Instituto Juan March
de Estudios e Investigaciones

95 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Neutrophil Development and Function

Organized by

F. Mollinedo and L. A. Boxer

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Introduction

F. Mollinedo and L. A. Boxer

Neutrophils are the most abundant leukocytes in blood and constitute the first line of defense against infectious diseases. These short-lived non-mitotic cells are generated at impressive numbers in the bone marrow as the result of a highly controlled process of myelopoiesis. Despite the huge number of neutrophils made daily in the bone marrow, many of them do not meet microorganisms but undergo apoptosis. Mature neutrophils are fully equipped with an arsenal of harmful agents stored in granules ready to be used to destroy phagocytosed microorganisms. The neutrophil availability for combating infections is regulated at different levels: proliferation and maturation of precursor cells in bone marrow, regulation of programmed cell death, migration through the endothelial cell barrier, activation of bactericidal systems. Failure to accomplish some neutrophil functions can lead to severe clinical disorders such as Chronic Granulomatous Disease (CGD) and Leukocyte Adhesion Deficiency (LAD). The genetic processes regulating neutrophil development in the bone marrow as well as the modulation of adhesion, diapedesis, migration, exocytosis, respiratory burst and apoptosis as well as the pathophysiological processes leading to clinical disorders were the focus of the workshop. This meeting dealt with novel trends in neutrophil structure, function and development. Thus, this workshop covered molecular events regulating the whole life-span of the neutrophil, including how it is generated, activated and removed after undergoing an apoptotic process. All of these processes are finely regulated and we are now starting to understand them at the molecular level.

Myeloid cell production is regulated by a complex interacting network of cytokines that modulate proliferation, differentiation, apoptosis and mobilization of hematopoietic stem and progenitor cells. Chemokines, an emerging family of cytokines, are being increasingly implicated in the production, mobilization and activation of neutrophils, showing a high redundancy in their actions. One of the major decisions leading to neutrophil generation is made by stem cells committed to the myeloid lineage, when granule protein coding genes are turned on to provide the synthesis of the granule constituents that will endow the neutrophil with its battery of microbicidal proteins. CCAAT enhancer binding proteins (C/EBPs), in particular C/EBPα, play a central role in neutrophil development and synthesis of neutrophil granule proteins. CCAAT displacement protein, a negative regulator, must be down-regulated to allow expression of certain granule proteins. However, regulation of granule protein biosynthesis is individually controlled, and differences in timing of granule protein synthesis during maturation of neutrophil precursors results in formation of granule subsets with different protein content. Mature neutrophils are very short-lived leukocytes that die by spontaneous apoptosis. This process is not simply a way of getting rid of unused neutrophils or of cells after having performed their task, but may control the number of neutrophils available in combating infections.

Experiments of nature (LAD) as well as man-designed studies have demonstrated that neutrophil extravasation depend on its ability to adhere, via integrins, to the endothelium. Integrins behave both as docking and signaling molecules that mediate leukocyte adhesion as well as actin-cytoskeletal rearrangement and regulation of several neutrophil functional responses, such as migration, degranulation and respiratory burst. Neutrophils tend to adhere and transmigrate at endothelial cell borders, where P-selectin acts as a target for neutrophil migration. When ICAM-1 is highly expressed on activated

endothelial cells, neutrophil adhesion proceeds via direct β_2-integrin-ICAM-1 recognition. Studies using bone marrow-derived neutrophils from $fgr^{-/-}/hck^{-/-}$-knockout mice indicate that Fgr and Hck are not required for myeloid cell development, but are critical in controlling integrin-mediated responses such as generation of superoxide anion, adhesion, migration and degranulation. Adherence and motility are non-static processes and firm-loose cycles would allow cell movement. In order to explain the observed cycles in movement and respiratory burst, a novel mechanism was proposed involving metabolic clocks in neutrophils with cycles in the free concentration of essential metabolites (ATP and NADPH) which regulate downstream activities. Chemoattractants promote changes in neutrophil shape with the formation of a uropod at the rear of the cell, which can attract other neutrophils during neutrophil transmigration. Also, the most abundant neutrophil proteins MRP8/14, which together account for 45% of the cytosolic neutrophil protein were suggested to play a role, once released from cells, in neutrophil migration and adhesion.

