endothelium in synovial tissue from rheumatoid arthritis patients has provided evidence for complex interplay of several adhesion molecules: CD44, LFA-1, LECAM-1, VLA-4 or other ß1-integrins were found to contribute to binding. In case of VLA-4 inhibitory as well as stimulating effects of antibodies were observed.

Signalling and regulation of interaction mechanisms by adhesion molecules is also indicated by studies on the CD44 molecule in the mouse, which provided evidence that this molecule not only serves as a receptor for endothelial hyaluronidate, but also regulates adhesion mechanisms distinct from this function.

In conclusion, these data indicate that tissue immigration is governed rather by sets of adhesion molecules than by single homing receptors and that signalling through surface receptors may regulate binding and migration events in addition to the mere contribution to adhesion.

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PADGEM: A LEUKOCYTE RECEPTOR ON PLATELETS AND ENDOTHELIAL CELLS--ROLE IN INFLAMMATION AND THROMBOSIS

PADGEM, recently designated CD62, is an adhesion molecule that is located on the surface of platelets and endothelial cells. This protein is stored in storage granules within unstimulated cells, and is translocated to the surface during cell activation. The protein, with a molecular weight of 140,000, is a member of the selectin family of adhesion molecules, and is characterized by a domain structure of lectin-EGF-consensus repeats-transmembrane-cytoplasmic domains.

PADGEM binds specifically to monocytes and neutrophils, and mediates cell-cell interaction between activated platelets and leukocytes, and stimulated endothelial cells and leukocytes. PADGEM is a lectin that recognizes specific lineage-specific carbohydrates on the leukocyte surfaces; components of the PADGEM ligand include Lex, sialic acid, and possibly sialic acid linked 2,6 to galactose. The PADGEM ligand is similar but distinct from the ELAM-1 ligand.

PADGEM plays a role in inflammation and thrombosis, and may also play a role in metastasis. During thrombosis, platelets become part of the growing thrombus and express PADGEM. Antibodies specific for PADGEM have been used for in vivo diagnosis and localization of thrombi by nuclear imaging techniques. Leukocytes accumulate within the growing thrombus. Antibodies to PADGEM that block the interaction of PADGEM with leukocytes prevent the accumulation of leukocytes within the thrombus. Furthermore, PADGEM-mediated binding of monocytes to platelets enhances the expression of tissue factor activity. Monocytes may be the source of intravascular tissue factor for the activation of blood coagulation.

THE SUGAR COATING AND LEUKOCYTE TRAFFICKING

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Recruitment of leukocytes to sites of inflammation and tissue injury can be viewed as occurring in three identifiable steps. They include the rolling of the cells on the activated endothelium, firm adhesion and flattening, and finally, extravasation between endothelial cells into the surrounding tissue. Evidence accumulating from a number of laboratories suggest that the initial adhesion or "rolling" event is mediated by members of the newly described LEC-CAM family including Mel-14/LAM-1 (LEC-CAM-1, homing receptor) expressed on leukocytes, and ELAM-1 and CD-62 (GMP-140/PADGEM) expressed on endothelial cells and on endothelial cells and platelets, respectively. All three members of this receptor family contain a consensus lectin or carbohydrate binding domain at the distal tip of the protein. The carbohydrate ligand for ELAM-1 on leukocytes has been identified as sialyl-Le^x (NeuAcα2,3Galβ1,4 [Fucα1,3]GlcNac-) which is known to terminate carbohydrate groups of glycoproteins and glycolipids of neutrophils and other myeloid cells (1,2). The ligand for CD-62 was proposed by Larsen, et al. to be the related carbohydrate structure Le^x (3). However, results from several laboratories have suggested that sialic acid is an integral part of the carbohydrate ligand for CD-62 since sialidase treatment of neutrophils prevents their adhesion to CD-62 (1,2). We have obtained evidence that CD-62 also recognizes sialyl-Le^x as a high affinity ligand: 1) Soluble sugars with terminal sialyl-Le^x are 30-fold more potent inhibitors of CD-62 mediated adhesion than soluble sugars terminated with Le^x. 2) Glycolipid liposomes containing carbohydrate structures with sialyl-Le^x are potent inhibitors of CD-62 mediated adhesion while liposomes with related glycolipid structures

are not. 3) CHO glycosylation mutants that contain sialyl-Le^x (LEC-11) are bound by CD-62 while those with Le^x (LEC-12) are not. Thus, it appears that the carbohydrate specificities of ELAM-1 and CD-62 are overlapping, and that sialyl-Le^x on cell surface carbohydrate groups can mediate cell adhesion by both ELAM-1 and CD-62. Further investigation of the fine specificity of these LEC-CAMs for their carbohydrate ligands will be required to evaluate the potential for differences that may be relevant to differential recognition of various myeloid and lymphoid cell types. It will also be important to establish the degree to which presentation of carbohydrate ligands by specific glycoproteins or glycolipids allow for differential recognition of cell surface glycoconjugates by the two selectins. **References:** 1) T. Springer, et al. (1991) *Nature* **349**, 196-197; 2) T. Feizi (1991) *TIBS* **16**, 84-86; 3) Larsen, et al. (1990) *Cell* **63**, 467-474; 4) Phillips, et al. (1990) *Science* **250**, 1130-1132; 5) Polley, et al. (1991) *PNAS*, In Press.

POSTER SESSIONS

INTERACTIONS BETWEEN LYMPHOCYTES AND HIGH ENDOTHELIUM IN VITRO: KINETICS OF LYMPHOCYTE BINDING AND TRANSMIGRATION

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Previous studies have shown that cultured high endothelial cells (HEC) support high levels of lymphocyte adhesion. Light microscope analysis of HEC-adherent lymphocytes has identified two lymphocyte populations: type I cells which are bound to the upper HEC surface and type II cells which are underneath the HEC layer. Analysis of HEC-adherent lymphocytes following a 20 min pulse with PVG-RT7^a lymphocytes and a 40 min chase with lymphocytes from the congenic rat strain PVG-RT7^b showed that there was a time-dependent decrease in RT7^a cells in the type II fraction. Over this time period the transfer of RT7^a cells between type I and type II fractions was 80-90%. Type I cells selectively detached from HEC layers following 60 min incubation in lymphocyte-free medium and were collected in isolation from type II cells. Transfer of type I lymphocytes to a second HEC layer showed similar levels of binding and transmigration as control cells. These results indicate that type I and type II cells represent the same lymphocyte population at different stages of interaction with the HEC layer. In addition the number of adherent lymphocytes is controlled by the HEC layer rather than representing a subpopulation of highly adhesive lymphocyte. However transmigration of lymphocytes was not an inevitable consequence of binding to the upper surfaces of HEC in this model.

DIRECT CELL CONTACT IN COGNATE AND NOT COGNATE INTERACTIONS BETWEEN T CELLS AND B CELLS

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Cognate interactions between T and B cells is often vewed as an example of the specificity of the immune system. B lymphocytes process the antigen that have interacted with their specific receptor and present peptides in their own class II MHC antigens. Then, only T cells that recognize this new complex are activated and provide helper signals to the antigen presenting B cells.

