Instituto Juan March
de Estudios e Investigaciones

16 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Intra- and Extra-Cellular Signalling in Hematopoiesis

Organized by

E. Donnall Thomas and A. Grañena

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D. Cantrell
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R.J.O'Reilly - Cytokine Applications in the Treatment of Genetic Cytopenias.

INTRODUCTION

E. Donnall Thomas

In recent years there has been explosive progress in the understanding of hematopoiesis at the molecular level and an increasing application of new knowledge to clinical problems. As is often the case when new developments occur simultaneously on several fronts, sub-specialization develops and those individuals in the sub-specialty areas tend to talk to and be understood only by those in their own areas. The purpose of this symposium at the March Foundation was to bring together scientists working in many diverse areas related to intra- and extra-cellular signalling in hematopoiesis as well as those involved with the clinical application of this new information.

The opening sessions dealt with the molecular structure of cytokine receptors with emphasis on the similarities among receptors and how these can result in both overlapping and unique functions. The next series of presentations focused on the intracellular events following receptor signalling and possible ways to regulate or alter the intracellular pathways. Presentations and discussion then switched from intracellular molecular events to stem cell biology and the role of stromal cells in providing signals that regulate hematopoiesis. This area serves as an important bridge between molecular studies and the eventual use of signals to alter the functions of cells in hematological disorders. The final session consisted of a series of presentations about the clinical application of the new knowledge of hematopoiesis to marrow transplantation and the treatment of genetic diseases.

The sessions were conducted in a relaxed and friendly atmosphere conducive to the main objective of interdisciplanry exchange of information. The final session included many physicians from the Madrid area. The participating scientists were unanimous in expressing their gratitude to the March Foundation and the Carreras Foundation for making the very successful meeting possible.

E. Donnall Thomas, M.D.

CYTOKINE RECEPTORS

THE LIF/ONCOSTATIN M RECEPTOR SYSTEM: A MULTI-SUBUNIT CYTOKINE RECEPTOR FAMILY

Linda S. Park, Bruce Mosley, Della Friend, Michael R. Comeau, Steven F. Ziegler, Janis Wignall, Bettina Thoma, David Cosman, Heinz Baumann*, and David P. Gearing, Immunex Research and Development Corp., Seattle, WA and *Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, NY.

Leukemia inhibitory factor (LIF) and oncostatin M (OSM) are structurally and genetically related polypeptide hormones which mediate a broad range of biological activities. Both molecules can bind with high affinity to the same receptor complex which consists of the LIF receptor (LIFR) and gp130, both members of the Hematopoietin receptor family. Although this shared receptor complex is likely to underlie the overlapping biological activities of LIF and OSM, LIFR and gp130 are also at the center of a considerably more complex network of multi-subunit receptors. Based on both binding and signal transduction studies we have generated evidence that there is a second high affinity receptor complex for OSM which is not capable of binding LIF (and therefore does not contain the LIFR subunit). We believe that this receptor complex, at a minimum, is likely to contain an OSM specific binding subunit in addition to gp130. In addition to binding LIF and OSM, recent observations have suggested that LIFR and gp130 may also form the basis for a functional receptor for the neuro-active cytokine ciliary neurotrophic factor (CNTF). CNTF, which has structural features homologous to LIF and OSM, binds to a specific CNTF receptor subunit (CNTFRα) which is anchored in the cell membrane via a glycosylphosphatidylinositol linkage and has no cytoplasmic domain. We have now investigated the roles of LIFR, gp130, and CNTFRα in generating functional receptor complexes. High affinity binding of CNTF to neuronal cell lines can be competed with both LIF and OSM, suggesting that both LIFR and gp130 are involved in a CNTFR complex. By expressing combinations of receptor subunits in both hepatoma cell lines and BAF-BO3 cells we have demonstrated that the presence of gp130 and LIFR are sufficient (and necessary) to confer responsiveness to LIF, OSM and CNTF. The addition of CNTFRα confers both high affinity CNTF binding and enhanced responsiveness to CNTF without modifying the response to LIF or OSM. These studies demonstrate that the LIFR/gp130 complex is involved in mediating biological responses to at least three cytokines in a complicated network of molecules with overlapping biological roles.

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Interleukin 6 receptor and signal transduction

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The relationship between chronic inflammation and B cell malignancies has been implicated, but the molecular mechanisms(s) have not been well documented. We have proposed that inflammatory cell-derived IL-6 plays an essential role in B cell abnormalities leading to the eventual development of multiple myeloma (1). Actually, IL-6 was found to be a growth factor for plasmacytoma/myeloma cells and IL-6 transgenic mice developed a malignant monoclonal plasmacytoma with c-myc gene rearrangement (2, 3). Furthermore, Klien and his colleague reported that administration of anti-IL-6 monoclonal antibody to patients with multiple myeloma reduced the frequency of the myeloma cells in the S phase, clearly demonstrating the *in vivo* role of IL-6 and the possible clinical application of IL-6 inhibitors in the patients (4).

IL-6 receptor consists of two molecule, one is a 80kDa IL-6 binding molecule designated IL-6Rα (5) and another is a signal transducer, gp130 which by itself cannot bind IL-6 but is essential for the formation of high affinity binding site (6). The singal is generated through gp130 molecule which acts as a signal transducer not only for IL-6 but also for oncostatin M (Kaposi's sarcoma growth factor) leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) (7, 8). We demonstrated that IL-6 rapidly and selectively induces the expression of the junB gene in a wide range of cells including myeloma cell lines, hepatoma cell lines and myeloleukemic cell lines. The induction of the junB gene by IL-6 required at least two protein kinases, a tyrosine kinase and a H7-sensitive protein kinase. In fact, IL-6 stimulation increased the cytosolic tyrosine kinase activity in a variety of cells (9).

We further identified and characterized an IL-6 immediate early response element in the junB promoter (designated JRE-IL6) (10). The JRE-IL6, located at-149 to-124, consists of two motifs, an ETS binding site (CAGGAAGC) and a CRE-like aite (TGACGCGA). Both motifs are necessary and sufficient for IL-6 response of the promoter. We showed that IL-6 activates the JRE-IL6 through an H7-sensitive pathway independent of PKC, PKA, CA^{2+}/CM-dependent kinases, ras, raf-1, or NF-IL-6 (C/EBPβ). Specific combination of the ETS site binding protein and the CRE-like site binding protein complex appears to give a basis how the JRE-IL6 selectively and efficiently respond to IL-6 signals, but not to signals generated through Ha-ras, raf-1 or PKC. This is in contrast to the oncogene responsive element of the polyoma virus enhancer consisting of an ETS binding site and an AP-1 binding site which efficiently responds to signals generated through Ha-ras, raf-1 or PKC but not IL-6 signals.

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THE STRUCTURE AND FUNCTION OF THE IFNγ RECEPTOR

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Interferon-gamma (IFNγ) is an important immunomodulatory cytokine that induces a variety of biologic responses in a number of different cells. One of the major projects in the laboratory is to elucidate the signal transduction pathway(s) used by IFNγ and thereby identify the molecular basis for IFNγs pleiotropic actions. To achieve this goal we are currently studying the structure and function of IFNγ receptor. We have purified, cloned and expressed the human and murine IFNγ receptors. These proteins are single polypeptides of 472 and 450 amino acids respectively that do not have intrinsic kinase or phosphatase activity or sequence and are not closely related to any other known proteins. Attempts to express IFNγ receptors across species barriers revealed that this single polypeptide is all that is required for conferring upon cells the ability to bind and internalize ligand. However, these studies also showed that formation of functionally active IFNγ receptors required the presence of a second species specific component. Using somatic cell hybrids, the gene(s) for the receptor accessory component(s) have been localized to human chromosome 21 and murine chromosome 16. We are currently attempting to identify and clone these molecule(s).

In the meantime we initiated a structure function analysis of the human IFNγ receptor's intracellular domain by taking advantage of the fact that we could form a functionally active human IFNγ receptor in murine cells that contained a single copy of human chromosome 21. Using deletional and point mutation mutagenesis approaches, we have identified the specific amino acids within the human and murine IFNγ receptors' intracellular domains that are important for mediating receptor-dependent ligand internalization and biologic response induction. One of these residues is a tyrosine near the receptor's carboxyl terminus (Y-440 and Y-420 in the human and murine receptors, respectively). Mutation of this tyrosine to either alanine or phenylalanine does not effect receptor expression, ligand binding ability, or receptor recycling, but completely ablates the ability of the receptor to signal for all IFNγ induced biologic responses measured to date. Mutation or deletion of any of the other 4 tyrosine residues in the receptor's intracellular domain did not lead to defective signaling. We have recently shown that the IFNγ induces the tyrosine phosphorylation of its own receptor in functionally responsive cells. IFNγ dependent IFNγ receptor tyrosine phosphorylation requires the presence of the receptor accessory component and the phosphorylation is species specific. Phosphorylation is extremely rapid (maximum phosphorylation occurs within 15 seconds) and transient (no phosphorylated receptor is detectable by 30 minutes after stimulation). The dose dependency of the phosphorylation mirrors the dose dependency for induction of MHC class II proteins on cells. Finally, inhibitors of tyrosine kinases (such as herbimycin A, tyrphostin and genestein) inhibit both IFNγ dependent receptor tyrosine phosphorylation and MHC class II induction. Current work is focused on (a) identifying the specific tyrosine residue(s) that are the target of the tyrosine kinase, (b) defining the specific kinase responsible for receptor phosphorylation.

and (c) exploring the subsequent molecular interactions that occur between the phosphorylated IFNγ receptor and other intracellular signaling components.

In an attempt to learn more about the receptor's mechanism of action, we examined the effects of overexpressing inactive receptor mutants in their own homologous cells. Expression of IFNγ receptors either lacking 97% of the intracellular domain, or 39 carboxyl terminal amino acids, or containing a point mutation in the critical intracellular domain tyrosine inhibited responses in the cells to IFNγ. Overexpression of inactive receptors did not affect expression of the endogenous wild type receptor. Three types of control experiments showed that the inhibition was specific. First, cells transfected either with empty vector or with full length homologous receptor remained fully responsive to IFNγ. Second, cells that were made unresponsive to IFNγ by expression of inactive IFNγ receptor mutants remained fully responsive to IFNα. Finally, inhibition was dependent on the ratio of inactive to wild type receptor expressed in the cell. At an inactive : wild type ratio of 100:1, the cells became completely unresponsive to IFNγ even at ligand doses that were 30,000 times greater than that needed to induce a maximal response in control cells. Thus the inhibition was due to a dominant negative effect of the inactive receptors and not simply to competitive inhibition. Current work is aimed at using dominant negative cytokine receptor mutants and transgenic mouse technology to inactivate IFNγ responsiveness in specific tissues *in vivo*. If successful this new approach should provide novel insights into the specific roles that IFNγ plays in protective and immunopathological responses.