Phagocytosis and secretion, two critical functions of neutrophils, involve docking and fusion of intracellular vesicles with the plasma membrane modulated by specific proteins. To compensate loss of cell surface during phagocytosis, cells generate from the trans-Golgi network vesicles to fuse with the nascent phagosomes. Different intracellular granules are mobilized independently during neutrophil exocytosis, and granule constituents, secreted or translocated into the cell surface upon cell activation, affect neutrophil adhesion and diapedesis. The identification of SNARE proteins in human neutrophils as well as their differential subcellular localization (plasma membrane *vs* granule) and functional assays, suggest a role of these proteins in both neutrophil phagocytosis and secretion.

The power of gene transfection was shown to be useful in dissecting the role of Fcγ receptors and their signaling. Phospholipase D seems to play an important role in phagocytosis signaling. Phospholipase D activation triggers a signaling cascade, involving Raf-1 and ERK-2, which promotes generation of pseudopods required for phagocytosis. Phagocytosis may be terminated by the generation of ceramide which inhibits phospholipase D. The phospholipase D physiological product, phosphatidic acid, is able also to regulate the activity of type 1 phosphatidyl-1-phosphate-5-kinase, an enzyme that generates PI-(4,5)-P_2, thought to promote PMN degranulation through its ability to recruit lipid-binding proteins at the membrane.

The main function of human neutrophils is to combat infection and they are very well equipped to perform this task. This includes the presence of specific as well as more general antimicrobicidal systems. The antibacterial protein of bactericidal/permeabilty-increasing protein (BPI) is made only in neutrophils and stored in azurophilic granules. BPI binds LPS and is cytotoxic toward gram-negative bacteria, promoting their uptake by neutrophils, as well as destruction and detoxification of bacteria and endotoxin.

Phagocytic cells contain a NADPH dependent oxidase important for killing engulfed microbes. This oxidase transfers electrons from NADPH to O_2 to form O_2^- and consists of a membrane-bound flavocytochrome b composed of an α ($gp22^{phox}$) and β ($gp91^{phox}$) subunit. Three cytosolic proteins, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$, and a GTP-binding protein p21rac are required for activation of electron transport. Mutations in any of the genes encoding a component of the NADPH oxidase causes CGD, a rare immunodeficiency that can cause life-threatening infections. The majority of CGD

patients suffer from the X-linked form of the disease caused by mutations in $gp91^{phox}$. Over the past 5 years the mutations in several hundred kindred of X-linked CGD families have been identified. These mutations lead to instability of $gp91^{phox}$ mRNA and/or protein. In spite of recent insights in the molecular underpinnings of CGD, little is known about the mechanisms leading to granulomata formation.

With the advent of a better understanding of the molecular mechanisms leading to NADPH oxidase activation, gene therapy is being attempted to correct the functional defect in O_2^- generation in CGD. A gene transfer approach is being also launched to treat patients with LAD, characterized by severe recurrent bacterial infection in which the phagocytic leukocytes fail to firmly adhere to endothelial cells and subsequently migrate to infectious sites. Molecular defects in the leukocyte integrin CD18 subunit are responsible for the failure to synthesize a CD18 subunit capable of forming heterodimers with the leukocyte integrin CD11 subunits and becoming inserted into the plasma membrane to mediate neutrophil adhesion to the endothelium. These gene therapy approaches look promising even though they are at a rather early stage, and several problems must be circumvented. Better vectors, other means for recruiting stem cells as well as mild suppression ("conditioning") of the patient's bone marrow with radiation to enhance engraftment of gene-corrected stem cells will be required.

Neutrophil functions have long been described, but a great advance in the knowledge at the molecular level of these functions has been obtained only during the last recent years. This workshop highlighted the rapid progress made over the last years in trying to dissect the molecular events occurring in the manifold functionality of these cells. *In vitro* and *in vivo* studies have revealed a great signaling cross-talk and complexity as well as pleiotropic effects of molecules and receptors in the different stages of neutrophil differentiation and activation. In addition, neutrophils contain specific components and display peculiar characteristics that make them as a unique and perfect weapon to fight rapidly infection, playing a major role in the surveillance system of host organism against foreign invaders and in acute inflammation. Future molecular studies are expected to reveal mechanisms that will enable us to improve and modulate neutrophil bactericidal and inflammatory responses.