We have developed a system where human splenic B cells are activated by T cells clones with no aparent MHC restriction. Briefly, several T cell clones, differing on origen and phenotype, are added to resting B cells in the presence of IL2, but in the absence of any other B or T cell activators, B cells not only are induced to upregulate CD23 antigen, but also to differenciate into IgG and IgM secreting cells.

This T-B cooperation requires cell to cell contact, as is inhibited when the physical interaction between both subsets is avoided by a permeable membrane. More important, not only CD4 alfa,beta T cells behave as helper cells, but also CD8 alfa,beta T cells, gamma,delta T lymphocytes and even some NK clones have this capability.

These data support that once a T lymphocyte is activated, there is an upregulation or modification of some adhesion molecules facilitating the interaction with any B cell close enough, and resulting in B lymphocyte activation. As T cell receptor does not play a direct role in this T-B cell interaction, conventional cytotoxic CD8 positive T cells may behave as helper T lymphocytes.

This model of T-B interaction explains the T cell dependant antibody response to membrane antigens, for example tumoral antigens, that can not be processed by B cells nor presented in class II antigens.

REGULATED EXPRESSION ON HUMAN MACROPHAGES OF ENDOGLIN, AN RGD CONTAINING SURFACE ANTIGEN.

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Endoglin is an endothelial homodimeric membrane antigen containing the tripeptide arginine-glycine-aspartic acid (RGD), which is a recognition motif for adhesion receptors of the integrin family. We have investigated the expression of endoglin by monocyte/macrophage cells from different tissue compartments and at different stages of cell differentiation. Although endoglin is absent from peripheral blood monocytes, it is expressed by in vitro differentiated monocytes as determined by flow cytometry using the endoglin specific mAb 44G4 and 8E11. Furthermore, Northern blot analyses revealed a correlation between the presence of endoglin mRNA and the surface expression of the antigen by in vitro differentiated monocytes. Immunostaining of frozen tissue sections with the 8E11 mAb demonstrated the presence of endoglin not only in the endothelium of all the tissues studied, but also on the interstitial macrophages present in the red pulp of the spleen. Using as a model of macrophage differentiation monocytic cell lines treated with phorbol esters, we found that the reactivity of the 8E11 mAb is greatly increased on U-937 and HL-60 cells during their PMA-induced differentiation. These findings clearly demonstrate for the first time the regulated expression of the putative adhesion molecule endoglin by macrophages.

FINAL MATURATION OF HUMAN BONE MARROW CELLS CAPABLE OF SPONTANEOUS Ig-SECRETION: EVIDENCE FOR THE REQUIREMENT OF FIBRONECTIN-VLA-4 RECOGNITION.

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The human bone marrow (BM) cells capable of spontaneous and high-rate Ig-secretion in vitro consist of in vivo-induced and mature B lymphocytes. This cell subset has been involved in the serum Ig and antibody formation. We have recently shown that the final maturation of these cells is induced by IL-6 secreted by adherent BM stromal cells. This IL-6 signal is necessary but not sufficient, since, in cultures devoid of adherent BM cells, IL-6 could not induce any Ig-secretion. The inter-cellular contact between stromal cells and Ig-secreting cells was not needed since the Ig-secretion occurred in a culture where the two populations remained separated by a nucleopore membrane. In addition, the supernatant of stromal cell cultures induced Ig-secretion on BM cell cultures depleted of stromal cells, thus suggesting that an additional soluble factor secreted by the stromal cells was required. Interestingly, BM cells Ig-secretion was blocked by mAb to VLA-4 antigens, but not by mAb to LFA1, ICAM1, CD44 or VLA-1. It is well known that BM stromal cells produced large quantities of fibronectin (FN) and that VLA-4 can act as a FN-receptor. In accordance, the role of FN on the present system was investigated. Stromal cell-depleted BM preparations did not secrete Ig in the presence of FN or IL-6 alone. However, the addition of FN plus IL-6 to such cultures induced as much Ig-secretion as the co-culture with BM stromal cells. FN was more active when presented to the BM cells in a fixed form by pre-coating the culture-wells with the protein. The FN-inductive effect could be reversed by the inclusion of mAb to VLA-4. Additionally, mAb to FN inhibited the Ig-secretion of unfractionated BM cell cultures. These results strongly suggest that, in addition to IL-6, the secretion of FN by BM stromal cells is essential for the Ig-secretors to reach the high-rate Ig-producing stage characteristic of these cells, and that the FN-mediated signal appears to be driven through VLA-4 molecules.

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DIVALENT CATION REGULATION OF THE FUNCTION OF LEUCOCYTE INTEGRINS

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The leucocyte integrins LFA-1 (CD11a/CD18), CR3/Mac-1 (CD11b/CD18) and p150-95 (CD11c/CD18) are heterodimeric adhesion receptors that regulate many dynamic adhesion processes of leucocytes through their binding to specific ligand molecules. The interaction between LFA-1 expressed on T cells and its ligand molecule ICAM-1 is one of the best defined models for analysis of leucocyte integrin functional activity. This interaction shows a temperature dependence and is also known to require the presence of millimolar concentrations of the divalent cation Mg^{2+}. We have recently described a monoclonal antibody named 24 that recognizes an epitope on the three α subunits of leucocyte integrins. Expression of the 24 epitope is also temperature and Mg^{2+} dependent.

We have further assessed the role of divalent cations Mg^{2+}, Ca^{2+} and Mn^{2+} in the activation of LFA-1 on T cells which results in ligand binding and in the expression of the 24 epitope. Manganese strongly promoted both expression of the 24 epitope and T cell binding to ICAM-1 via LFA-1, suggesting that Mn^{2+} is able to directly alter the conformation of LFA-1 in a manner that favours ligand binding. Since Mn^{2+} also promotes functional activity of other integrins, parallels in mechanism of ligand binding may span the integrin family. In contrast, induction of 24 epitope expression by Mg^{2+} required removal of Ca^{2+} from T cell LFA-1 with EGTA. Furthermore, binding of mAb 24 to T cell LFA-1 in the presence of Mg^{2+} or Mn^{2+}, was found to be specifically inhibited by Ca^{2+}, suggestive of a negative regulatory role for Ca^{2+} in the control of leucocyte integrin function. Analysis of T cell binding to ICAM-1 via LFA-1 in the presence of Mg^{2+} or Mn^{2+}, confirmed that Ca^{2+} exerted inhibitory effects upon LFA-1 function. One implication of our findings is that Ca^{2+} bound with relatively high affinity to LFA-1 may serve to maintain an inactive state. Induction of function and 24 expression by Mn^{2+} or physiological activators may therefore occur as a result of displacement of Ca^{2+} from leucocyte integrins.

Expression and organization of integrins in mouse skin carcinogenesis.

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We have analysed the expression and organization of various members of the integrin β1 subfamily and the α6β4 member in several mouse epidermal keratinocytes cell lines representative of different stages of mouse epidermal carcinogenesis.

All the cell lines which exhibit an epithelioid phenotype express variable levels of α3β1 integrin, localized mainly at the regions of cell-cell contact, with independence of their tumorigenic capacity. High levels of the fibronectin receptor α5β1 have only been detected in two highly tumorigenic cell lines which exhibit a fibroblastoid phenotype and being localized at the focal contacts. The adhesion properties of the different cell lines to various extracellular matrix components are presently being investigated.