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Structure and Function of Leukemia Inhibitory Factor and Its Receptor

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Leukemia Inhibitory Factor (LIF) was first purified and cloned based on its capacity to induce terminal differentiation to macrophages in the M1 murine myeloid leukemic cell line. Subsequently it has been shown that LIF is a highly pleiotropic cytokine that shares biological activities with interleukin-6 and interleukin-11 (inducing acute phase responses, enhancing platelet production), Oncostatin M (OSM) (inhibition of embryonal stem cell differentiation) and ciliary neurotrophic factor (CNTF) (enhancing neuronal cell differentiation). In addition LIF increases bone deposition, inhibits fat accumulation in adipocytes, stimulates proliferation of myoblasts and is required for blastocyst implantation in the uterine wall.

At least part of this biological redundancy might be explained at the molecular level by the observation that these cytokines share at least one receptor subunit. The primary LIF-binding receptor subunit (LIFRα) binds only LIF and binds it with low affinity. Association of this ligand/receptor complex with a second receptor subunit (gp130) generates a high-affinity cell signalling complex. The gp130 subunit is shared with the IL-6, IL-11, OSM and CNTF receptors although each of these receptors also contains a ligand-specific α-chain. For OSM and CNTF receptors, both LIFRα and gp130 are part of the receptor complex. Thus at least three molecular interactions - LIF/LIFRα which is ligand specific, LIF/gp130 which is shared by IL-6, IL-11, OSM and CNTF and is responsible for high-affinity conversion and LIFRα/gp130 which may be responsible for cell signalling - are responsible for LIF-induced signalling. All of the receptor subunits defined for this class of ligands are structurally related and contain at least one copy of a hemopoietin receptor domain the structure of which has been determined for the related growth hormone receptor complex (a duplicated β-barrel structure homologous to papD and CD-4 domains).

LIF itself is predicted to take up the conformation of a four α-helical bundle (helices A-D) with connecting loops and three intramolecular disulfide bonds connecting the N-terminal loop to the C-D loop and the D-helix to the A-B loop. This structure is highly homologous to the experimentally determined structures of other cytokines (growth hormone, IL-2, GM-CSF, IL-4).

We have taken advantage of the one way species cross-reactivity of LIF (mouse LIF is active only on mouse cells but human LIF is active on both mouse and human cells) to generate interspecies chimeric LIF molecules and determine the site of interaction of human LIF with the human LIF receptor α-chain. We found that the C-D loop contributes the majority of the binding energy for this interaction with lesser contributions from two residues in the C-helix and B-C loop. Surprisingly these same residues in the human LIF molecule contribute to the unusually high binding affinity of human LIF to the mouse LIF receptor α-chain. The definition of this binding site interaction along with others will aid in the design of agonistic and antagonistic analogues of this important family of cytokines.

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INTRACELLULAR SIGNALLING

The role of Ras proteins in T cell antigen receptor signal transduction.

Manolo Izquierdo, Melissa Woodrow, Sally J. Leevers,* Chris J. Marshall*,
Doreen Cantrell

It has previously been shown in T cells that stimulation of protein kinase C (PKC) or the T cell antigen receptor(TCR) induces the rapid accumulation of the active GTP-bound form of the guanine nucleotide binding protein $p21^{ras}$. Ras function is crucial for TCR induction of T cell activation .As well,signals generated by Ras can synergise with calcium signals to induce expression of the transcriptional factors that regulate the Interleukin 2 gene. TCR and PKC stimuli also induce the activation of extracellular signal-regulated kinase 2 (ERK2), a serine/threonine kinase that is rapidly activated via a kinase cascade in response to a variety of growth factors in many cell types. In this study, we show that $p21^{ras}$ is a component of the T cell antigen receptor signaling pathway that controls ERK2 activation. In the human Jurkat T cell line, transient expression of constitutively active $p21^{ras}$ induces ERK2 activation, measured as an increase in the ability of an ERK2-tag reporter protein to phosphorylate myelin basic protein (MBP). Thus, constitutively active $p21^{ras}$ bypasses the requirement for PKC activation or TCR triggering to induce ERK2 activation. In addition, activation of PKC or the TCR produces signals that cooperate with activated $p21^{ras}$ to stimulate ERK2. Conversely, expression of a dominant negative mutant of ras, Ha-*ras* N17, blocks ERK2 activation following TCR stimulation, indicating that endogenous $p21^{ras}$ function is necessary for the TCR-stimulated ERK2 activation. Taken together, these results demonstrate that the activation of $p21^{ras}$ is both necessary and sufficient to induce ERK2 activation in T cells.Ras regulation of the ERK kinase cascade may explain Ras control of T cell activation.

Molecular Circuitry Underlying T Cell Receptor Signaling

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T lymphocytes express a variety of specialized receptor structures that direct appropriate responses to parasitism. For example, ligand occupancy of the T cell antigen receptor provokes a sterotyped set of biochemical alterations culminating in lymphokine release and cell proliferation. The signaling mechanisms responsible for regulating this activation process are incompletely described, however recent studies permit strong inferences regarding the coupling mechanisms that link antigen receptor stimulation to changes in transcription. We have pursued a molecular dissection of lymphocyte signaling pathways by manipulation of the repertoire of signal transduction elements in vivo This approach is especially advantageous in the analysis of lymphocyte responses since (1) signaling mechanisms can be simultaneously evaluated in all lymphocyte subsets, (2) the signaling process is observed in otherwise normal lymphoid cells, obviating the need to study transformed cell lines (which frequently manifest aberrant activation responses), and (3) the importance of signaling pathways in lymphocyte development is readily addressed. Using this approach, we have generated mice in which expression of the hematopoietic form of p59^{fyn}, a *src*-family protein kinase, is either eliminated or substantially augmented. The T lymphocytes from these mice behave as one might expect if this protein tyrosine kinase delivers a pivotal signal in the propagation of antigen-specific responses. At the same time, our evidence suggests that multiple signaling pathways simultaneously emanate from the T cell antigen receptor. In complementary experiments, the expression of four additional protein tyrosine kinase genes, two serine/threonine kinase genes, genes encoding classical heterotrimeric G proteins, and other genes encoding small molecular weight G proteins of the *ras* type, have been manipulated. A molecular dissection of the circuitry underlying T cell activation in whole animal model systems appears feasible.

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Synthetic inhibitors of a phospholipid signal transduction pathway selectively decrease cytokine mediated cytotoxicity and stimulate hematopoietic recovery after cytotoxic therapy. Jack W. Singer, M.D., Cell Therapeutics, Inc.

A phosphoslipid signaling pathway described by Bursten and coworkers (J. Biol. Chem. 266:20732, 1991) transduces signals by IL-1 and TNFα through type 1 receptors. In addition this pathway is activated directly by endotoxin and is a component of the complex signaling induced by PDGF, EGF, the B cell IgM receptor and the T cell CD3 receptor. The pathway is not involved in signaling by hematopoietic growth factors including G-CSF, IL-3, erythropoietin, and c-kit ligand and is not active in resting cells. Pentoxifylline, a methylxanthine derivative is a weak inhibitor of this pathway with an IC_{50} of 30μM in ras-transformed NIH-3T3 cells and approximately 100μM in P388 cells. CT-1501R, an unnatural metabolite of pentoxifylline formed in man only when a P450 inhibitor is coadministered, has an IC50 of 0.26μM and 10μM, respectively for these cells. CT-1501R inhibits the release of TNFα from macrophages induced by IL-1 or LPS, and suppresses adhesion of U937 cells to human umbilical vein endothelial cells following activation with TNFα or endotoxin. When CT-1501R was given to groups of at least 20 mice at 100 mg/kg IP TID starting simulatenously or at 2 or 4 hrs following a lethal dose of endotoxin, survival was enhanced. Sixty percent of mice survived when CT-1501R was started at the same time as endotoxin, 55% survived when it was started after 2 hours, and 37% when treatment was started after 4 hrs. A single dose of CT-1501R administered simulatenously with endotoxin allowed 68% of mice to survive compared to no survivors in the control arm. CT-1501R produced a 75% reduction in the one hour peak circulating levels of TNFα following endotoxin and also suppressed the increase in IL-1α that occurred after 6 hours but had no effect on IL-1α levels at earlier time points. These data suggest that CT-1501R may protect against adverse clinical events associated with inflammatory cytokine cascades without affecting normal homeostatic functions. Although CT-1501R has no effects on basal hematopoiesis, when it was administered to mice treated with high dose 5-fluorouracil (190 mg/kg), marked enhancement of multilineage hematopoietic recovery was observed. Compared to vehicle control treated animals, CT-1501R treated mice had earlier neutrophil, platelet and CFU-GM/femur recovery. Platelet and CFU-GM/femur nadir count were significantly higher in animals treated with CT-1501R. Both platelet counts and CFU-GM/femur overshot basal values

by more than two times by day 15 whereas neutrophil counts recovered only to control values. Significantly more mice treated with CT-1501R than control mice survived ($p<0.04$; log rank). Taken together, these data suggest inhibition of a specific, proinflammatory phospholipid signal transduction pathway can yield a number of potentially beneficial clinical effects including inhibition of toxic cytokine cascades and enhancement of recovery of hematopoiesis following a cytotoxic challenge.

STEM CELL BIOLOGY

MOLECULAR ANALYSIS OF THE SYNERGY OF IL-3 AND TNF-α IN STIMULATING PROLIFERATION OF HEMATOPOIETIC PROGENITOR CELLS.

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An optimal proliferative response is achievable in both normal CD34+ hematopoietic progenitor cells and blast cells of patients with acute myelogenous leukemia (AML) by combinational treatment with interleukin (IL)-3 and tumor necrosis factor (TNF)-α. Analysis of the molecular mechanisms mediating this synergism revealed that TNF-α, but not IL-3, enhanced binding activity of the c-jun/AP-1 transcription factor. In order to further elucidate the importance of c-jun/AP-1 for the capacity of TNF-α to synergize with IL-3, the antisense-technique was employed. Treatment of AML blasts with an antisense oligomer directed against the translation initiation site of c-jun, but not with the corresponding sense or an unrelated nonsense oligonucleotide resulted in intracellular RNA/oligomer duplex formation followed by efficient inhibition of c-jun/AP-1 protein synthesis. Elimination of c-jun/AP-1 by antisense oligomers relieved TNF-α/IL-3-mediated synergism, while IL-3-induced growth stimulation remained unaffected. Molecular analysis of the mechanisms governing TNF-α-induced c-jun/AP-1 binding revealed that TNF-α induced a signalling cascade leading to posttranslational modification of pre-existing c-jun/AP-1 protein and thereby allowed binding of c-jun/AP-1 to its recognition site, while IL-3 did not. Moreover, TNF-α transcriptionally activated the c-jun gene by enhancing binding activity of the NF-jun transcription factor which recognizes a palindromic sequence within the c-jun promoter located immediately 5' of the SP-1binding site. Activation of NF-jun and thus expression of c-jun/AP-1 is a prerequisite for TNF-mediated growth-stimulation of IL-3 treated hematopoietic progenitor cells in that deletion of the NF-jun recognition sequence abolished TNF-mediated activation of a reporter gene linked to the c-jun promoter. Moreover, accumulation of c-jun mRNA was achievable in TNF-stimulated progenitor cells but not in cultures that had received IL-3 only. In contrast, more mature myelopoietic cells - though responding to TNF with functional activation - failed to synthesize DNA on exposure to TNF and also did not exhibit NF-jun binding activity.