Faustino Mollinedo and Laurence A. Boxer

Session 1: Neutrophil development and apoptosis

Chair: Laurence A. Boxer

Regulation of the Production and Movement of Myeloid Progenitor Cells by Chemokines

Hal E. Broxmeyer, Ph.D.

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Blood cell production is regulated by a complex interacting network of accessory cell produced cytokines that act to stimulate, co-stimulate, suppress, or co-suppress the proliferation, differentiation, apoptosis and movement of hematopoietic stem and progenitor cells.[1] An emerging family of cytokines called chemokines, for their chemotactic activities, are being increasingly implicated in the production and movement of myeloid stem and progenitor cells.

Chemokines are currently categorized into four subfamilies (CC, CXC, C, and CX_3C) denoted by cysteine residues and their spacing in the molecule. Over 50 chemokines have been identified and recombinant protein is available for their use in evaluating functional activities.[2,3] Of a large number of chemokines that have been tested, more than 20, crossing the CC, CXC, and C groups, are suppressive for immature subsets of multipotential (CFU-GEMM), erythroid (BFU-E), and granulocyte-macrophage (CFU-GM) progenitors that respond in vitro to stimulation by combinations of growth factors such as erythropoietin, IL-3, GM-CSF and steel factor.[2,3] This suppression has been confirmed in vivo in mice for most of these chemokines, and also in a human clinical trial for one, macrophage inflammatory protein (MIP)-1α.[4] Over 14 chemokines crossing the CC, CXC and CX_3C groups do not manifest this myelosuppressive activity in vitro and in vivo.[2,3] In contrast, of the numerous chemokines tested, only the CXC chemokine, stromal derived factor (SDF)-1[5], which binds CXCR4, and the two CC chemokines CKβ-11[6] and SLC, which bind CCR7, have the capacity to chemotax myeloid progenitor cells.

The difficulty in sorting out dominant vs. redundant effects is complicated by the fact that there are currently 9 receptors for CC chemokines (CCR1 to CCR9), 5 receptors for CXC chemokines (CXCR1 to CXCR5) and one receptor each for the one C and one CX_3C chemokine.[2,3] Many of the chemokine receptors bind more than one chemokine and some chemokines bind more than one receptor. To begin defining dominant chemokine receptor functions, we have collaborated with others to assess hematopoiesis in mice deleted in either CXCR2,[7] CCR2,[8] or CCR1. Results from each of these receptor gene knockout mice highlighted the important but different roles of these receptors in either steady-state and/or stress-induced conditions.

While some information is beginning to emerge regarding intracellular signaling events mediating some of the above mentioned chemokine functions, it is not yet clear how important these intracellular molecules are to these actions. To evaluate this, we have collaborated with others to assess chemokine actions in mice deleted in expression of SHIP and SHP-1. Results implicate these phosphatases in both positive and negative effects of different chemokine functions.

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Regulation of Neutrophil Development by C/EBP transcription factors.

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The CCAAT enhancer binding proteins play critical roles in myeloid differentiation. In particular, C/EBP alpha is important for early granulocytic development, while C/EBP epsilon is critical for terminal granulocytic differentiation. Other C/EBP proteins, such as C/EBP beta (NF-IL6), to date have not shown to be involved in neutrophil maturation.

C/EBP alpha is a basic leucine zipper transcription factor which has been shown to serve as a master regulatory switch in several differentiation systems, including adipocyte and liver development, and also as a tumor suppressor gene which can inhibit cell proliferation by activating p21. C/EBP alpha regulates a number of myeloid-specific genes, and analysis of C/EBP alpha knockout mice provided evidence for a necessary and specific role of this factor during hematopoiesis in vivo: the animals were absolutely deficient in granulocytes, whose differentiation was blocked at an early stage, while the development of other hematopoietic lineages remained undisturbed.