Expression of α6β4 integrin is restricted to the cell lines with epithelioid phenotype. The levels of this integrin appear to be directly related to the tumorigenicity of the various cell lines. We have also observed that α6β4 is localized at the cell to substratum contact areas in large adhesion structures apparently similar to the "stable anchoring contacts" (SAC), recently described by Carter et al. (*J.Cell Biol.*, 111:3141, 1990). In the highly tumorigenic HaCa4 cell line, a considerable amount of those adhesion structures remain associated to the cell surface after treatment of the cells with high salt buffer where the cytokeratin component remain insoluble. These preliminary results suggest an association of α6β4 integrin to the cytoskeleton trough interaction, direct or indirectely, with the cytokeratin intermediate filaments.

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TITLE: ***Restricted diversity of the γδ TCR in a subset of autoreactive T cells using the VNR as an accessory molecule.***

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It has been shown that the Vitronectin receptor (VNR) acts as an accessory molecule for the autorreactivity of a series of $\gamma 4^+$ skin intraepithelial lymphocytes (sIEL) cell lines (Roberts, K., et al. 1991. J. Exp. Med. 173:231.). In this work we present the molecular analysis of the TCR expressed by those sIEL cell lines and by a series of newborn thymus $\gamma\delta$ T cell hybridomas displaying the same type of autoreactivity.

Every autoreactive cell line or hybridoma expresses a $C\gamma 4^+$ γ-chain with very restricted junctional diversity. All but one also express a $V\delta 6$ δ-chain. The diversity of the δ-chains, although more extensive than that of the γ-chains, shows also restricted patterns of variability. In addition 2 of the 3 sIEL cell lines and 3 of the 6 hybridomas express a second γ-chain.

In conclusion: 1) the restricted diversity of the $\gamma\delta$ TCR in these cells is compatible with recognition of a unique or very restricted set of self molecules. 2) The similarity of TCR expressed in the thymic hybridomas and sIEL cell lines suggests a thymic origin for the autorreactive sIEL.

The possible role of the VNR in the scape of the sIEL precursors from thymic deletion and the acquisition of the autoreactivity will be discussed.

IDENTIFICATION AND CHARACTERIZATION OF ICAM-2 AND ICAM-3

Antonin de Fougerolles and Timothy A. Springer

The leukocyte adhesion molecule LFA-1 mediates a wide range of lymphocyte, monocyte, natural killer cell, and granulocyte interactions with other cells that are critical in both the immune and inflammatory responses. Two counter-receptors for LFA-1 have previously been discovered; ICAM-1, a highly inducible 5 Ig domain molecule, and a constitutively expressed 2 Ig domain structure, ICAM-2, which is 35% homologous to ICAM-1.

In an endeavor to further characterize human ICAM-2, two murine mAb were generated to ICAM-2 transfected COS cells, and designated CBR-IC2/1 and CBR-IC2/2. Immunoprecipitated, reduced ICAM-2 migrated as a broad band of Mr 60,000 in SDS-PAGE. Treatment with N-glycanase revealed a peptide backbone of Mr 31,000, consistent with the size predicted from the cDNA. ICAM-2 had a broad distribution on hematopoietic cell lines and little expression on other cell lines, the sole exception being cultured endothelial cells which possess high levels of ICAM-2. Resting lymphocytes and monocytes expressed ICAM-2, while neutrophils did not. Staining of tissue sections with anti-ICAM-2 mAb confirmed their strong reactivity to vascular endothelium, but demonstrated a lack of expression on other tissues. Small clusters of ICAM-2 positive cells were, however, seen in germinal centers. In constrast to ICAM-1, there was little or no induction of ICAM-2 expression on lymphocytes or cultured endothelium upon stimulation with inflammatory mediators.

One of the two mAb, CBR-IC2/2, was found to totally inhibit ICAM-2:LFA-1 interactions. Using this mAb, several LFA-1-dependent, ICAM-1-independent phenomenon were investigated. LFA-1-dependent binding to both stimulated and unstimulated endothelium was found to be totally accounted for by ICAM-1 and ICAM-2. Using a cell binding assay to purified LFA-1, in conjunction with anti-ICAM-1 and anti-ICAM-2 mAb, we demonstrated that an LFA-1-dependent, ICAM-1-, ICAM-2-independent pathway of adhesion existed on many lymphoid cell lines, including the T cell lymphoma cell line, SKW3.

To further characterize this pathway of adhesion, mAb were raised to SKW3 and screened to inhibit binding of this cell line to purified LFA-1. One mAb, CBR-IC3/1, was selected which completely inhibited this novel pathway of adhesion. Immunoprecipitates revealed this third ligand to run similarly under reduced and nonreduced conditions at an Mr of 120,000 by SDS-PAGE. Treatment with N-glycanase resulted in a reduced Mr of 75,000, indicating that, like ICAM-1 and ICAM-2, this is a highly glycosylated protein. This ligand, putatively called ICAM-3, due to its functional relationship with ICAM-1 and ICAM-2, is restricted to the hematopoietic lineage, being highly expressed on lymphocytes, neutrophils, and monocytes. Expression of ICAM-3 on resting T cells was found to increase upon activation with PHA. Consistent with its functional characterization, ICAM-3 was not expressed on either resting or stimulated endothelium.

The function of ICAM-1 in inflammation and immune responses has been well documented. What roles ICAM-2 and ICAM-3 play in these phenomena are not yet known. Since ICAM-2 is the predominant LFA-1 ligand on resting endothelium, this pathway of adhesion between lymphocytes and resting endothelium may have important consequences for normal recirculation of lymphocytes through tissue endothelium. The fact that ICAM-3 is the only LFA-1 ligand that is expressed at considerable levels on resting lymphocytes and neutrophils implies an important role in the generation of immune responses.

PMA INDUCED DIFFERENTIATION OF U937 CELLS ENHANCES ADHESION TO FIBRONECTIN AND INVERSELY REGULATES THE $\alpha 5\beta 1$ AND $\alpha 4\beta 1$ FIBRONECTIN RECEPTORS