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GENE EXPRESSION IN PURIFIED MURINE HEMATOPOIETIC STEM CELLS.

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We have demonstrated that pluripotent hematopoietic stem cells (PHSC) can be purified from bone marrow using a combination of counterflow centrifugal elutriation, immunological subtraction of lineage positive cells and fluorescence activated cell sorting (FACS) procedures. The fraction containing small, low density PHSC that eluted at a flow rate of 25 ml/min (FR25) was relatively depleted of CFU-S whereas the large, high density PHSC fraction that eluted at a flow rate of 35 ml/min (FR35) contained numerous CFU-S. We used an anti-lineage marker monoclonal antibody cocktail and immunobeads to subtract the lineage positive cells from these 2 subsets. The Lin- cells were stained with anti c-*kit* monoclonal antibody and sorted by FACS into c-*kit* bright, c-*kit* dull and c-*kit* negative fractions. A single injection of 200 FR25 Lin- c-*kit* bright cells repopulated the entire lymphohematopoietic system in *W/W*^{v} mice whereas no PHSC were present in the c-*kit* dull and c-*kit* negative cells.

We have also examined the effects of stem cell factor (SCF) on the number and distribution of PHSC in normal mice. SCF increased the absolute number of PHSC 3-fold/mouse with a 10-fold increase in PHSC in the peripheral blood and in the spleen. Peripheral blood PHSC were Lin- c-*kit* bright and like bone marrow PHSC they completely repopulated the bone marrow and thymus of irradiated and *W/W*^{v} recipients. An injection of 1500 and 100 c-*kit* bright cells repopulated 4/4 mice and 1/5 mice, respectively. In contrast, no repopulation was observed in 6 mice after an injection of 1500 c-kit dull or 2 x 10^5 c-*kit* negative cells from peripheral blood. More recently we have demonstrated that co-injection of SCF and G-CSF into splenectomized mice produced a 35-fold increase in peripheral blood PHSC.

The FR25 and FR35 Lin- c-*kit* bright, dull and negative cells were analyzed by reverse transcriptase-PCR for specific mRNA expression. The FR25 Lin- c-*kit* bright cells had GATA-1 mRNA but not NFE-2 or erythropoietin receptor (EpoR) mRNA. GATA-1, NFE-2 and EpoR mRNA were present in FR25 Lin- c-*kit* dull cells and in all FR35 subsets. Recently, a form of c-*kit* mRNA containing an additional 12 nucleotides in the region coding for the extracellular domain has been identified (Alan Bernstein et al.). This message, termed c-kit_A, arises by alternative splicing of the primary c-*kit* transcript. We have examined purified FR25 and FR35 PHSC populations

for expression of c-*kit* and c-kit_A mRNA. Both mRNAs were expressed in c-*kit* bright and c-*kit* dull cells whereas c-*kit* negative cells had little or no message. The ratio of c-*kit*/c-kit_A was approximately 2:1 in FR25 Lin- c-*kit* bright cells and 4:1 in FR35 Lin- c-*kit* bright cells. We conclude from these data that GATA-1 is required to initiate erythropoiesis, and that differences in the relative amount of c-*kit*/c-kit_A mRNA may indicate a functional difference among PHSC.

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The genes that govern differentiation and self-renewal: analysis in single hematopoietic precursors by polyA PCR and subtractive hybridization

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Clonal methods have defined a hierarchy of hematopoietic precursor cells differing stage-to-stage in their ability to self-renew and in their differentiative and growth potentials. Although differences in biological potential can be predicted to derive ultimately from the transcriptional activity of key regulatory genes, the relevant mechanisms and genes remain unknown at the present time. The quest for such information has been a driving force behind efforts at stem cell purification. However, purifications using currently available markers continue to yield fractions containing cells with diverse biological potentials and thus unsuitable for identification of differentially expressed genes that might determine specific differentiative states.

My laboratory has developed approaches that allow comprehensive study of the spectrum of genes expressed at various stages of the precursor hierarchy. The strategy has been two-fold. First, we devised a novel technique providing unbiased PCR amplification of all polyadenylated mRNA's present in single cell samples. The second and crucial element is an experimental design which allows unequivocal identification of the biological potential of single micromanipulated cells by analysis of the developmental fates of their sibling cells in colony starts. We have accumulated an extensive collection of amplified cDNA samples from single cells with multiple representatives at various points in the precursor hierarchy, including samples from pluripotential, oligopotential and unipotential cells. The sample set provides a means for rapid mapping of the expression patterns of any known genes over much of the hierarchy. Mapping experiments with probes for genes with lineage-specific expression and for various cytokine receptors have helped to validate the sample set as well as to provide novel information. Novel subtractive hybridization protocols have also been developed to exploit the power inherent in the PCR-based approach. Pilot experiments have identified mRNA sequences expressed uniquely in precursors at defined early stages in the hierarchy and not represented in the existing sequence data banks. These techniques are expected to allow rapid expansion in knowledge of genes and their products that specify the biological potential of hematopoietic precursor cells.

TOWARDS IN VITRO EXPANSION OF PURIFIED HUMAN HEMATOPOIETIC STEM CELLS

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Despite rapid and significant advances in the understanding of cytokines, cytokine receptors, signalling pathways, transcription factors and factors in control of the cell cycle, many basic questions related to the role of these various factors in the biology of early hematopoietic cells remain. Studies to address these questions are hampered by difficulties to obtain sufficient normal hematopoietic cells for study and the extensive heterogeneiety of immature hematopoietic cells in normal hematopoietic tissues. Studies of the biology of human hematopoietic cells are further complicated by uncertainties with respect to the significance of *in vitro* assays in relation to *in vivo* function. The initial goal of our studies was to separate cells capable of initiating long-term cultures (LTC-IC) from colony-forming cells that can be assayed in semi-solid medium and use such purified cell fractions to study the biology of early hematopoietic cells. It was found that LTC-IC could be highly enriched (at a good yield) separate from the large majority of clonogenic cells by sorting bone marrow cells on the basis of $CD34^+$ expression and low levels of both CD71 (transferring receptor) and CD45RA (1). When such $CD34^+CD45RA^{lo}CD71^{lo}$ cells were cultured in serum-free medium supplemented with IL-3, IL-6, Steel factor and erythropoietin, a 10^6-fold increase in cell numbers was observed over a period of four weeks. Interestingly, the absolute number of $CD34^+CD71^{lo}$ cells did not appear to change in such cultures. When $CD34^+CD71^{lo}$ cells

were sorted from expanded cultures and recultured, extensive cell production was repeated, again without significant changes in the absolute number of cells with the $CD34^+CD71^{lo}$ phenotype that were used to initiate the (sub) cultures. These results document that primitive hematopoietic cells can generate extensive progeny, apparently without depleting the size of a percursor cell pool. Several models were proposed to explain the observed maintenance of $CD34^+CD71^{lo}$ cells in our cultures were considered: 1) survival of non-dividing cells; 2) self-renewal balanced by loss of cells; 3) asymmetrical divisions and 4) combinations of the above. Two experimental strategies were explored in order to discriminate between these models. In the first, sorted $CD34^+CD45RA^{lo}CD71^{lo}$ cells were labeled with the fluorescent tracking dye PKH26, followed by analysis of PKH26 fluorescence of $CD34^+CD71^{lo}$ and other cells present in the cultures at various time points (up to 11 weeks). In the second approach, single $CD34^+CD45RA^{lo}CD71^{lo}$ cells were directly sorted into individual wells, and growing cells were then analysed by flow cytometry. Results from these experiments indicated a considerable variability in 1) the number of surviving input cells (ranging from 30 to 80%); 2) the proportion of cells that contributed significantly to the total cell production measured at day 20 (ranging from 1 to 5%), and 3) the number of $CD34^+$ cells present in individual clones. Taken together, the observed maintenance of primitive $CD34^+$ cells in our cultures apparently involves a combination of survival of $CD34^+CD71^{lo}$ cells with a very low turn-over together with a very limited production of $CD34^+$ cells (2). In order to examine the effect of serum-free culture of purified candidate stem cells in the presence of IL-6, IL-3, Steel factor and erythropoietin on cells capable of reconstituting hematopoiesis *in vivo*, these studies were repeated in a murine model. For this purpose, $Sca^+Lin^-WGA^+$ cells before and after culture were injected into lethally irradiated recipients. Results with these primitive hematopoietic cells of murine origin were remarkably similar to those observed with the purified human cells: extensive production of cells without an increase (or decrease!) of cells with long-term repopulating potential. These results suggest that primitive hematopoietic cells either lack self-renewal potential, or that

the culture conditions used to not support production of primitive hematopoietic cells (3). If self-renewal of adult bone marrow stem cells is indeed limited, a prediction would be that the actual *production* of stem cells is confined to early stages of development (i.e. in fetal liver). In order to test this hypothesis, we have recently started to compare the behaviour of $CD34^+CD45RA^{lo}CD71^{lo}$ cells purified from fetal liver and umbilical cord blood in our culture system with those purified from adult bone marrow. The results of these studies have shown extensive functional differences in the proliferative response between primitive hematopoietic cells from various tissues that are compatible with the production (self-renewal) of such cells being largely restricted to early stages of development (4).

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MURINE STROMAL CELLS TRIGGER THE DEVELOPMENT OF VERY EARLY CD34++/CD38- HUMAN STEM CELLS IN SHORT -TERM AND LONG-TERM ASSAYS.

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Most of our knowledge on the mechanisms regulating stem cell self-renewal and differentiation relies on in vivo studies performed in the murine system where a hierarchy of transplantable stem cells with different potential has been defined. Evidence that such long-term reconstituting cells exist in the humans is only indirect and comes from the observation that multiple lineages are derived from neoplastic clones and that hematopoietic reconstitution observed after marrow transplantation may be monoclonal. Experimental approach to study the biology of the most primitive cells in vitro is hampered by the very low incidence of these cells and by their inability to form visible clones in standard short-term colony-assays. *In vitro,* the only available strategy consists of growing human marrrow cells for periods up to 8-10 weeks in the presence of a competent underlayer of marrow stromal cells. In these conditions, it is assumed that most of clonogenic cells present at the initiation of the culture will either differentiate or die. Therefore, clonogenic cells recovered after several weeks in the coculture with stromal cells represent the progeny of more immature progenitor cells present in the input population.

Our goal in this study was to design an in vitro method for maintaining and expanding primitive human progenitor cells that could be subsequently used to investigate molecular events associated with the regulation of early hematopoiesis. We constructed a long-term culture assay by coculturing primitive CD34++/CD38- cells with both human stromal cells and with a murine stromal cell (MS-5) known to support mouse long-term reconstituting stem cells. We reasoned that the use of murine stromal cells might have two advantages: (1) favor the proliferation of early human stem cells over their differentiation as most of the murine growth factors do not cross-react with human cells; (2) favor the identification of novel activities regulating early stem cells development.