Previously, we demonstrated that granulocyte colony-stimulating factor receptor (G-CSFR) mRNA was downregulated and granulocyte maturation blocked in C/EBP alpha -/- mice (Zhang et al, PNAS 94:569, 1997). This phenotype is distinct from that of G-CSFR -/- mice, which have a quantitative defect in granulopoiesis, but mature granulocytes are detected at a reduced level (12% of wild type) in the peripheral blood (Liu et al, Immunity 5:491, 1996). These results suggest that other C/EBP alpha regulated cytokine receptors in addition to the G-CSFR regulate granulocyte development. We now demonstrate loss of expression of interleukin-6 receptor (IL-6R), but not the IL-6 transducer (gp130), by Northern blot analysis. Quantitative RT-PCR demonstrated that IL-6R mRNA in C/EBP alpha -/- fetal liver is 170 fold less than that observed in wild type, and that G-CSFR mRNA is decreased 17 fold. FACS analysis using biotinylated G-CSF and IL-6 revealed no detectable G-CSF or IL-6 receptor protein on the surface of C/EBP alpha -/- spleen or fetal liver hematopoietic cells. Concordant with the drastically reduced receptor levels, no colonies were obtained in methylcellulose CFU assays in response to IL-6 and G-CSF stimulation of hematopoietic precursors from C/EBP alpha -/- fetal liver. Finally, we demonstrated that the block in granulopoiesis in the C/EBP alpha -/- fetal liver hematopoietic cells can be rescued in vitro by adding soluble IL-6R and IL-6 into the CFU assay, or alternatively by retroviral transduction of the G-CSF receptor and subsequent G-CSF administration. These studies suggest that in addition to G-CSF receptor, C/EBP alpha directly or indirectly regulates the expression of the IL-6 receptor. Furthermore, the failure of granulopoiesis in vitro observed with C/EBP alpha -/- fetal liver hematopoietic cells in response to IL-6 and G-CSF can be compensated by restoration of IL-6 or G-CSF receptor expression, suggesting that loss of these two receptors alone play a significant role in the absolute block in granulocyte maturation observed in vivo.

In order to further delineate the role of C/EBP alpha in human granulopoiesis, we studied its expression and function in human myeloid cell lines and primary hematopoietic cells. Using purified populations of human bone marrow cells, we show that the expression of C/EBP alpha during hematopoietic development is initiated with the commitment of multipotential precursor cells to the myeloid lineage. It is also specifically upregulated during granulocytic differentiation of normal human CD34+ cells and myeloid cell lines. Conversely, differentiation along the monocytic pathway results in rapid downregulation of C/EBP alpha gene expression. Conditional expression of C/EBP alpha from a zinc-inducible metallothionein promoter in stably transfected U937 results in granulocytic maturation over a period of two weeks, as determined by cell morphology, NBT assay and upregulation of mRNA for the myeloid maturation marker CD18 and the granulocyte-specific G-CSF receptor. Strikingly, induced C/EBP alpha was also capable of activating neutrophil-specific genes encoding the secondary granule proteins lactoferrin and human neutrophil collagenase, which cannot otherwise be induced in these cell lines, even after chemical stimulation of granulocytic differentiation. Granulocytic differentiation was also observed in stable lines treated with zinc for only two days. Conditional expression of C/EBP alpha does not prevent monocytic differentiation by TPA if cells express C/EBP alpha for less than 7 days. However, cells expressing C/EBP alpha for over 7 days enter an irreversible stage of neutrophilic differentiation resistant to monocytic induction. In summary,
upon upregulation of C/EBP alpha, U937 cells progress into stages of differentiation which are more mature than those invoked by known chemical inducers, such as retinoic acid. These results indicate that C/EBP alpha can serve as a myeloid differentiation switch acting on bipotential myeloid precursors and directing them to mature to granulocytes.

Current efforts in the laboratory focus on understanding regulation, signal transduction pathways, and interacting partners of C/EBP alpha. We are in the process of screening myeloid leukemias for abnormalities in structure and expression of these two genes, as well as mutations. Other projects directed at leukemogenic mechanisms include analysis of the effects of translocations found in AML on C/EBP alpha function. Our long term goals are to understand the abnormalities seen in acute myelogenous leukemia (AML), in which differentiation of myeloid blasts is blocked.