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The interaction of monocytes with the extracellular matrix component fibronectin (Fn), involves specific cell surface receptors and results in cell attachment and differentiation. We are studying the regulation of these receptors during cell differentiation using the pro-monocytic cell line U937 and the inducing agent PMA. We previously reported that U937 cells interact with two sites in Fn, RGD and CS-1, via two independent receptors, the $\alpha 5\beta 1$ and $\alpha 4\beta 1$ integrins respectively (O. C. Ferreira, A. Garcia-Pardo and C. Bianco, J. Exp. Med. 171, 351, 1990). In this study we have determined the effects of PMA on the interaction of U937 cells with both sites in Fn. PMA treated U937 cells show an enhanced adhesion to Fn and to an 80 kDa Fn fragment that contains the RGD sequence. This enhancement parallels a 2-3 fold increase in the surface expression of the RGD-dependent receptor $\alpha 5\beta 1$. An anti-$\alpha 5\beta 1$ mAb completely inhibits cell adhesion to Fn and to the 80 kDa fragment. $\alpha 5\beta 1$ receptors from untreated and PMA treated cells were isolated by affinity chromatography on 80 kDa-Sepharose matrices and shown to contain a similar complex of 152/125 kDa, although proteins from PMA treated cells had slightly faster mobility on SDS-gels. In contrast, the total number of cells adhering to a 38 kDa En fragment (containing the CS-1 site), is lower for PMA treated U937 cells than for undifferentiated cells. This decrease is accompanied by a 50% loss of cell surface $\alpha 4\beta 1$, the specific receptor for CS-1. Furthermore, PMA differentiated cells specifically adhere to another Fn fragment of 58 kDa, which does not contain CS-1 but contains the Hep II domain of Fn, while untreated U937 cells do not attach to the 58 kDa fragment. Our results indicate that differentiation of U937 cells enhances adhesion to Fn primarily bu up-regulating the $\alpha 5\beta 1$ integrin. PMA also induces a down regulation of $\alpha 4\beta 1$, and possibly affects the ligand specificity of this integrin. We postulate that these two integrins may play different roles during monocyte differentiation.

Sulfated glycans bind directly to Thy-1 and block the Thy-1-mediated thymic lympho-epithelial cell-adhesion.

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Thy-1 is a cell-surface molecule distributed in a variety of tissues, including mammalian brain and rodent thymus on which it represents a major membrane glycoprotein. The Thy-1 gene has been well conserved during the evolution; it codes for a polypeptide of about 110 aa which is similar to the variable (V) domain of immunoglobulin. Although previous studies have detailed the protein structure and gene organization of Thy-1, the functional role of this molecule remains ill defined. Recently, we have shown that Thy-1 can play a role as a cell adhesion molecule mediating the binding of mouse thymocytes to thymic epithelial cells (1). This Thy-1-dependent adhesion, which is heterophilic and Ca^{2+} independent, seems to be responsible in part for the initial cell-contact during lympho-epithelial thymic interactions.

In the present study, we have investigated the interaction between Thy-1 and sulfated glycans which are known to interact with proteins (including adhesion molecules such as laminin, collagen, fibronectn, NCAM etc.) and to regulate a number of biological processes. These interactions play an important role in cell-substrat and cell-cell adhesion. It is known that the binding of heparin to collagen and fibronectin inhibits cell adhesion to these proteins. Heparin also modulates the self-assembly of laminin. Lymphocyte adhesion receptors as MEL14, GMP140, CD2 and CD4 are known to bound to sulfated glycans. For the two LEC-CAMs, exogenously added sulfated glycans inhibit their binding to the cognate receptors. Parish et coll. (2) have recently reported the likely interaction between Thy-1 and sulfated glycans. Now we confirm this study showing Thy-1 binds directely to sulfated glycans, and further indicating that sulfate glycans can completely block the Thy-1 depentent thymic lympho-epithelial cell adhesion.

Purified Thy-1 from mouse thymocytes was used to demonstrate this molecule binds directly to sulfated glycans under physiological conditions. We have set up an Iodination protocol, in order to protecte Thy-1 against the loss of sulfated glycan binding activity during the radiolabling. A panel of sulfated glycans has been tested for their ability to bind to ^{125}I-Thy-1 molecules. They could be grouped into 3 classes with strong (A), weak (B) and non (C) interactions, respectively. These results reveal that although the negatively charged sulfate groups of sulfated glycans are critical for this interaction, their carbohydrate moieties also play an important role. In an effort to evaluate the role of sulfated glycans on Thy-1-dependent lympho-epithelial thymic interaction, we developed a Thy-1-mediated cell adhesion system, using the T-lymphomas AKR1 and its Thy-1-negative mutant AKR1 (*Thy-1$^-$ d*). We were able to show that the presence of sulfated glycans from the A class (at 50 ug/ml) completely abolished the Thy-1-mediated adhesion. This inhibition is likely to result from the direct interaction between sulfated glycans and Thy-1 because i) only preincubation of the T-lymphoma cells, but not the epithelial cells, with sulfated glycans showed inhibitory effect; ii) the ability of sulfated glycans to inhibit adhesion correlated with their "overall" affinities to the purified Thy-1 molecule. Currently, the mechanim by which sulfated glycans exert this inhibitory effect is under investigation.

1. *He et al. 1991 J. Exp. Med. 173:515.*
2. *Parish et al. 1988 Immunol. Cell Biol. 66:221.*

THE ROLE OF ICAM-1 IN CLASS II-RESTRICTED ANTIGEN RECOGNITION BY T CELL CLONES

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The recognition of antigen by class II-restricted T cells involves specific interactions between antigenic peptides, MHC molecules and T cell receptors as well as independent interactions between adhesion molecules and their ligands. We have analized the influence of one of these adhesion molecules, ICAM-1, in the interactions of allospecific and antigen-specific T cells with their target cells at the clonal level.

We have used as antigen presenting cells, (i) DR1 or DR2 positive B cell lines; (ii) murine L cells tranfected with DR1, DR2a or DR2b and (iii) the same L cells overtransfected with ICAM-1. The transfections were carried out by calcium phosphate precipitation of the ICAM-1 cDNA inserted in the CDM8 vector (donated by B. Seed). Transfected cells were cloned by limiting dilution in selection medium. ICAM-1 expression was determined by indirect immunofluorescence using the RR1/1 mAb (a gift from T. A. Springer). As effector cells we used HLA-DR1 or DR2-restricted allospecific and antigen-specific (influenza or MBP) T cell clones.

Our results show:

1) ICAM-1 expression on the APC enhanced recognition of specific peptides by DR1-restricted influenza-specific T cell clones by a 20-30%;

2) the effect of ICAM-1 expression in the presenting cells was variable for MBP-specific DR2-restricted T cell clones: some clones unable to recognize MBP presented by L cells lysed MBP-pulsed L cells if they expressed ICAM-1; some other clones showed a 90-100% enhancement of the cytotoxic response and in a third group there was a 20-30% increase of the cytotoxicity of MBP-pulsed ICAM-1 positive L cells;

3) the presence of ICAM1 did not significantly modify the proliferative response of DR1-specific alloreactive clones. The same clones were tested with an L cell transfectant expressing low levels of DR1. The response to these cells if they also expressed ICAM-1was enhanced in only one of the clones, whereas the response of other clones was not affected.

4) the proliferation of DR2-allospecific clones (DR2a or DR2b) to L cells expressing ICAM-1 showed some enhancement.

5) none of the DR1 or DR2 allospecific clones unable to recognize the DR molecules expressed in the L cells became positive by the additional expression of ICAM-1 in the stimulator cells. This could not be explained by the lack of an endogenous human peptide, since all but one also failed to recognize a human fibroblast transfected with DR1 +/- ICAM-1. The proliferation of the responsive clone was clearly enhanced by the expression of ICAM-1 in the stimulators.

These data confirm that the LFA-1/ICAM-1 interaction has a role in the antigen-specific recognition by some of the T cell clones, increasing the efficiency of the recognition. However, this influence varies widely among different T cell clones, probably depending on the intrinsic efficiency of the recognition by each of the clones.