The effect of MS-5 murine stromal cells was tested both in semi-solid assays and in long-term cultures.

- A striking growth-promoting effect of MS-5 was observed in short-term clonogenic assays: addition of 10,000 MS-5 cells to methylcellulose increased 2-5 fold the cloning efficiency of CD34+/CD38- with a selective effect on primitive pluripotent progenitors and triggered the emergence of CFU-blast with a high replating efficicency. Interestingly, MS-5 cells alone did not promote CD34+/CD38-

development, but synergized with any combination of human growth factors and particularly with c-kit ligand and IL-3. Interestingly, the synergistic effect of MS-5 could not be substituted by any combination of growth factors, including bFGF, IGF-1 or IL-11, neither by human stromal cells.

- In order to assess whether MS-5 would support or not long-term hematopoiesis, cocultures were established in 24-wells plates by incubating 5,000 CD34+/CD38- cells on preestablished human and murine stromas. Production of clonogenic progenitors was regularly assessed in methylcellulose assays during the 6-10 weeks of the coculture. We show that murine cells allow the expansion of very early progenitor cells during at least 5 weeks but as expected did not promote their terminal differentiation. Limiting dilution experiments (2, 5, and 50 cells /well) were set up to precisely determine the *frequency* of LTC-IC in the initial CD34++/CD38- suspension and their *proliferation capacity* expressed as the *number of clonogenic progenitors generated per LTC-IC* after 5 weeks coculture. Five percent of CD34++/CD38- cells met the criteria required for LTC-IC (defined as a cell which gives rise to at least one progenitor after 5 weeks) and this frequency was identical for cells cultured on human stroma and murine stroma.The average number of progenitors produced per LTC-IC however was higher on MS-5 stroma (2.7 with a very wide distribution ranging from 1 to 30)) than on human stroma (1.6). Our data thus extend previous observations which support the idea that non-human stromal cells support human LTC-IC maintainance despite the fact that most growth factors known to act on early human cells are not species cross-reactive.

- In an attempt to characterize the growth-promoting activity of the murine MS-5 cell line, we designed an in vitro screening assay easier to control than the LTC-IC assay. We selected as a target cell the human factor-dependent cell line UT-7, whose proliferation normally depends on the addition of GM-CS, IL-3 or Epo. Interestingly, MS-5 cells also support the long-term growth of this cell line in the absence of any human growth factors, and none of the known cross-reactive growth factors would substitute for this effect. A similar growth pattern was obtained on Sl/Sl cell line, genetically unable to produce c-kit ligand, or on MS-5 in the presence of anti-c-kit antibodies which excludes that this cytokine plays a crucial role. Interestingly, MS-5 supernatant was inactive and UT-7 proliferation was negligible if a physical contact between UT-7 and MS-5 was prevented. These data strongly suggest that additional activities active on early human stem cells are synthesized by murine stromal cells and function either as cell-associated molecules or in synergy with components of the extracellular matrix.

STEM CELL-STROMAL CELL CULTURES

CYTOKINE MODULATION OF THE HEMATOPOIETIC MICROENVIRONMENT

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The ability to isolate relatively pure populations of stem cells and progenitor cells, coupled with the application of molecular techniques to identify functional activities in the microenvironment have allowed us to begin to unravel the complexities of hematopoietic regulation. The emerging picture suggests that numerous factors work in concert to control proliferation and differentiation of stem cells, as well as the maturation and release of endstage cells. These activities act in a concentration dependent manner which can be controlled at the level of receptor-ligand gene transcription, which in turn may be regulated by signals provided by other cells. Therefore, the hematopoietic microenvironment is truly a paracrine system, where changes in any one cytokine may be reflected by changes in others to maintain an appropriate balance between stimulatory and inhibitory factors.

Currently, a long-term culture system is used to functionally dissect the microenvironment. However, as this work proceeds it becomes imperative that we establish relevance between our observations made in vitro and those made in vivo. At present, several cytokines such as granulocyte colony-stimulating factor (G-CSF) are being evaluated in clinical trials to shorten or overcome periods of marrow dysfunction or to recruit into the periphery hematopoietic progenitors. Yet little is known with regard to how many of these cytokines exert their effects. In a number of experimental models, G-CSF appears, in addition to affecting the number and function of the mature granulocytes, to act also on less committed hematopoietic progenitors as it promotes endogenous recovery of hematopoiesis following lethal irradiation. The question arises whether G-CSF exerts its effect directly on the hematopoietic progenitors and/or on the microenvironment.

Our laboratory has recently evaluated the effect of exogenous G-CSF on the expression of other cytokines in the long-term marrow culture (LTMC) system. We initially evaluated the expression of interleukin-3 (IL-3) and kit ligand (KL), as they have been reported to affect the more immature hematopoietic progenitors. Using standard conditions, confluent LTMC were established from normal human bone marrow donors. LTMC were then switched to serum-deprived conditions in the presence or absence of recombinant human G-CSF. At various timepoints, stromal cells were lysed and mRNA procured. The mRNA preparations were analyzed by either ribonuclease protection assays or semi-quantitative PCR. Consistently, in comparison to control samples, there was an increase in the amount of IL-3 mRNA shortly after the addition of G-CSF. The expression of KL mRNA in response to G-CSF peaked much later. Using a semi-quantitative PCR assay that could distinguish transmembrane versus soluble forms of KL, it appeared that G-CSF stimulated a preferential expression of transmembrane KL mRNA over soluble KL mRNA. Whether this increased level of detectable RNA is translated into protein is not firmly established at this time. In addition, it is not clear whether the increased amount of detectable RNA can be attributed to increased transcription rates or stabilization of message. Nevertheless, it seems reasonable to hypothesize from this data that some of the in vivo effects of G-CSF are not a result of G-CSF acting directly on stem cells or progenitor cells, but rather the effect of modulating the expression of other factors in the microenvironment.

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BONE MARROW STROMAL REGULATION OF HEMATOPOIESIS: A PROBLEM OF SYNTHESIS

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Studies of murine Dexter adherent cells have provided a model for stromal regulation of hematopoiesis. We have previously reported that irradiated stromal layers were sources of IL-6, G-CSF, GM-CSF and Steel factor. We could only detect mRNA for IL-3 by RT-PCR but found evidence of biologically active IL-3 by studies showing blocking of 32D cell growth on stroma by monoclonal antibodies to IL-3. Interpretation of these data are complicated by observations that IL-3 exposure and/or high density turns on RT-PCR detectable mRNA for IL-3 in 32D cells. In situ hybridization studies indicate that anywhere from 5-23% of adherent cells express IL-3. Following on the seminal observations of Brecher and colleagues that normal marrow could be transplanted into normal non-myeloablated hosts, we have evaluated the ability of male Balb/C marrow to engraft into non-myeloablated female Balb/C host mice. Using either 40 million cells per day for five days or 2 tibias and femurs per day for five days, we have obtained engraftment rates in bone marrow at 1 month to 25 months up to 78%. Similar engraftment rates have been seen in spleen and thymus with minimal engraftment in liver and none in brain or muscle. We have detected engraftment using Southern blot analysis probed with the PY2 probe which recognizes repetitive sequences on the Y chromosome. Similar results were obtained using cytogenetic approaches and evaluating the Y chromosome by c-banding. We have also compared the ability of marrow harvested 6 days after 150 mg/kg 5-Fluorouracil with normal marrow to engraft into normal non-myeloablated hosts. In this setting we found that post-5-FU marrow was markedly deficient at 1,2,3,10 and 12 months post engraftment as compared to normal bone marrow. We have also evaluated the schedule dependency of marrow engraftment in this male/female model. We have found that engrafting a total of 200 million cells either in one injection, over 5 days or over 10 injections resulted in progressively increasing percent engraftments as determined by Southern blot analysis and densitometry. When 200 million cells were engrafted at day 1, the cumulative percent engraftment was 10 ± 4% while with a 5 day injection schedule engraftment was 61 ± 15 and with a 10 day injection schedule engraftment was 76 ± 24%. This suggests that there is a scheduled dependency of engraftment and further indicates that there may be 5 to 10% of marrow niches available at any one time in a normal host animal. These data suggest the potential importance of scheduling for autologous marrow engraftment and its possible utility in gene transfer approaches and further suggest that local stromal cytokine production may determine the geography of marrow cell production.

G-CSF AND GRANULOCYTIC MATURATION

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G-CSF is a growth factor largely responsible for the development of the granulocytic lineage, and is essential for the survival, proliferation and differentiation of granulocytic precursors. Bone marrow cells are capable of granulocytic maturation under the influence of G-CSF alone, although in combination with IL3, GM-CSF, and/or SCF this response is enhanced. The cytokine acts through its cognate receptor, which is most likely present as a homodimer with a single high affinity binding site on mature granulocytes and their precursor cells.

Several disorders are known where a maturation arrest exists in the granulocytic lineage. Acute myeloid leukaemia is characterized by an excessive amount of myeloid precursor cells (blast cells). In approximately 50% of the cases these blast cells are still capable of a proliferative response upon in vitro stimulation with G-CSF, but they hardly mature. Exceptions are AML-cases with a t(8,21)(q22;q22). Blast cells from these patients respond to G-CSF stimulation, as evidenced by induction of proliferation, but the leukaemic cells also mature into neutrophils when cultured in the presence of high concentrations of the cytokine.

Severe congenital neutropenia or Kostmanns syndrome is characterized by a persistent severe absolute neutropenia and recurrent infections. In a series of patients with congenital neutropenia, different in vitro responses to G-CSF were seen. Some patients had a normal G-CSF response, with respect to the number and the dose-response curve of myeloid progenitor cells to G-CSF. Several patients had a reduced responsiveness to G-CSF, but less when G-CSF and GM-CSF were assayed together. In one patient hardly any myeloid growth could be detected, whatever combination of myeloid growth factor was used.

These variable patterns of G-CSF response indicate that in some cases at least an impaired or mutated G-CSF receptor could cause the maturation block seen in AML and/or neutropenia. Studies to investigate this mechanism are currently undertaken.

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APPLICATION OF CYTOKINES

AN OVERVIEW OF CYTOKINES IN MARROW TRANSPLANTATION

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Following myeloablative therapy and the intravenous administration of hematopoietic precursor cells, a period of three to four weeks is required before repopulation of the marrow by donor cells is sufficient for the production of useful numbers of granulocytes, platelets and red cells. During that time patients must be supported by the transfusion of these formed elements of the blood. Despite transfusions and antibiotics, the recipients are at risk of death from infections and bleeding. The administration of G-CSF and GM-CSF have already been shown to accelerate the recovery of granulocytes and to significantly shorten the time required for hospitalization of the patient.

Current research directed toward the clinical application of cytokines includes the following:

The exploration of the clinical usefulness of a series of other cytokines and cytokine combinations, particularly those that may accelerate platelet recovery.

The use of cytokines to stimulate the release of hematopoietic precursors into the circulation for collection for subsequent transplantation.