Transcriptional Regulation of Neutrophil-Specific Gene Expression
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During myeloid differentiation, the pluripotent hematopoietic stem cell passes through several well-defined morphologic stages within the bone marrow. These changes include progressive nuclear segmentation and the acquisition of stage specific granules. Primary granules appear at the myeloblast stage, and are found in both neutrophils and monocytes. At the myelocyte stage, neutrophil precursors acquire specific granules, a marker of commitment to terminal neutrophil differentiation. The fully mature granulocyte is then released into the blood where it circulates only briefly before migrating into the peripheral tissues; it then survives for only a few days before undergoing apoptosis.

The regulatory mechanisms governing this complex differentiation pathway are beginning to be elucidated. Current evidence suggests that the growth factors governing granulocyte lineage commitment and maturation act through a combination of general and lineage-specific transcription factors that influence patterns of gene expression and direct downstream changes in proliferation and phenotype. Several transcription factors have been identified which are necessary for myeloid differentiation, and which have also been shown to influence myeloid-specific gene expression at several stages of granulocyte differentiation, including PU.1, C/EBPα, and retinoic acid receptors. Furthermore, negative regulators such as CCAAT displacement protein (CDP) have also been found to interact with genes at multiple stages of differentiation.

Studies in our laboratory have focused on the regulation of a late myeloid specific gene, lactoferrin (LF). LF is one of the secondary granule protein (SGP) genes, a group of unlinked and functionally diverse genes expressed late in neutrophil maturation. SGP gene expression is coordinately regulated at the transcriptional level, and is uniformly absent from leukemic cells induced with chemical agents to undergo differentiation toward neutrophils. We have studied the transcriptional regulatory pathways that regulate LF gene expression. We have determined that both negative and positive regulatory factors play a role in determining the tissue and stage specific expression of the LF gene, and have developed evidence that these pathways of transcriptional regulation are shared among the secondary granule proteins.

We have demonstrated that CCAAT displacement protein (CDP/cut), a conserved silencing protein in many diverse systems, binds the LF promoter, and that expression of LF is associated with loss of CDP binding. Furthermore, constitutive overexpression of a transfected CDP cDNA, is associated with repression of LF expression. Although CDP binding sites have not been defined in the other SGP promoters, overexpression of CDP coordinately represses expression of all SGP genes. This suggests that CDP may be a shared negative regulator partially responsible for the stage specific expression of these genes. However, CDP is ubiquitously and constitutively expressed; consequently, the means by which its binding is modified in a stage-specific manner remains to be determined.

In further studies, we have sought to identify factors responsible for the upregulation of LF in an attempt to identify potential shared positive regulators of SGP expression. Reporter gene assays in 32Dwt18 (32D) cells and MEL cells indicated that the first 89bp of the promoter are capable of directing myeloid-specific LF gene expression. This region contains a C/EBPα binding site. Co-transfection of the LF-89luc plasmid with increasing concentrations of a C/EBPα expression vector in 32D cells results in a linear transactivation of luciferase reporter activity which was abolished by mutation of the C/EBP binding site. Similarly, U937 cells induced to express C/EBPα from a stably transfected inducible transgene showed morphologic maturation and increased LF gene expression which correlated with increased binding of C/EBPα to the LF promoter in EMSA analysis. However, several other findings suggest that C/EBPα alone is insufficient to induce LF gene expression. EMSA analysis of 32D cells demonstrate binding of C/EBPα to the LF promoter in uninduced, LF-nonexpressing cells, which does not change with

induction of maturation and concomitant LF expression. Induced HL60 and NB4 cells show abundant C/EBPα binding to the LF promoter, but do not express LF. Furthermore, CDP overexpression in 32D cells blocks LF expression despite unchanged C/EBPα binding.

We have investigated possible explanations for this discrepancy. Because C/EBPα is known to readily heterodimerize with other C/EBP family members, we investigated potential interactions of C/EBPα, C/EBPβ, and C/EBPε. Preliminary studies suggest that a combination of C/EBPα and C/EBPβ together transactivate the LF-89luc plasmid more efficiently than either C/EBPα or C/EBPβ alone. We have also demonstrated that the region of the LF promoter spanning -167bp to -89bp appears to harbor both positive and negative regulatory elements. Thus, it appears that C/EBPα alone cannot induce the maturation-induced increase in LF gene expression. An analysis of C/EBP and CDP binding support the hypothesis that an inverse binding of these factors is necessary for LF induction. We therefore hypothesize that LF expression provides a model for a complex interaction of positive and negative regulatory factors influencing tissue- and stage-specific gene expression in the myeloid lineage.