The Genomic Organisation of the Human CD18 Gene and RFLP studies.

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The gene for the β-subunit of the human leukocyte integrins (CD18) has been located to near the q telomere of chromosome 21. We have obtained genomic clones covering the CD18 gene spanning a region of about 40 kb. The gene is organised into 16 exons. The first exon consists entirely of 5' untranslated sequences. Using both hybridisation/protection and primer extension type techniques, we have demonstrated that there are multiple initiation sites of transcription. The first exon is therefore of variable length with the majority of between 50 to 90 bases. The longest one detected by RACE-PCR is of 127 bases. No TATA or CCAAT boxes are found immediately 5' to this region suggesting that the transcription of the CD18 gene is probably not regulated by these elements. The polyadenylation site is also heterogeneous within a span of nine bases although the most 3' site is used most frequently. This is perhaps due to the absence of a pronounced GT-rich sequence about 25 bp 3' from this region. All exon/intron boundaries conform to the GT/AG splicing consensus. Most of boundaries are similar to those of the β3 integrin gene.

The CD18 gene is polymorphic by RFLP analysis. From 20 unrelated healthy individuals three loci can be defined. Locus A (2 alleles) and locus B (2 alleles) are within the CD18 gene and are likely to arise from the insertion/deletion of intronic sequences. Locus C is due to the presence/absence of two 3' extragenic Bgl-II sites and three alleles can be detected. There appears to be very weak linkage between the alleles. Of the 12 possible haplotypes, only one is not found in the 20 individuals. Four unrelated LAD patients were also studied. Six different haplotypes are found among them indicating that LAD is not associated with any particular haplotype. In addition, the presence of six haplotypes suggests that the deficiencies are different. This conjecture is supported by preliminary evidence obtained from the molecular characterisation of the CD18 specific cDNA derived from the LAD cell lines. Four point mutations and a splicing defect have been identified.

THE INFLUENCE OF RECEPTOR MOBILITY IN CELL ADHESION STRENGTHENING

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We have used an in vitro model system of glass-supported planar membranes to study the effects of lateral mobility of membrane-bound receptors on cell adhesion. Egg phosphatidylcholine (PC) bilayers were reconstituted with two anchorage isoforms of the adhesion molecule lymphocyte function-associated 3 (LFA-3). The diffusion coefficient of glycosyl-phosphatidylinositol (GPI) anchored LFA-3 approached that of phospholipids in the bilayers, whereas the transmembrane (TM) anchored isoform of LFA-3 was immobile. Both static and laminar flow assays were used to quantify the strength of adherence to the lipid bilayers of the T lymphoma cell line Jurkat which expresses the counter-receptor CD2. Cell adhesion was dependent on LFA-3 density and was more efficient on membranes containing the GPI isoform than the TM isoform. Kinetic measurements demonstrated an influence of contact time on the strength of adhesion to the GPI isoform at lower site densities (25-50 sites/μm^2), showing that the mobility of LFA-3 is important in adhesion strengthening. At higher site densities (1500 sites/μm^2) and longer contact times (20 minutes), Jurkat cell binding to the TM and GPI isoforms of LFA-3 showed equivalent adhesion strengths, although adhesion strength of the GPI isoform developed 2-fold more rapidly than the TM isoform. Reduction of CD2 mobility on Jurkat cells at 5°C greatly decreased the rate of adhesion strengthening with the TM isoform of LFA-3, resulting in a 30-fold difference between the two LFA-3 isoforms. Our results demonstrate that the ability of a membrane receptor and its membrane-bound counter-receptor to diffuse laterally enhances cell adhesion both by allowing accumulation of ligands in the cell contact area and

Stimulation of adhesion of U-937 promonocytic line to human endothelial cells by monoclonal antibodies to endoglin and VLA-5.
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Endoglin is a major glycoprotein of human endothelial cells, a homodimer of Mr=170,000. The sequence deduced from that of the isolated cDNA revealed a type I integral membrane protein with no homology to known proteins or motifs. As an RGD tripeptide is accessible at the surface of each chain, we postulated that this protein might be implicated in RGD mediated interactions. Adhesion of U-937 cells, labelled with the fluorescent dye BCECF, to monolayers of endothelial cells derived from human umbilical vein (EC) was monitored using the FCA multiwell microfluorimeter. Pretreatment of EC with monoclonal antibodies (Mab) to endoglin was shown to increase the adhesion event by 5 to 20-fold, in a dose-dependent fashion and with maximum IgG concentrations of 20 μg/ml. The increased adhesion was inhibited in the presence of RGD but not by RGE peptides (200-400μg/ml) and by cytochalasin B. Pretreatment of U-937 cells with Mab to endoglin also sfimulated their adhesion to EC. We also analysed the reactivity of Mab to known integrins on U-937 and EC by flow cytometry and in the adhesion assay. Although VLA-2, VLA-3, VLA-4, VLA-5 and VLA-6 were present on EC, only Mab to VLA-5 and to β1 chain could stimulate adhesion when preincubated with EC. On U-937 cells, VLA-4, VLA-5, VLA-6, LFA-1, and Mac-1 were present but only Mab to VLA-5 and β1 could stimulate adhesion. Thus, increased adhesion in this system can be triggered on either cell type by Mab to VLA-5 or endoglin.suggesting that both molecules are implicated in this interaction. Since VLA-5 binds to fibronectin via RGD, it is challenging to speculate that it might also bind to endoglin. Alternatively, endoglin might be a constituent of the mutivalent complex which mediates interaction between monocytes and endothelial cells. Future biochemical studies should test these hypotheses.

THE HUMAN LEUKOCYTE ADHESION MOLECULE ICAM-2. GENERATION OF A MONOCLONAL ANTIBODY, CHARACTERIZATION OF THE PROTEIN AND REGULATION OF THE EXPRESSION.

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Leukocyte adhesion plays a fundamental role in leukocyte functions. The binding between cells involves participation of several cell surface glycoproteins, and among the most important are the CD11/CD18 integrins and their ligands CD54 (ICAM-1) and ICAM-2. It has been shown that ICAM-2 is a cellular ligand for CD11a/CD18. We have synthesized the ICAM-2 DNA by PCR, and expressed it in E. coli and COS-1 cells. The protein made in bacteria was used to obtain polyclonal and monoclonal antibodies. ICAM-2 is a glycoprotein with an apparent MW of 55 000. It is strongly expressed on endothelial cells, and less in hematopoietic cells.

The regulation of the expression of ICAM-2 was studied by using the anti-ICAM-2 MAb. Results from flowcytometry experiments and immunoblotting shows that while the expression of CD54 (ICAM-1) can be regulated by different cytokines, the expression of ICAM-2 seems to be constitutive in all cells examined.