The use of G-CSF and GM-CSF for elevation of the granulocytes in normal donors for granulocyte transfusions, and the use of EPO to stimulate red cell production before surgery for autologous transfusions.

The use of cytokines, particularly the kit-ligand, for stimulation of stem cells to permit gene insertion by retroviruses.

The use of cytokines for in vitro expansion of hematopoietic precursors for subsequent transplantation or gene transfer.

These and other aspects of the clinical application of cytokines will be summarized.

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"RECOMBINANT HUMAN GROWTH FACTORS: FROM THE BENCH TO THE CLINIC?"

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Cellular proliferation and differentiation in the living organisms are regulated,at least in part,by a complex network of interacting peptides or growth factors. The purification and molecular cloning of these growth factors has now led to the exciting task of ascertaining their physiological role in vivo and to verify their value as therapeutic substances worthy of clinical use.Rigorous methods of drug evaluation are required to prove the efficacy and safety of these new biological agents,particularly now that so many of them are being produced by the new genetic engineering techniques.Diseases involved include cancer,infectious diseases,inflammatory processes,autoimmune disorders,developmental defects and aging.In cancer, they are being tested as direct antitumor agents,as enhancers of immunological reactivity against tumor cells,as potentiators of chemotherapy and radiotherapy,as mitigators of the myelotoxicity associated with current cancer therapies and as aids to gene therapy and the development of new "tumor vaccines".Some of these agents (erythropoietin,interleukin-2,G-CSF,GM-CSF) have already been approved for clinical use by several countries,undoubtedly adding to the therapeutic armamentarium of cancer clinicians,but also increasing significantly the costs of already overstretched clinical budgets (even if their judicious use might actually decrease overall costs by saving hospitalization costs or other expensive drugs).At the same time,many antagonistic factors are being developed for use in those diseases that may be initiated or mediated by cytokines (interleukin-1 antagonists or monoclonal antibodies anti-TNF,for example).It should therefore come as no surprise that cytokines and recombinant growth factors have become important research topics for both pharmaceutical companies and experts in this new but rapidly growing subject of "pharmaco-economics".

Of all the cellular systems of proliferation and differentiation in human beings,the best understood in cellular and molecular terms is "hemopoiesis". This is probably due to a number of historical and technical reasons (easy sampling of mature cells from peripheral blood and of progenitor cells from the bone marrow;good morphological and molecular markers;good and reproducible functional and kinetic assays, etc). In spite of this, the identification and purification of putative specific inhibitors of progenitor and pluripotent stem cell proliferation and differentiation is proving particularly difficult. Within the bone marrow one can find (in soluble form or attached to stroma or cell membranes) more than ten different stimulatory substances,colony-stimulating factors or interleukins, some of which are fairly lineage-specific,whereas others are more wide-acting and less selective. The selectivity of action can be regarded as a potential pharmacological advantage, although the clinical toxicity will ultimately depend on the cellular target/s and the subsequent release of toxic cytokines or active inflammatory mediators.

If one considers that in our organism co-exist (more or less peacefully) some 200 different cell types,and assuming that for each tissue (according to the hemopoietic model) there might be at least 5-10 glycoproteins regulating cell growth and differentiation, it is easy to conclude that several hundred molecules required for survival,proliferation and maturation of human cell types still await to be discovered. It would probably be unrealistic to expect that all of these will deserve the category of "drugs" and finding diseases or clinical situations to suite each individul cytokine or growth factor (or growth inhibitor) will remain the main challenge for clinical research for some time.

Future Applications of Hematopoietic Growth Factors in Bone Marrow Transplant Patients

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Several cytokines are being evaluated in preclinical and clinical trials. These molecules have a variety of activities and will alter current techniques of medical practice. Listed below are ten key future directions for utilization of cytokines:

1. To enhance pluripotent cell recovery.
2. To enhance phagocyte function against infection.
3. To reduce non hematologic toxicity.
4. To mobilize peripheral blood progenitor cells.
5. To increase tumor cell sensitivity to chemotherapy.
6. To reduce normal hematopoietic cell toxicity to chemotherapy.
7. To facilitate umbilical cord blood transplant.
8. To enhance tumor cell cytotoxicity.
9. To expand progenitor cell pools invitro before infusion with transplant.
10. To facilitate gene insertion and to be utilized as a gene insertion product.

Definitive determination of each of the above future directions by clinical trials which are currently ongoing are critical in determining the rate with which the utilization of cytokines will impact standard medical care. Furthermore, in order to optimize the impact cytokines will have on survival therapeutic applications of one or several combinations of the above future directions may be required.

Determination of the full spectrum of activities with cytokines already approved for clinical use in the United States or Europe (rhGM-CSF, rhG-CSF, rh-EPO, rh-INFalpha, rh-IL2) and the toxicities and activities of novel cytokines will be required to definitively determine the potential clinical efficacy of the future directions listed above. A list of hematopoietic growth factors being explored for potential clinical applications are listed below.

CYTOKINES	POTENTIAL APPLICATIONS
rhGM-CSF	Neutrophilia, monocytosis, infection
rhG-CSF	Neutrophilia, infection
rh-EPO	Erythrocytosis
rh-INFalpha	Anti-tumor
rh-IL2	Anti-tumor
rh-IL3	Neutrophilia, monocytosis, thrombopoiesis
rhM-CSF	Infection, anti-tumor
rh-IL1	Chemo-radio protectant, neutrophilia, thrombopoiesis
rh-FM	Neutrophilia, thrombopoiesis, infection
rh-KL	Pluripotent progenitor proliferation
rh-IL6	Thrombopoiesis, neutrophilia
rh-TNF	Anti-tumor
rh-IL4	Anti-tumor
rh-IL7	Lymphocytosis, anti-tumor
rh-IL11	Thrombopoiesis
rh-IL5	Eosinophilia
rh-IL10	Macrophage inhibitor
rh-MIF	Macrophage inhibitor
rh-LIF	Pluripotent progenitor proliferation
rh-IL8	Toxic mediator

In conclusion, advanced technology has opened the door to several exciting areas of exploration using cytokines. However, other than in unique patient populations the use of cytokines has currently not effected patient survival. Trials exploring future clinical utilization of cytokines must be directed to impact survival.

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CYTOKINE APPLICATIONS IN THE TREATMENT OF GENETIC CYTOPENIAS

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The identification and cloning of genes encoding a variety of hematopoietic and lymphopoietic growth factors and the subsequent generation of these factors by recombinant genetic techniques has stimulated extensive evaluation of these factors in the treatment of drug-induced, acquired and genetic cytopenias. Our Center has conducted a series of studies examining the effects of different recombinant cytokines on hematopoietic progenitor cell growth and differentiation both in vitro and in vivo in children afflicted with severe congenital disorders or neutrophil, red cell and platelet production. In clinical trials, GCSF has induced and sustained recovery of neutrophil populations in Kostmanns' agranulocytosis, myelokathexis and Schwachman-Diamond syndrome. Interleukin-3 (IL-3) has induced full or partial remissions in a proportion of patients with Blackfan-Diamond anemia and certain forms of congenital amegakaryocytosis. This cytokine has also induced partial recovery of neutrophil counts in a proportion of patients with Fanconi's anemia and dyskeratosis congenita. Recently, evidence has been generated indicating that certain lymphopoietic cytokines may also induce partial corrections of certain genetic disorders of T-cell activation, including genetic deficiencies of IL-2 production. Prospective analyses of the effects of these and other cytokines on marrow and lymphoid progenitors in vitro have, in certain diseases, proven to be predictive of in vivo response and have provided surprising new insights into disease pathogenesis. For other disorders, such as Diamond-Blackfan anemia and Fanconi's anemia, correlations between in vitro and in vivo responses to cytokine have not been observed. These studies will be critically reviewed and new results detailed.

POSTERS

INTERLEUKIN-3 AND ERYTHROPOIETIN INDUCE THE ACTIVATION OF THE 70 kDa S6 KINASE AND THIS ACTIVATION IS BLOCKED BY THE IMMUNOSUPPRESSANT RAPAMYCIN.

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Interleukin-3 (IL-3) and erythropoietin (EPO) are cytokines that are critical regulators of the proliferation and differentiation of cells of the hematopoietic system, but their intracellular mechanisms of action are poorly understood. Changes in intracellular phosphorylation are triggered by binding of IL-3 or EPO to their receptors and some of these changes are also triggered by interleukin-2 (IL-2) receptor stimulation. IL-2 activates the 70 kDa S6 kinase, a serine/threonine kinase whose activity is regulated by serine/threonine phosphorylation. The immunosuppressant rapamycin inhibits IL-2-dependent proliferation and IL-2-triggered activation of the 70 kDa S6 kinase. We have investigated whether these features of IL-2 intracellular signalling are shared by IL-3 and EPO signal transduction pathways. In a cell line whose proliferation can be supported by either IL-3 or EPO (Ba/F3-EPOR), we found that IL-3 or EPO activated the 70 kDa S6 kinase and that rapamycin inhibited IL-3 or EPO-supported proliferation and activation of the 70 kDa S6 kinase. Two different cell lines (Ba/F3-EPORgp55 and MEL) containing constitutively activated EPO receptors were analyzed. Rapamycin inhibited the activation of the 70 kDa S6 kinase in both cell lines, but the proliferation of the cell line possessing a more malignant phenotype (MEL) was unaffected by rapamycin. This is probably due to the constitutive activation of signalling molecules that overcome the inhibition of the 70 kDa S6 kinase by rapamycin in MEL cells. These results indicate that the 70 kDa S6 kinase activation pathway is a common element triggered by the stimulation of IL-3, EPO, and IL-2 receptors.

THE EFFECT OF SOME GROWTH FACTORS ON THE CD34+ 33- BLAST CELL POPULATION IN ACUTE MYELOID LEUKEMIA.

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In recent years there increasing knowledge has been gained about the effect of Hematopoietic Growth Factors (HGFs) on normal hematopoiesis and also on leukemic blast cells in Acute Myeloid Leukemia (AML). This active research work has shown that many blast cells in AML can respond to certain HGFs by increasing their proliferative rate. However, few studies have been performed on the action of HGFs acting on the theoreticaly more immature CD34+ 33- blast cell population. The present study was designed with two aims:

A.- To verify the action of IL-3, G-CSF, and both combined on the CD34+ 33- blastic fraction. We wished to explore whether both HGFs have a synergistic effect on the more immature blast cells.

B.- To see whether the IL-4 is able to modulate the proliferative response and the autonomous growth of that cellular fraction.

In 20 cases of AML we have sorted the CD34+ 33- blast cells with a magnetic cell sorter (MACS). This sorter uses surface antigens for the cellular selection. following this wehave made cellular cultures in parallel, using both populations: Cd33+ and CD34+ 33- blast cells.

1.- To analyze the proliferative response to IL-3, G-CSF and IL-3 plus G-CSF we studied ten cases of AML using semisolid cultures.

2.- To study the effect of IL-4 in another ten cases of AML we made liquid cultures with IL-4. After 24h. the cells were plated in semisolid cultures.