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NEUTROPHIL APOPTOSIS IN RESOLUTION OF INFLAMMATION

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Neutrophils are programmed for constitutive death by apoptosis which leads to their safe recognition and clearance by macrophages (1, 2). The molecular basis of constitutive apoptosis in neutrophils is still poorly understood but involves endogenous proteases including calpains and caspases (3). The rate that neutrophils undergo apoptosis can be regulated by exogenous factors, being promoted by ligation of cell surface Fas, tumour necrosis factor-alpha acting via interaction of the 55 and 75 kD receptors and by beta-2 integrin-mediated phagocytosis followed by release of reactive oxygen species. Conversely, apoptosis may be inhibited by lineage-specific survival factors (GM CSF, GCSF), pro-inflammatory mediators (eg. LPS and IL-1-beta) and adhesion to extracellular matrix proteins or other cells. However, the main focus of this talk is the clearance of neutrophils once they have undergone apoptosis.

In vivo data indicate that extravasated neutrophils can be cleared by professional phagocytes, (macrophages) and by semi-professional phagocytes such as glomerular mesangial cells (1,4). Indeed, even in the intravascular space neutrophils undergoing apoptosis may be taken up by macrophages (5). Even before the cells are ingested apoptosis leads to injury-limiting reduction in the capacity to secrete injurious mediators (6), but phagocytic clearance is also injury-limiting in three ways. Firstly, this prevents release of injurious contents from apoptotic granulocytes undergoing secondary necrosis, which not only threatens direct tissue injury but can also trigger pro-inflammatory responses once the debris has been ingested by macrophages. (7). Secondly, not only does the uptake by macrophages and mesangial cells of apoptotic neutrophils fail to cause release of a range of pro-inflammatory mediators including chemokines (2,8) but exciting recent data indicate that ingestion of apoptotic cells in fact triggers anti-inflammatory effects such as release of TGF beta-1 and reduction in TNF-alpha secretion by activated macrophages (9). Lastly, uptake of apoptotic cells by macrophages appears to prevent the capacity of co-cultured immature dendritic cells to present antigen derived from ingested apoptotic cells to T-cells (10).

Phagocyte recognition of apoptotic neutrophils is complex and may involve many receptors (for a recent review see ref 11). Some details will be given in the talk. However, there are now exciting new data which point to pathogenetic perturbation of apoptotic cell clearance in inflammatory and immune disorders. Firstly, gene-targetted mice deleted for C1q which may bridge apoptotic cells to phagocytes, exhibit spontaneous and severe renal inflammation coupled with evidence of failed clearance of apoptotic cells (12). Secondly, antiphospholipid autoantibodies which recognise phosphatidylserine exposed on the surface of apoptotic cells appear able to opsonise such cells for macrophages, thereby converting the safe post-phagocytic response to a pro-inflammatory response including release of TNF-alpha (13). Whether such adverse consequences of perturbed clearance can be avoided is not yet clear, but there is obvious therapeutic promise in the recent observation that glucocorticoids markedly upregulate phagocytosis of non-phlogistic phagocytosis of apoptotic neutrophils (14). Finally, there may also be therapeutic opportunities in exploiting the capacity of macrophages to direct apoptosis in bystander leucocytes, particularly when stimulated by phagocytosis of opsonised particles or apoptotic cells (15).

Thus apoptosis in the neutrophil may be regulated by exogenous agents for therapeutic gain, and makes an important contribution to resolution of inflammation. Furthermore, perturbation of safe clearance appears likely to contribute to persistent inflammatory and immune responses.

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Session 2: Adhesion and recruitment of neutrophil leukocytes

Chair: Faustino Mollinedo

NEUTROPHIL ADHESION UNDER CONDITIONS OF FLOW.