REGULATED EXPRESSION AND FUNCTION OF CD11c/CD18 INTEGRIN ON HUMAN B LYMPHOCYTES. RELATION BETWEEN ATTACHMENT TO FIBRINOGEN AND TRIGGERING OF PROLIFERATION THROUGH CD11c/CD18.[1]

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CD11c/CD18 (p150,95) is a β2 integrin expressed by myeloid, NK and certain lymphoid cells such as some cytotoxic T cell clones and B cell malignancies. We have studied the expression and function of CD11c on resting and activated B lymphocytes. Flow cytometry, immunoprecipitation and mRNA analyses showed that cell activation with phorbol esters or with a variety of stimuli such as *Staphylococcus aureus* or anti-μ antibodies in combination with cytokines induced "de novo" CD11c/CD18 cell surface expression on most B cells while CD11b expression was not affected. Functional analysis of CD11c/CD18 on B cells revealed that it plays a dual role: first, CD11c/CD18 is implicated in B cell proliferation, as demonstrated by the ability of several anti-CD11c mAb to trigger comitogenic signals, and second the newly expressed CD11c/CD18 mediates B cell binding to fibrinogen (FG). Our data data conclusively demonstrate the role of CD11c/CD18 on both B cell activation and adhesion processes and suggest that FG may be involved in B cell activation.

FUNCTIONAL EVIDENCE FOR THREE DISTINCT AND INDEPENDENTLY INHIBITABLE ADHESION ACTIVITIES MEDIATED BY THE HUMAN INTEGRIN VLA-4: CORRELATION WITH DISTINCT α4 EPITOPES.

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The human integrin VLA-4 (CD49d/CD29), the leukocyte receptor for both the CS-1 region of plasma fibronectin (Fn) and the vascular cell surface adhesion molecule-1 (VCAM-1), also mediates homotypic aggregation upon triggering with specific anti-VLA-4 mAb. Epitope mapping of this integrin on the human B-cell line Ramos, performed with a wide panel of anti-VLA-4 mAb by both cross-competitive cell binding and protease sensitivity assays, revealed the existence of three topographically distinct epitopes on the α4 chain, referred to as epitopes A, B and C. By testing this panel of anti-VLA-4 mAb for inhibition of cell binding to both a 38 kDa Fn fragment containing CS-1 and to VCAM-1, as well as for induction and inhibition of VLA-4 mediated homotypic cell adhesion, we have found overlapping but different functional properties associated with each epitope. Anti-α4 mAb recognizing epitope B inhibited cell attachment to both Fn and VCAM-1, whereas mAb against epitope A did not block VCAM-1 binding and only partially inhibited binding to Fn. In contrast, mAb directed to epitope C did not affect cell adhesion to either of the two VLA-4 ligands. All mAb directed to site A, as well as a subgroup of mAb recognizing epitope B (called B2), were able to induce cell aggregation, but this effect was not exerted by mAb specific to site C and by a subgroup against epitope B (called B1). Moreover, although anti-epitope C and anti-epitope B1 mAb did not trigger aggregation, those mAb blocked aggregation induced by anti-epitope A or B2 mAb. Also, anti-epitope A mAb blocked B2-induced aggregation, and conversely, anti-epitope B2 mAb blocked A-induced aggregation. Further evidence for multiple VLA-4 functions is that anti-Fn and anti-VCAM-1 antibodies inhibited binding to Fn or to VCAM-1, respectively, but did not affect VLA-4-mediated aggregation. In summary, we have demonstrated that there are at least three different VLA-4-mediated adhesion functions, we have defined three distinct VLA-4 epitopes, and we have correlated these epitopes with the different functions of VLA-4.

EA-1, A NOVEL ADHESION MOLECULE INVOLVED IN HOMING OF PROGENITOR T LYMPHOCYTES TO THE THYMUS

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Cell-cell interaction between mature hemopoietic cells and endothelium has been extensively studied, but the interaction of hemopoietic precursors with endothelium during development is still an untapped field and nothing is known about the adhesive molecules involved.

We are interested in the homing molecules involved in the binding of hemopoietic precursors present in the blood stream on the thymus endothelium . In order to carry out biochemical studies we established an in vitro model using both hemopoietic precursors (1) and endothelial cell lines (2-3). Monoclonal antibody, EA-1, raised against a mouse embryonic cell line, blocks adhesion of the mouse pro-T cell line FT-F1 to the thymus blood vessels (4), thymic capsule and liver from newborn mice, aswell as to a thymic endothelial cell line. It has no effect on the adhesion of mature T cells or myeloid cells. The antigen recognized by EA-1 is present on the vascular endothelium of various mouse tissues but is absent on pro-T cells. It precipitates molecules with apparent molecular weight of 140 and 200 kD and the band pattern is the same under reducing and non reducing conditions. When endothelial cell lines were treated with neuraminidase their affinity for EA-1 antibody was reduced; endoglycosidase F, hyaluronidase and phospholipase C showed no influence. Thus, there is likely to be an O-linked sialic acid near the epitope. Immunoprecipitation of native protein from FPLC column fractions suggests that the EA-1 antigens are in a complex of 700-1000 kD. The 140 kD band was digested by CNBr, the peptides were separeted on a HPLC reverse phase column and 10 amino acids were sequenced. We are now attempting to isolate a cDNA encoding the cell adhesion molecule recognized by EA-1.

On the basis of inhibition studies, immunofluorescence staining and immunoprecipitation experiments with antibodies against known adhesion molecules, we suggest that EA-1 is a new molecule involved in the adhesion of pro-T cells to the thymic endothelium (5).

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REGULATION OF LFA-1 BINDING TO ICAM-1 BY TYROSINE KINASES AND PHOSPHATASES.

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The adhesion of T cells mediated by LFA-1 and ICAM-1 has been shown to be finely regulated and closely associated with T cell activation. In order to elucidate the mechanisms that regulate the ICAM-1/LFA-1 binding, we generated a soluble form of murine ICAM-1 and examined its binding to murine LFA-1. The murine ICAM-1 cDNA that we had previously cloned was modified to remove the transmembrane and cytoplasmic domains, subcloned into the pBCMGS mammalian expression vector and transfected into the murine myeloma cell line NS-1. The transformed NS-1 cells secreted up to 10 μg/ml soluble ICAM-1. It was purified to homogeneity and its binding to murine LFA-1 was studied in two ways. First, sICAM-1 was immobilized on microtitre wells and the binding of LFA-1^+ cells to the wells were quantitated. Second, the purified sICAM-1 was radio-iodinated and its binding to LFA-1^+ cells was measured. The former would be a measurement of the avidity (multivalent interaction) of LFA-1 while the latter would quantitate the affinity (monovalent binding) of LFA-1 for ICAM-1. The binding of sICAM-1 to LFA-1 was readily detected by both methods and it was inhibited by antibodies to LFA-1 and ICAM-1 or an excess amount of unlabelled sICAM-1, indicating that the binding was specific. Scatchard plot analysis showed two forms of binding sites, ie high (Kd = $3X10^{-10}$M) and low (Kd = $2.5X10^{-8}$M) affinity forms. On the murine T cell line MBL-2 or helper T cell hybridoma line, the majority of LFA-1 molecules seem to be in the low affinity form since only less than 10^4 high affinity receptor per cell can be detected whereas total LFA-1 molecules detected by anti-CD11a antibody is over 10^5/cell. Phorbol ester, which activates protein kinase C and induces cell aggregation, did not increase ^{125}I-sICAM-1 binding to LFA-1. Cytochalasin B, which inhibits actin filaments, and staurosporin, a protein kinase C inhibitor, significantly inhibited the binding of LFA-1^+ cells to plate immobilized sICAM-1 but they did not affect the binding of ^{125}I-sICAM-1 to the cells. Cross-linking of the TCR/CD3 significantly increased the binding of sICAM-1 within 2 min followed by a rapid decline in 5 min. Since T cell receptor cross-linking is known to activate tyrosine kinases, the effects of tyrosine kinase inhibitor genistein and tyrosine phosphatase inhibitor metavanadate were examined. Genistein significantly enhanced the binding of ^{125}I-sICAM-1 whereas metavanadate almost completely inhibited the binding. These results indicate that LFA-1 mediated cell adhesion seems to be regulated by at least two distinct mechanisms. One involves the protein kinase C pathway that regulates cell adhesion without changing the affinity of LFA-1 for ICAM-1 and possibly involves cytoskeleton. The other pathway appears to be mediated by tyrosine phosphorylation and dephosphorylation of unidentified molecule(s). This pathway induces changes in the affinity of LFA-1 most likely due to a change in its conformation.