Our results show that IL-3 and G-CSF had a synergistic effect on the CD34+33- blastic population in five cases, but was inhibitory in two cases. When the combination of IL-3 and G-CSF was used with CD33+ blast cells, the synergistic effect was observed only in two cases. When these HGFs were used alone, we observed that G-CSF was more effective than IL-3 in inducing the CD34+33- cellular fraction to form colonies.

When the cultures with IL-4 were analyzed, we observed that, in most cases the proliferative capacity of the most immature blast cell population studied decreased. Moreover, autonomous growth was also lower; indeed in two cases autonomous growth was completely abolished.

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A. Macedo is a fellow from JNICT , Portugal

LONG-TERM HEMOPOIETIC CULTURES FROM THE PRONEPHROS OF RAINBOW TROUT, *Oncorhynchus mykiss* W.

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Some *in vivo* studies have pointed out that the hemopoietic tissues of lower vertebrates contain microenvironments which seem to be structurally homologous to those found in the mammalian bone marrow. In order to obtain further data, we have developed culture conditions that allow the establishment of long-term cultures of renal hemopoietic tissue of the rainbow trout, *Oncorhynchus mykiss*. In addition, we have established a stromal cell line (Trout Pronephric Stroma, TPS), obtained from a long-term pronephric culture, which is able to support hemopoiesis.

Cultures of explants of the trout pronephros were initiated in 24-well culture plates, incubated at 18 ± 1°C in air. The culture medium contained RPMI-1640 (Gibco) with 25mM Hepes buffer, 10% FCS and 10% trout serum. Long-term pronephric cultures (LTPCs) were established using confluent cultures grown in the wells, which were scraped off and transferred to 25 cm^2 flasks, the same medium been used. From 4 weeks onwards, LTPCs appeared to be made up of fibroblastic reticular cells and epithelioid cells, which formed multilayered networks. After 18 weeks, they contained scattered hemopoietic foci formed by granulocytic cells at several stages of differentiation, which were closely associated with the fibroblastic reticular cells. Free granulocytic cells, most of them heterophils, were present in the culture supernatants. The ultrastructural and cytochemical features of the stromal cells in the cultures were similar to those found *in vivo* for the fibroblastic reticular cells of the pronephros of *O. mykiss*, and of the mammalian bone marrow. In addition, this cell type is structurally similar to that forming the microenvironment in bone marrow cultures.

A stromal cell line (TPS) was established by successive weekly passages of an LTPC. Successive passages were done by trypsinization of the cultures. Cells were plated in 25 cm^2 flasks at 9.4×10^4 to 10^5 cell/ml (5 ml / flask). The culture medium for TPS cultures (TPS-M) was similar to that indicated above, but contained 10% FCS and 5% trout serum. The cell line is now at passage number 57 (May 10th, 1993). The TPS cultures consist of fibroblastic reticular cells and epithelioid cells, resembling those found in the long-term pronephric cultures, but do not contain hemopoietic cells.

Hemopoiesis in TPS cultures was induced by co-cultivating them with allogenic pronephric cell suspensions. Subconfluent cultures of the cell line (5×10^5 cells / 25 cm^2 flask) were inoculated with 4.5×10^7 pronephric cells in 5 ml of TPS-M. In some experiments, the medium was supplemented with 10^{-5}M hydrocortisone 21-hemisuccinate (Sigma). In others, the trout serum supplement consisted of 2.5% normal trout serum plus 2.5% serum from stressed trout (containing endogenous cortisol). After three weeks, hemopoiesis was evident in the co-cultures, including myelopoiesis and erythropoiesis. The hemopoietic activity was higher in cultures supplemented with serum from stressed trout.

BLOOD AS A SOURCE OF STEM CELLS FOR HEMOPATOPOIETIC RECONSTITUTION

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Blood is a usable source of hematopoietic stem cells in clinical situations where their recovery from the bone marrow is not feasible or convenient. Different factors may influence the stem cell yield of collection procedures and there are practical difficulties to quantitate the potential for hematopoietic reconstitution of the cellular fractions obtained from blood. The growth of CFU-GM is widely used as an indirect index of this potential. Evaluation based on quantitation of cells phenotypically characterized with MoAb as stem cells, or in cell growth in long term cultures is at present more difficult. We rely on cuantitation of CFU-GM as a guide of the hematopoietic potential of the cellular fractions collected from circulating blood to be used for auto or allo-transplant; we show yields obtained in different clinical situations.

I.- Collection of hematopoietic stem cells from peripheral blood in patients with hematological malignancies.

Data pertain to collections obtained from 13 patients: 6 with advanced Non-Hodgkin Lymphoma (NHL), 4 with advanced Hodgkin Disease (HD) and 3 with Multiple Myeloma (MM), all responsive to therapy and previously treated with chemotherapy ± radiotherapy to the point of inapparent or highly reduced bone marrow involvement. Collections have been obtained under different conditions: steady state with no previous "mobilizing" maneouvre, following courses of MOPP and Daunorubicin-AraC (3/5 days) (1 HD patient); following courses of Vincristine + Prednisone + Cyclophospamide (CTX)(1/5/4 days) (1 NHL patient); and after one course of VAD therapy (1 MM patient). All these without CSF. All other colletions were performed after treatment with CTX (either 1 g/m/dx4 or 4 g/m/dx1) followed by GM-CSF (1 MM patient) or G-CSF (5 NHL, 1 HD and 1 MM). When done in the recovery phase of myelosupresive actions, apheresis were initiated when recovering WBC reached $1x10^9$/l and continued daily with the aim of collecting a minimum of $6x10^8$/Kg mononuclear cells (MNC) and $15x10^4$/Kg CFU-GM. After CTX (4g/m/dx1) followed by G-CSF the interval to collections was quite constant (10-11 days).

Highest yields of both MNC and CFU-GM were obtained in patients with NHL in whom older age, heavy previous therapy and short interval from preceding chemotherapy seem to unfavorably influence yields both of MNC and CFU-GM. Patients with HD produced lower yields of MNC and even lower of CFU-GM, what may be related either to previous chemotherapy received by these patients and/or to the nature of HD. Intermediate yields were obtained from patients with MM. Higher yields were obtained in collections performed after courses CTX followed by daily injections of CSF, G or GM. In all cases but 1 MM patient, no tumor cells were detected in the collected fractions as assessed by cytogenetic or molecular genetics methods. Harvested cells cryopreserved in liquid nitrogen have been used for hematopoyetic reconstitution after myeloablative therapy in 8 of these

patients, all successful. Intervals to >0.5x10^9/l granulocytes have been 9 to 21 days (mean 16, median 14) and to 50x10^9/l platelets 12 to 165 days (mean 38, median 15). At this time follow-ups are too short to evaluate effectiveness of the procedure in terms of disease control.

II.- Colletion of hematopoyetic stem cells from cord blood.

Collections have been done by spontaneous drainage of the cord blood inmediately after section and before placental delivery and by syringe aspiration from the vessels of the fetal face of the placenta after its delivery; for anticoagulation, additive-free heparin, 10-20 U/ml, has been used. Collected blood has been frozen at a velocity of 1ºC/min after addition of an equal volume of Medium-199 and 20 % DMS, without any previous fractionation.

Results.- Cord blood has been collected from 5 placentae of normal newborn males. Mean values and ranges have been as follows: Total volume of collected blood, 133 ml (100-206); red cells volume, 45 ml (34-66); nucleated cells, 1.7 (1.1 - 3.2) and MNC 0.6 x 10^9 (0.4 - 1.2); CFU-GM, 43.5 x 10^4 (12.7 - 106). Proportions of nucleated cells and CFU-GM obtained from the placental veins were respectively 10.6% (4.1 - 19.2) and 17.5% (5.3 - 23) of the total harvests. CFU-GM were 72.5 per 1000 MNC in the average. Caryotype analysis showed no female metaphases and microbiological cultures were negative. Cell viability in unfrozen samples (Trypan blue) was 88 % (80-90) and the recovery of nucleated cells 96 % (91-100). CFU growth was 98% (81-180) of the pre-freezing growths. Collections have also been performed from placentae of haploidentical newborn siblings of 2 children with leukemia with similar results.

Although the potential for bone marrow engraftment of our collected fractions of cord blood remains unproved, based on our data we believe that cord blood may and must be collected as a usable source of cells for allo-transplant in cases of newborn siblings of pediatric patients eligible for this type of treatment, more so were it confirmed that, when using cord blood cells, the HLA barrier can be overcome up to a certain point.

COMMENTS: Our data show the feasibility of obtaining: 1) cellular fractions with capacity for hematopoyetic reconstitution after myeloablative therapy and with a low burden or apparently free of tumor cells from the circulating blood of patients with bone marrow involvement by hemoproliferative disease; and 2) cellular fractions relatively rich in CFU-GM of fetal origin and free of contaminating maternal cells from cord and placental blood in numbers that could be enough for hematopoyetic reconstitution after myeloablative therapy in pediatric patients.

In the clinical setting there is need for reliable and easy to perform methods that could provide a more direct evaluation of the primitive hematopoietic stem cells content of cellular fractions. It is foreseeable that with the availability of other CSF's the efficiency of the collection procedures for autologous transplants could be improved, making them safer (less risk of infection during myelosupression) and less time consuming and costly.

YIELD OF MNC AND GM-CSF OF COLLECTION PROCEDURES FROM PERIPHERAL BLOOD IN PATIENTS WITH NHL, HD AND MM AFTER DIFFERENT MOBILIZING MANEOUVRES

Dx	N	Mobiliz. therapy	Collect. cycles	Daily apher-esis	Total MNC x 10^8/Kg	Total CFU-GM x10^4/Kg	CFU-GM/ 10^3 MNC
			Range Median Mean	Range Median Mean	Range Median Mean	Range Median Mean	Range Median Mean
NHL	1	VCR, PRED 1g/m/dx5 CTX 1g/d/dx4	2	14	10.8	37.4	34.5
NHL	5	CTX	1	3-8	6.4-7.6	31-187	84.5-247
		4g/m/dx1	1	6	6.9	39.6	63.6
		G-CSF	1	5.6	6.9	79.3	101.
HD	1	None MOPP DNM-AraC (3/7d)	3	18	17.1	4.8	2.8
HD	3	CTX	2-3	10-16	13-17.7	8.4-28.1	5.6-16.3
		4g/m/dx1	2	13	17.2	11.1	6.5
		G-CSF	2.3	13	16.7	15.9	9.5
MM	1	CTX 1g/m/dx4 GM-CSF	2	10	4.0	14.4	35.6
MM	1	1st CTX 1g/m/dx4 G-CSF 2nd, VAD	2	10	5.5	11.8	55.5+
MM	1	CTX 4g/m/dx1 G-CSF	1	5	6.2	13	21

(*) Other members of the team directly involved in this work have been: Medical staff: J.R.Cabrera, J.L Díez, R.Forés, I.Sanjuán and M.Briz. Technicians and nurses: M.D.Sánchez-Mora, N.Polo, B.Alvarez, C.Moreno, M.S.Marco, F.Fernández and M.A. Redondo.