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Using homotypic and heterotypic aggregation as models of neutrophil adhesion, we have analyzed kinetics and molecular mechanisms of adherence and disaggregation over a physiologically relevant range of shear rates. The techniques employ rotational viscometry to apply precise and uniform shear rates, and flow cytometry to resolve the size distribution of aggregates. The kinetics are mathematically modeled based on a theory that describes the interaction of spherical particles mixed in a linear shear field. In this experimental setting, three phases of homotypic adhesion can be defined. The first is detected within seconds of addition of agonists (e.g., chemotactic peptide, f-Met-Leu-Phe), and peaks within 30 seconds. The efficiency (i.e., the percent of encounters that result in stable adhesion) is remarkably high and optimum at shear rates of 400-800 s^{-1} where the estimated duration of contact between cells without adhesion is ≤6 msec. The second phase is a plateau observed over a period of 2 minutes in which adhesion is stable when exposed to much higher shear rates (e.g., 3000 s^{-1}) than those allowing optimum aggregation. The third phase is characterized by a rapid transition to disaggregation, the rate of which is directly proportional to the magnitude of the shear stress at rates >400 s^{-1}. At shear rates of 400 s^{-1} or less, aggregation is sustained.

Current evidence indicates that the following adhesion molecules are involved in the peak efficiency at optimal shear rates of 400-800 s^{-1}: L-selectin (CD62L) interacting with PSGL-1 (CD162), Mac-1 (CD11b/CD18) interacting with an unknown ligand, and LFA-1 (CD11a/CD18) interacting with ICAM-3 (CD50). If the L-selectin pathway is blocked, aggregation fails to occur when shear rates are 400 s^{-1} or higher, though a low efficiency aggregation occurs at shear rates of 100 s^{-1} where the estimated duration of cell contact in the absence of adhesion is 25 msec. If either the Mac-1 or LFA-1 pathway is blocked, adhesion efficiency at 400 s^{-1} is reduced by ~40%, and if both are blocked, aggregation fails to occur at any shear rate tested. Distinctions between LFA-1 and Mac-1 functions can be demonstrated. Aggregation dependent on LFA-1/ICAM-3 is rapid and transient, lasting <2 minutes, while aggregation dependent on Mac-1 is slower to develop but sustained for at least 10 minutes. These adhesion pathways form a necessary cascade enabling stable adhesion to occur efficiently at physiologic shear rates of 400-1600 s^{-1}. The L-selectin-dependent step functions within the brief contact durations (<6 msec) of these shear rates, and prolongs intercellular contact long enough (25 msec) for LFA-1/ICAM-3 adhesion to occur. Mac-1-dependent adhesion then follows and allows more

prolonged stable adhesion. In addition to the tethering function, L-selectin may signal enhanced function of Mac-1.

In a heterotypic model employing these techniques, 300.19 cells expressing human ICAM-1 at a density comparable to that of cytokine-stimulated endothelial cells were allowed to interact with human neutrophils at various shear rates from 90-1500 s^{-1}. Peak efficiency of adhesion following addition of the agonist f-Met-Leu-Phe was seen between 90-300 s^{-1}, diminishing proportionally with higher shear rates. Distinctions between LFA-1- and Mac-1-dependent adhesion were evident. LFA-1 adhesion was rapid and transient, peaking in 1 minute and returning to baseline within 3 minutes. Mac-1 adhesion was much slower (peaking at 5 minutes). The efficiency of LFA-1-dependent adhesion was ~2.5X greater than that of Mac-1, accounting for most of the initial peak adhesion at 1 minute. These results indicate that CD18 integrins Mac-1 and LFA-1 can cooperate to support adhesion to ICAM-1 under flow, with LFA-1 providing the efficiency needed for rapid adhesion, and Mac-1 sustaining the attachment.