INTRACELLULAR Ca^{2+} LEVELS CAN DETERMINE TRANSIENT OR SUSTAINED ACTIVATION OF LFA-1

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Besides triggering of the CD2 or CD3 receptor, LFA-1 can be activated by several other receptors. Our latest data show that triggering of cloned T lymphocytes by MHC class II, CD43 or CD44 can lead to an increased adhesion of these cells to purified ICAM-1. The induced adhesion shows distinct kinetics, in which we can discriminate two different activation routes: a sustained and a transient activation of LFA-1. Extracellular (Ca^{2+} and Mg^{2+}) cations contribute to these distinct kinetics. Interestingly we found that the kinetics of aggregation correspond to the kinetics of intracellular Ca^{2+} fluxes generated by these antibodies. We have evidence that at least two distinct signalling routes with different cation requirements can activate LFA-1 through a raise in intracellular Ca^{2+}.

VLA-4 is Involved in Lymphocyte Binding to Brain Endothelium in Experimental Autoimmune Encephalitis.

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Experimental autoimmune encephalitis (EAE) is an inflammatory disease of the central nervous system that can be induced in the Lewis rat by a single intraperitoneal injection of a CD4+ T cell clone specific for myelin basic protein. Disease is manifested by tail and limb paralysis within six days of the injection. At this time, lymphocytes and monocytes form large cuffs around venules within the animal's brain stem and spinal cord, and in some areas infiltrate into the brain tissue itself. The immune cells appear to arrive from the circulation since large numbers of lumenally bound and migrating cells can be seen in tissue sections. In order to characterize the receptors that mediate the interaction of circulating lymphocytes and monocytes with this endothelium we adapted the Stamper-Woodruff in vitro binding assay to EAE brain tissue. The assay measures the binding of viable leukocytes to profiles of vessels exposed in unfixed cryostat tissue sections. Normal rat and human lymphocytes, as well as several human lymphoid cell lines were found to bind only to the inflamed vessels in diseased brain. The binding was not affected by antibodies against Lecam-1, ß2 Integrin, or CD44, but was largely inhibited by anti-ß1 integrin. Of the ß1 integrins, only antibodies that block the VCAM-1 binding activity of VLA-4 were inhibitory; an antibody against the fibronectin binding domain of VLA-4 enhanced binding to brain vessels. These results indicate that VLA-4 is important for lymphocyte adherence to inflamed brain endothelium. However, VLA-4 expression alone is not sufficient for this interaction since some cell lines that express high levels of functional VLA-4 bound poorly to EAE brain vessels. Anti-VLA-4 was also found to inhibit lymphocyte binding to TNF-stimulated brain endothelium in culture and preliminary experiments indicate that anti-VLA-4 may prevent the development of EAE when administered in vivo.

LIST OF INVITED SPEAKERS

Workshop on
ADHESION RECEPTORS IN THE IMMUNE SYSTEM

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Efectos anómalos de transporte eléctrico en conductores a baja temperatura.

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Técnicas de prolongación analítica en el problema de reconstrucción del objeto en óptica.

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Encefalopatía portosistémica experimental.

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Aspectos faunísticos y ecológicos de carábidos altoaragoneses.

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Estudio de procesos bioquímicos implicados en la abscisión de la aceituna.

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La verdadera morfología y fisiología de Azoyobacter: células germinales.

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Papel modulador de los glucocorticoides en la población de receptores para insulina y glucagón.

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Paleoecología del yacimiento del Neógeno continental de Los Valles de Fuentidueña (Segovia).

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Estudio de la fosforillasa kinasa de hígado y leucocitos: purificación, características y regulación de su actividad.

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Reimplantación experimental de la extremidad posterior en perros.

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Secreción y reutilización de trifosfato de adenosina (ATP) por sinaptosomas colinérgicos.

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Contribución a la fauna de esponjas del litoral catalán. Esponjas córneas.

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Propiedades dinámicas y termodinámicas de superficies de sólidos.

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Ciclo anual de zooplancton costero de Gijón.

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Receptores insulínicos en hipotálamo de rata: localización subcelular y mecanismo(s) de regulación.

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Estudio del mecanismo secretor de hormona juvenil en oncopeltus fasciatus.

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Fusariosis del gladiolo: un estudio preliminar.

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Análisis estructural en sabinares de la provincia de León.

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Citokinas en agrios. Actividades endógenas, efectos fisiológicos y aplicaciones.

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Estudios ecológicos en jarales (cistion laurofilii): Variación anual de algunos factores del entorno y manifestaciones fenológicas.

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Estudios espectroscópicos de fosfolípidos polimerizables.

183 Herrero Ruiz de Loizaga, V. J.:
Estudio de reacciones químicas por haces moleculares. Aplicación a la reacción $C_2H_5Br + K$ $Brk + C_2H_5$.

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Utilización sintética en química orgánica de metales pesados como catalizadores. Oxidación asimétrica.

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Receptores presinápticos en el conducto deferente de rata.

196 Estévez Toranzo, A.:
Supervivencia de patógenos bacterianos y virales de peces en sistemas de cultivo.

197 Lizarbe Iracheta, M.ª A.:
Caracterización molecular de las estructuras de colágeno.

203 López Calderón, I.:
Clonación de genes de «Saccharomyces cerevisiae» implicados en la reparación y la recombinación.

211 Ayala Serrano, J. A.:
Mecanismo de expresión de la PBP-3 de «E. coli»: Obtención de una cepa hiperproductora de la proteína.

240 **Genetic Strategies in Development.**
Symposium in honour of Antonio García Bellido. Lectures by S. Ochoa, S. Brenner, G. S. Stent, E. B. Lewis, D. S. Hogness, E. H. Davidson, J. B. Gurdon and F. Jacob.

244 **Course on Genome Evolution.**
Organized by E. Viñuelas. Lectures by R. F. Doolittle, A. M. Weiner/N. Maizels, G. A. Dover, J. A. Lake, J. E. Walker, J. J. Beintema, A. J. Gibbs, W. M. Fitch, P. Palese, G. Bernardi and J. M. Lowenstein.