The amplification of CD34+ human haemopoietic cells by SCF and other cytokines.

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The research aims to develop the background to novel treatments for leukaemia and related blood disorders by the manipulation of CD34+ haemopoietic cells.

The procedure has two main components: purification and amplification.

Purification is being assessed by Cellpro immunoaffinity separation, FACS and magnetic bead technology. Each technique is being assessed on a starting volume of 10ml of bone marrow.

Amplification of CD34+ cells in vitro is being assessed by exposure of purified cells to a variety of cytokines including SCF, IL3, IL6, G-CSF and the hormone Epo. Various dose rates and cytokine combinations are being assessed to establish the optimum dose and cytokine combination for CD34+ amplification. Novel culture systems are being assessed in order to optimise amplification cultures. Amplification is assessed by classical in vitro colony forming assays and also by immunocytochemistry of amplified cultures to identify CD34+ cells.

Once reliable reproducible amplification procedures have been obtained, it is proposed to assess retroviral gene transfer into purified CD34+ cells. This will involve the transfer of 'marker' genes, eg: Neo^r into CD34+ cells and the possible 'piggy-back' clinical transplantation of these cells along with normal bone marrow cells. This experiment will give important data on the fate of transfected CD34+ cells on transplantation. Further retroviral gene transfer will investigate the possibility of gene therapy in disorders such as SCID and sickle cell anaemia.

Monocyte differentiation induced by specific Mab interactions with membrane receptor molecules.

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Monocytes and macrophages play an important role in the immune response and obtain their effector and regulatory functions upon activation and subsequent differentiation of myeloid progenitor cells. *In vivo* this situation is accompanied by a rapid increase in cytokine production (e.g. IL-1, IL-6, TNF) and augmented cellular adhesion, which facilitates migration of neutrofils and monocytes from peripheral blood into inflamed tissues.

Adhesion to both cellular and extracellular substrates is facilitated through a family of α/ß heterodimeric glycoproteins of cell surface receptors, termed integrins. In the present study we investigated monocyte differentiation in relation to the expression of members of the Integrin family. Therefore monocytes were purified by counterflow elutriation and cultured in the presence of monoclonal antibodies (MAb) specific for several membrane receptor molecules, after which levels of interleukin-6 (IL-6) in the cultures were determined as indication for cellular activation.

We observed that MAb against the α-chain of the β2 integrins LFA-1, MAC-1 and GP150,95 (CD11a,b,c/CD18) induced IL-6 secretion within 16 h., as well as MAb against the monocyte specific marker CD14 and CD45 (T200/leucocyte common antigen), a molecule present in all hemetopoietic cells and implicated in modification of lymphocyte activation. This coincided with the finding that one of the natural ligands of MAC-1 and GP150,95, fibrinogen, also induced IL-6 in monocytes. Moreover, we observed a homotypic aggregation of monocytes upon incubation with MAC-1 and CD45 MAb, which could be blocked by MAb to LFA-1 and its ligand ICAM-1, and not by MAb to the MAC-1 ligand, ICAM-3. Homotypic aggregation was not seen in the presence of bacterial lypopolysaccharide (LPS), a potent activator for monocytes and which was used as a positive control for high level IL-6 secretion.

These observations suggest that integrins play an important role in early monocyte activation. Moreover, crosslinking of MAC-1 and CD45 results in affinity maturation of other adhesion structures (LFA-1, ICAM-1), which *in vitro* leads to homotypic aggregation, and *in vivo* could facilitate adherence of activated monocytes to endothelial cells and subsequent extravasation.

DOWN-REGULATION OF C-MYC IS NOT OBLIGATORY FOR GROWTH INHIBITION AND DIFFERENTIATION OF HUMAN MYELOID LEUKEMIA CELLS.

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The produot of the c-myc gene localizes in the cell nucleus and is expressed in many cell types. Suppression of c-myc expression is observed during induced differentiation of several myeloid cell lines and it has been attributed to the cell growth arrest that accompanies terminal differentiation. To dissect the role of c-myc in the proliferation-differentiation switch we have studied its expression in K562 cells exposed to a battery of chemical agents. This model system allowed us to discriminate between the growth arrest and differentiation phenomena as well as the induction of differentiation along two different lineages (erythroid and myelomonocytic). Our results showed that c-myc expression did not significantly decrease when growth inhibition is reversible, either by treatment with a differentiating agent as hydroxyurea (0.5 mM) (which induced erythroid differentiation) or by a non-differentiating agent as interferon-α (2000 u/ml). In contrast, c-myc expression decreased when cells underwent terminal differentiation. This result was observed treating the cells with 1-β-D-arabinofuranosylcytosine (araC, 1 μM), which induces irreversible erythroid differentiation and with 12-O-tetradecanoylphorbol-13-acetate (TPA, 10 nM) which induces irreversible myelomonocytic differentiation. This c-myc downregulation was not a direct effect of protein kinase C (PKC) activation as was also detected in cells treated with staurosporin (100 nM), a PKC inhibitor that also induced myelomonocytic differentiation. Induction of myelomonocytic differentiation resulted in a greater loss of c-myc expression than induction of erythroid differentiation. Thus, the K562 model demonstrates that c-myc down-regulation is not obligatory for growth arrest and non-terminal differentiation of human myeloid cells. In contrast, c-myc down-regulation occurred in terminal differentiation. We are isolating K562 sublines expressing ectopic c-myc under inducible promoters to asses Myc function during K562 differentiation. Preliminary results indicate that TPA-induced differentiation occurs in the presence of high Myc levels.

TRANSFORMING GROWTH FACTOR-β_1 MEDIATES ITS INHIBITION OF INTERLEUKIN-4 INDUCED PROLIFERATION BY INTERFERING WITH INTERLEUKIN-4 SIGNAL TRANSDUCTION. A R Mire-Sluis, L Randall, M Wadhwa and R Thorpe. Div. Immunobiology, NIBSC, Blanche Lane, South Mimms, Herts. EN6 3QG. UK.

Interleukin-4 (IL-4) is a T-lymphocyte derived 20kDa glycoprotein exhibiting a broad spectrum of biological activity towards many of the cellular components of the haemapoietic system. IL-4 can induce the proliferation of several haemapoietic cell types including the leukaemic cell lines TF-1 and MO-7. We have used whole cell labelling procedures and a permeabilised system designed to detect rapid changes in dynamic phosphorylation events and show that IL-4 induces the activation of a phosphotyrosine specific phosphatase which leads to the rapid dephosphorylation of an 80kDa protein (p80) from tyrosine residues. TGF-β_1 is a complex molecule possessing potent anti-proliferative effects on many cell types and addition of TGF-ß to both TF-1 and MO-7 cells simulaneously with IL-4 strongly inhibits IL-4 induced proliferation. The inhibitory effects of TGF-β_1 on IL-4 induced proliferation are time dependent, as preincubation with TGF-β_1 markedly increases the inhibitory capacity of the cytokine towards either TF-1 or MO-7 cells. Data shows that cells preincubated with TGF-β_1 in this way no longer incorporate radiolabelled phosphate onto p80 suggesting that TGF-β_1 has downregulated either production of the 80kDa protein or the phosphatase activity required for IL-4 induced proliferation. If TGF-β_1 is added directly to cells it can mimic the IL-4 mediated dephosphorylation of p80 in a dose responsive manner closely correlating with its inhibition of proliferation, suggesting that TGF-β_1 interferes with the early events of IL-4 signal transduction.

DIFFERING TRANSDUCTION PATHWAYS BETWEEN INTERLEUKIN-5, GM-CSF OR IL-3 SUGGEST A DISTINCT ROLE FOR THE ALPHA CHAINS OF THEIR RECEPTORS IN MEDIATING CYTOKINE SIGNALLING.

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Cytokines have now been shown to play a pivotal role in the control of haematopoiesis and in both pathogenesis and treatment of disease. Although the biological properties of many cytokines have been well defined, the biochemical mechanisms underlying cytokine activities are not clearly defined and often remain phenomenological. We have therefore implemented a scientific strategy to link the biochemical effects seen in in vitro signal transduction assays to biological events seen with cells growing in culture. We and others have shown, using protein labelling and immunoblotting, that IL-3 and GM-CSF utilise tyrosine phosphorylation as part of their signal transduction mechanisms. We now show that IL-5 utilises phosphotyrosine dephosphorylation as an integral signalling mechanism, with particular reference to the dephosphorylation of an 80kDa tyrosine containing protein. These divergent biochemical events are further confirmed by the striking dichotomy between the effects of specific signalling enzyme inhibitors (Lavendustin A, Genistein, Calphostin C etc.) in cell culture. GM-CSF and IL-3 induced proliferation is enhanced by tyrosine phosphatase inhibitors and reduced by tyrosine kinase inhibitors - whilst the exact reverse occurs with IL-5. Inhibitors of PKC inhibit IL-3 and GM-CSF but not IL-5 induced proliferation. It has recently been shown that all three cytokines share the same beta chain of their receptor although the role alpha or beta chains play in signal transduction remains enigmatic. The data presented here suggests that the alpha chain of each receptor links to the individual signal transduction pathways responsible for mediating the activity of these cytokines.

THE SIGNAL TRANSDUCTION MECHANISMS MEDIATING TUMOUR NECROSIS FACTOR-α INDUCED CYTOTOXICITY INVOLVES PROTEIN PHOSPHORYLATION AND ACTS THROUGH THE TYPE 1 (p60) RECEPTOR. A R Mire-Sluis and A Meager. Division of Immunobiology, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Herts., EN6 3QG, U.K.

Tumour Necrosis Factor-α (TNF-α) was originally characterised by its antitumour activity but is now recognised to be a cytokine which exerts diverse effects on many cells, realising a role as an important mediator of immunological and inflammatory responses. TNF-α mediates its effects by binding to specific receptors on the cell surface that exist as a high affinity 80kDa receptor (type II) and a lower affinity 60kDa receptor (type I). Using specific anti-receptor antibodies (M Brockhaus, Roche, Welwyn, U.K.) we have probed the biological functions of these two receptor types and investigated the signal transduction mechanisms of TNF-α. The KYM-1 rhabdosarcoma cell line has been developed for the bioassay of TNF-α as it is sensitive to the cytotoxic action of this cytokine. We have developed a subclone KYM-1 D4 that is especially sensitive to TNF-α. We also have a panel of TNF-α resistant clones, one of which KYM-1 37B8R is very resistant to the activity of TNF-α. KYM-1 D4 cells have shown that the anti-type I receptor McAb htr-9 is a potent agonist towards these cells, showing TNF-α like cytotoxicity. This suggests that type I receptors alone are able to transduce the signal required for TNF-α induced cytotoxicity. An anti-type II receptor antibody McAb utr-1, did not appear to have any antagonistic effect on TNF-α cytotoxic activity. This suggests that type II receptors, although possessing high affinity for TNF-α are not critically involved in the cytotoxic response pathway. We show that both TNF-α and htr-9 induce the rapid phosphorylation of several proteins of molecular weights 130, 65, 48, 42 and 30kDa within 5 minutes of addition to KYM-1 D4 cells. This confirms that the binding of either TNF-α or htr-9 to the type I receptor induce identical signals and lead to the same biological response. The anti-type II McAb utr-1 however had no effect on phosphorylation patterns. The KYM-1 37B8 TNF-α resistant clone retains type I receptors, but has completely lost type II receptors. However, in this cell line addition of TNF-α did not elicit any changes in phosphorylation patterns, indicating that resistance was due to an uncoupling of the TNF-α receptors from the normal signal transduction pathways.

HEMATOLOGICAL AND IMMUNOLOGICAL RECONSTITUTION IN PATIENTS UNDERGOING AUTOLOGOUS BONE MARROW TRANSPLANTATION SUPPORTED WITH G-CSF. PRELIMINARY RESULTS.

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It was the objective of our study to evaluate the influence of rHu G-CSF in the hematopoietic and immunological recovery following ABMT. Five patients who were undergoing ABMT for malignant lymphoma were treated whith G-CSF 5 μg/kg/d/s.c. from day +1 until their WBC reached $10x10^9$/L. Conditioning regimen was Bu+Cy in two patiens and BEAM in three, followed by infusion of bone marrow criopreserved cells (median $2.8 \pm 1.2 \times 10^8$ CN/kg). All patients engrafted. By flow citometric analysis using a FACScan, we studied samples from bone marrow and peripheral blood obtained before ABMT and by days +15, +30, +45, +60, +100 postransplant. Monoclonal antibodies against CD3, 4, 5, 7, 8, 10, 13, 14, 15, 19, 20, 22, 29, 33, 34, 41a, 42b, 45, 56, 71 and HLA-DR were used in various combinations. Marrow cells with T- lymphoid markers were increased by day +15 and myeloid precursors from day +30, whereas monocytic precursors reached their highest level and slowly lowered down from that day. Conversely B-lymphoid population was first detected by day +30 growing up from then. Cells with megacaryocityc markers increased from day +15 and eritroid precursors had a erratic behavior. CD34+ cells maintained a moderate level from the begining of the study.

Peripheral blood studies showed that CD3+ cells decreased progresively with time. An inverted CD4/CD8 subset was observed all over the study. Although the absolute number of CD4+ cells increased moderately, their percentage was constant. The CD4/CD29 population showed a decreasing trend after ABMT, whereas CD4/CD45 and CD8/CD56 populations maintained stable numbers. From day +60 cells with CD19/CD5 markers were detected in progression.

Althoug the series is very small and precludes proper conclusions, the preliminary data obtained on the bone marrow immunological analysis and the peripheral blood lymphoid subsets (T, B, NK), suggest that treatment with G-CSF may facilitate the recovery of myeloid and monocytic progenitors and aparently does not play a significant role in the immunological recovery after ABMT. Further studies are needed to asses this question.

SERUM ERYTHROPOIETIN IN ANEMIA OF CHRONIC DISORDERS.

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Serum Erythropoietin (sEp) was studied in two groups of patients with anemia of chronic disorders (ACD). In 43 cases, ACD was due to rheumatoid arthritis (ACD-RA) and in 58 patients to other pathologies (ACD-O). Levels OF SeP were compared with 82 cases with iron deficiency anemia (IDA) and 67 healthy controls (reference group, RG). Serum Ep was increased in the three forms of anemia ($p<0.0001$). Although Hb concentrations were similar in all three types of anemia, sEp was lower in ACD-RA and ACD-O than in IDA ($p<0.00001$). As expected, a negative correlation between Hb and sEp was demonstrated in all forms of anemia, even though Ep response was lower in ACD-RA ($r=-0.45$, $p=0.0004$) and ACD-O ($r=-0.42$, $p=0.006$) than in IDA ($r=-0.72$, $p<0.00001$). In conclusion, these results suggest that Ep response to anemia in ACD is blunted in relation to that of other forms of anemia. This may contribute to development of ACD. Ep treatment should therefore be kept in mind for patients with moderate to severe ACD.

EFFECT OF CYCLOSPORIN A ON ANTI CD69 mAb AND INSULIN-INDUCED TYROSINE PHOSPHORILATION IN HUMAN LYMPHOCYTE MEMBRANE FRACTIONS.

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Tyrosine phosphorylation in resting and activated (cultured for 3 days with PHA) human peripheral blood lymphocytes (PBL) was studied. Treatment of PBL with 20 µl/10^6 PBL of anti CD69 mAb or 10^{-7} M insulin stimulated plasma membrane tyrosine kinase activity of these cells when using poly (Glu^{80} Tyr^{20}) as exogenous substrate. Plasma membranes of resting PBL displayed a basal tyrosine kinase activity that was not modified following activation with PHA. Culture of resting PBL with anti CD69 mAb increased the tyrosine kinase activity in a time dependent manner. Exposure of resting and activated PBL to insulin for 3 min stimulated the tyrosine kinase activity, being the stimulation higher in activated PBL. Resting PBL cultured with 20 ng/ml phorbol ester (PMA) showed the highest tyrosin kinase activity and no addtional increase in its activity was detected after culture of PBL with anti CD69 mAb and PMA. The tyrosine kinase activity reached its maximun when was stimulated with 20 µM sodium vanadate together with anti CD69 mAb or insulin. 80 µM Genistein reduced the anti CD69 mAb or insulin-induced phosphorylation. Following incubation of human PBL with anti CD69 mAb or insulin, two membrane phosphotyrosil protein of 59 and 95 kDa were respectively detected by immunobloting. Both tyrosine kinase activity and tyrosine phosphorylation of specific membrane proteins induced by anti CD69 mAb and insulin were blocked by 0.5 µg/ml Cyclosporin A (CsA). Whereby a novel action of CsA as suppressive function is thus described.

PERIPHERAL BLOOD STEM CELLS TRANSPLANTATION AFTER PRIMING WITH GM-CSF.

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Between January-91 and February-93, 18 patients entered a peripheral blood stem cell transplantation program in our center. 17 of them were diagnosed of haematological malignancies (7 Multiple Myeloma, 4 Hodgkin's disease, 2 non-Hodgkin Lymphoma, and 4 Acute non-Lymphoblastic Leukemia) and one patient had advanced stage Breast Cancer.

After one month without any chemotherapy, patients received rhuGM-CSF at doses of 5 µg/Kg/day via central venous catheters by continuous infusion. On the day 5th, the apheresis were started, and GM-CSF infusion was maintained until the last procedure was completed.

Patients underwent a mean of 3.8 leukaphereses (range 3-6) in consecutive days, using every time a continuous flow cell separator COBE Spectra. The collection rate was 0.9 mL/min, the inlet flow ranged between 40 and 55 mL/min, and the plasma pump rate was set by visual inspection to adjust the hematocrit of the product at 3-5%.

The mean number of mononuclear cells collected in our patients was 4.4 x 10^8/Kg (range 3.9-5.6) and the mean of CFU-GM collected was 9.9 x 10^4/Kg (range 0.3-58).

Among the 18 patients, 15 received myeloablative chemoradiotherapy followed by rescue with autologous peripheral blood stem cells. We noticed one death before engraftment due to septicemia (day +7), and 14 patients were evaluable for engraftment. The mean day to reach 500/mm3 granulocytes was +13 (9-22) and to reach 50.000/mm3 platelets was +17 (10-30).

We conclude that the continuous infusion of GM-CSF in patients in complete remission of hematological malignancies allows the recruitment of enough stem-cells to ensure a complete and durable engraftment with only a few leukaphereses procedures.

G-CSF IN THE NEUTROPENIC PERIOD AFTER AUTOLOGOUS AND ALLOGENIC BONE MARROW TRANSPLANTATION: DETERMINING THE BEST SCHEDULE OF ADMINISTRATION.

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From February-1992 a program of G-CSF use after autologous and allogeneic bone marrow transplantation (BMT) was started. The proposal of this study is optimize the therapeutic schedule of G-CSF in the neutropenic period after BMT, regarding the benefits and costs.

We have administered G-CSF in intravenous continuous infusion at doses of 5 μg/Kg/day. The patients are being randomized in 2 groups (Group A: infusion is started on day 0 post-BMT; Group B: infusion is started on day 7 post-BMT) and stratified with regard to allogeneic or autologous BMT. The G-CSF infusion is stopped after three consecutive days with neutrophil count (NC) >1000 /mm³.

At the moment, the study includes 26 patients, 14 in group A, and 12 in group B.

Preliminary results are as shown:

	Group A	Group B	
Day > 500 NC/mm³ (x) (range)	11.6 (7-20)	14.4 (11-18)	p < 0.01
Day > 1000 NC/mm³ (x) (range)	13.6 (10-23)	15.8 (12-22)	p < 0.06
Day>50.000 PLT/mm³ (x) (range)	25.2 (13-45)	26.6 (17-45)	p < 0.23
Hospitaliz. days (x) (range)	27.7 (15-65)	23.8 (17-38)	p < 0.16
Infusion days (x) (range)	16.1 (13-28)	11.9 (7-18)	p < 0.001

We conclude that there aren't statistical differences between the two schedules in regard to days of hospitalization and time to reach a NC > 1000/mm³,* in spite of a longer period of treatment in group A, which may rise the costs of procedure. Since patient numbers are still small, more patients will have to be recruited to validate these results.

THE ROLE OF INTERLEUKIN-2 RECEPTOR SUBREGIONS IN INTERLEUKIN-2 DEPENDENT SIGNAL TRANSDUCTION.

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Growth and differentiation signals induced by interleukin-2 (IL-2) are mediated through the heterotrimeric interleukin-2 receptor (IL-2R) complex. The β-chain subunit of the complex, IL-2Rβ, has a 286 amino acid cytoplasmic domain that is essential for signal transduction. We are studying the role that subregions of IL-2Rβ play in the signal transduction process using a series of IL-2Rβ chain mutants transfected into the IL-3 dependent Baf-B03 pro-B cell line. Cells transfected with wild-type receptors display normal signaling responses to IL-2, that is, increased protein tyrosine kinase (PTK) and phosphatidylinositol 3-kinase (PI3K)activities, induction of the protooncogene c-myc, cell cycle progression and proliferation. Cells transfected with receptors that have a deletion of the serine-rich domain, or receptors that are truncated at amino acid residue 323, do not show any of these responses when cultured with IL-2. In contrast, cells transfected with receptors that are truncated at amino acid residue 350 show an increase in PTK and PI3K activities, but have no c-myc mRNA accumulation, and do not proliferate following IL-2 stimulation. These studies define a role for the region between amino acid residues 323 and 350, which we have named the Amp region, in IL-2 dependent signal transduction. Further, they demonstrate that the serine-rich region of IL-2Rβ is essential for PTK and PI3K activation, but activation requires the presence of the Amp region. The results also indicate that PTK and PI3K activation do not provide a sufficient signal to induce IL-2 dependent proliferation. Overall these results support a two signal model of IL-2 induced cellular proliferation.

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INTRA- AND EXTRA-CELLULAR SIGNALLING IN HEMATOPOIESIS

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

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