246 **Workshop on Tolerance: Mechanisms and implications.**
Organized by P. Marrack and C. Martínez-A. Lectures by H. von Boehmer, J. W. Kappler, C. Martínez-A., H. Waldmann, N. Le Douarin, J. Sprent, P. Matzinger, R. H. Schwartz, M. Weigert, A. Coutinho, C. C. Goodnow, A. L. DeFranco and P. Marrack.

247 **Workshop on Pathogenesis-related Proteins in Plants.**
Organized by V. Conejero and L. C. Van Loon. Lectures by L. C. Van Loon, R. Fraser, J. F. Antoniw, M. Legrand, Y. Ohashi, F. Meins, T. Boller, V. Conejero, C. A. Ryan, D. F. Klessig, J. F. Bol, A. Leyva and F. García-Olmedo.

248 Beato, M.:
Course on DNA - Protein Interaction.

249 **Workshop on Molecular Diagnosis of Cancer.**
Organized by M. Perucho and P. García Barreno. Lectures by F. McCormick, A. Pellicer, J. L. Bos, M. Perucho, R. A. Weinberg, E. Harlow, E. R. Fearon, M. Schwab, F. W. Alt, R. Dalla Favera, P. E. Reddy, E. M. de Villiers, D. Slamon, I. B. Roninson, J. Groffen and M. Barbacid.

251 **Lecture Course on Approaches to Plant Development.**
Organized by P. Puigdoménech and T. Nelson. Lectures by I. Sussex, R. S. Poethig, M. Delseny, M. Freeling, S. C. de Vries, J. H. Rothman, J. Modolell, F. Salamini, M. A. Estelle, J. M. Martínez Zapater, A. Spena, P. J. J. Hooykaas, T. Nelson, P. Puigdoménech and M. Pagès.

252 **Curso Experimental de Electroforesis Bidimensional de Alta Resolución.**
Organizado por Juan F. Santarén. Seminarios por Julio E. Celis, James I. Garrels, Joël Vandekerckhove, Juan F. Santarén y Rosa Assiego.

253 **Workshop on Genome Expression and Pathogenesis of Plant RNA Viruses.**
Organized by F. García-Arenal and P. Palukaitis. Lectures by D. Baulcome, R. N. Beachy, G. Boccardo, J. Bol, G. Bruening,

J. Burgyan, J. R. Díaz Ruiz, W. G. Dougherty, F. Garcia-Arenal, W. L. Gerlach, A. L. Haenni, E. M. J. Jaspars, D. L. Nuss, P. Palukaitis, Y. Watanabe and M. Zaitlin.

254 **Advanced Course on Biochemistry and Genetics of Yeast.**
Organized by C. Gancedo, J. M. Gancedo, M. A. Delgado and I. L Calderón.

255 **Workshop on The Reference Points in Evolution.**
Organized by P. Alberch and G. A. Dover. Lectures by P. Alberch, P. Bateson, R. J. Britten, B. C. Clarke, S. Conway Morris, G. A. Dover, G. M. Edelman, R. Flavell, A. Fontdevila, A. García-Bellido, G. L. G. Miklos, C. Milstein, A. Moya, G. B. Müller, G. Oster, M. De Renzi, A. Seilacher, S. Stearns, E. S. Vrba, G. P. Wagner, D. B. Wake and A. Wilson.

256 **Workshop on Chromatin Structure and Gene Expression.**
Organized by F. Azorín, M. Beato and A. A. Travers. Lectures by F. Azorín, M. Beato, H. Cedar, R. Chalkley, M. E. A. Churchill, D. Clark, C. Crane-Robinson, J. A. Dabán, S. C. R. Elgin, M. Grunstein, G. L. Hager, W. Hörz, T. Koller, U. K. Laemmli, E. Di Mauro, D. Rhodes, T. J. Richmond, A. Ruiz-Carrillo, R. T. Simpson, A. E. Sippel, J. M. Sogo, F. Thoma, A. A. Travers, J. Workman, O. Wrange and C. Wu.

257 **Lecture Course on Polyamines as modulators of Plant Development.**
Organized by A. W. Galston and A. F. Tiburcio. Lectures by N. Bagni, J. A. Creus, E. B. Dumbroff, H. E. Flores, A. W. Galston, J. Martin-Tanguy, D. Serafini-Fracassini, R. D. Slocum, T. A. Smith and A. F. Tiburcio.

258 **Workshop on Flower Development.**
Organized by H. Saedler, J. P. Beltrán and J. Paz Ares. Lectures by P. Albersheim, J. P. Beltrán, E. Coen, G. W. Haughn, J. Leemans, E. Lifschitz, C. Martin, J. M. Martínez-Zapater, E. M. Meyerowitz, J. Paz-Ares, H. Saedler, C. P. Scutt, H. Sommer, R. D. Thompson and K. Tran Thahn Van.

259 **Workshop on Transcription and Replication of Negative Strand RNA Viruses.**
Organized by D. Kolakofsky and J. Ortín. Lectures by A. K. Banerjee, M. A. Billeter, P. Collins, M. T. Franze-Fernández, A. J. Hay, A. Ishihama, D. Kolakofsky, R. M. Krug, J. A. Melero, S. A. Moyer, J. Ortín, P. Palese, R. G. Paterson, A. Portela, M. Schubert, D. F. Summers, N. Tordo and G. W. Wertz.

260 **Lecture Course Molecular Biology of the Rhizobium-Legume Symbiosis.**
Organized by T. Ruiz-Argüeso. Lectures by T. Bisseling, P. Boistard, J. A. Downie, D. W. Emerich, J. Kijne, J. Olivares, T. Ruiz-Argüeso, F. Sánchez and H. P. Spaink.

261 **Workshop on The Regulation of Translation in Animal Virus-Infected Cells.**
Organized by N. Sonenberg and L. Carrasco. Lectures by V. Agol, R. Bablanian, L. Carrasco , M. J. Clemens, E. Ehrenfeld, D. Etchison, R. F. Garry, J. W. B. Hershey, A. G. Hovanessian, R. J. Jackson, M. G. Katze, M. B. Mathews, W. C. Merrick, D. J. Rowlands, P. Sarnow, R. J. Schneider, A. J. Shatkin, N. Sonenberg, H. O. Voorma and E. Wimmer.

263 **Lecture Course on The Polymerase Chain Reaction.**
Organized by M. Perucho and E. Martínez-Salas. Lectures by D. Gelfand, K. Hayashi, H. H. Kazazian, E. Martínez-Salas, M. Mc Clelland, K. B. Mullis, C. Oste, M. Perucho and J. Sninsky.

264 **Workshop on Yeast Transport and Energetics.**
Organized by A. Rodríguez-Navarro and R. Lagunas. Lectures by M. R. Chevallier, A. A. Eddy, Y. Eilam, G. F. Fuhrmann, A. Goffeau, M. Höfer, A. Kotyk, D. Kuschmitz, R. Lagunas, C. Leão, L. A. Okorokov, A. Peña, J. Ramos, A. Rodríguez-Navarro, W. A. Scheffers and J. M. Thevelein

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