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Neutrophil Mechanisms of Adhesion

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A neutrophil can be recruited to a site of injury within two hours. This migration is a multistep process employing adhesion receptors acting in a specific sequence. First the neutrophil rolls in a loosely tethered fashion along the vessel by means of the selectins. Then following receipt of an activating stimulus, it uses the β2 integrins to firmly adhere to and migrate across the vascular endothelium under the influence of chemoattractants. These factors include chemokine family members such as human IL-8 (CXC) or the murine homologue MIP-2, the bacterial peptide fMLP, activated complement factor C5a, leukotriene LTB4 and PAF. Attention has recently turned to a subset of the S100 family which consist of low molecular mass (approximately 10kDa), calcium binding proteins with restricted tissue distributions (1). Some members of the S100 family have been demonstrated to have proinflammatory effects by acting as chemoattractants. For example psoriasin (S100A7) exhibits chemotactic activity towards neutrophils and and T cells but not monocytes and S100L (S100A2) has chemotactic activity for eosinophils. CP-10 (S100A8) is reported to be an extremely potent chemotactic factor for murine myeloid cells with an activity of 10^{-13}M (2).

In man, MRP-14 (S100A9) is coexpressed in myeloid cells with a closely related molecule, MRP-8 (S100A8), with which it forms an MRP-8/14 heterodimer and together they account for 45% of the cytosolic protein in human neutrophils (3). Human MRP-14 (huMRP-14) stimulates neutrophil adhesion to ligand fibrinogen by activating the β2 integrin

Mac-1 (4). MRP-8 negatively regulates the proadhesive effect of MRP-14 by forming a heterodimer with a 1:1 stoichiometry. This proadhesive effect is very selective since huMRP-14 fails to induce any chemotactic activity and also fails to exhibit other features of the stimulated neutrophils such as neutrophil shape change, L-selectin shedding, Ca^{2+} flux or respiratory burst. In order to discover whether the findings obtained previously with human MRP-14 translate to the murine version of the protein and also to further understand the biology and proinflammatory properties of the S100 proteins in general, we have cloned and characterised the activity of murine MRP-14 (muMRP-14) both *in vitro* and *in vivo* (R. May-see poster). MuMRP-14 is also proadhesive and seems not to act as a chemoattractant. However, it does cause a Ca^{2+} flux in unprimed neutrophils suggesting that it can signal into the cell. Unexpectedly, muMRP-14 induced an *in vivo* chemotactic response similar in magnitute to fMLP. At present we are investigating whether this response might be indirect. Further features of these interesting neutrophil proteins will be discussed.

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METABOLIC CLOCKS AS SIGNALING DEVICES IN NEUTROPHIL ACTIVATION AND MOTILITY

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The hallmark of signal transduction is generally believed to be the appearance of a phosphorylated protein, cAMP, or calcium in a cell's cytosol. However, recent studies have suggested that calcium oscillations may encode information in their frequencies (1-3). Oscillations in pyridine nucleotides (NADH and NADPH) have also been observed. I propose a new model of neutrophil signaling during activation and motility wherein amplitude and frequency modulation of intracellular metabolite concentrations (e.g., NADPH) encode signaling information (4-6).

Resonance energy transfer (RET) studies of CR4 (complement receptor type 4)-to-uPAR (urokinase receptor) interactions revealed oscillatory receptor proximity relationships, which suggests a long range (~2 μm) temporal coherence in receptor properties. The oscillation period was 20 sec. for unstimulated cells and 10 sec. for stimulated cells (FMLP, fibrinogen, immune complexes, etc.). The receptor proximity oscillations appear to be directly associated with the signal transduction apparatus because sub-saturating doses of kinase inhibitors increased the oscillation's risetime whereas phosphatase inhibitors increased the oscillations' declination time. Control experiments indicate that these receptor oscillations were not due to other physical effects, such as changes in cell thickness. Why then does the signaling apparatus oscillate? Signaling oscillations are apparently due to oscillations in metabolite concentration. By measuring the autofluorescence of NAD(P)H, we found that it oscillates with the same periods as receptor proximity oscillations under all experimental conditions. However, the phase of receptor oscillations was shifted 180° relative to NAD(P)H, suggesting that receptor proximity oscillations may be associated with ATP oscillations. Thus, cell metabolite flux is a chemical signal for a cell function to appear whereas the associated kinases may be the conduit through which signals pass (4). This concept contrasts sharply with current reaction-diffusion models of phosphorylation signaling.

Oscillations in oxidant production and pericellular proteolysis have been studied during cell migration in gel matrices containing reporters for these functions. Oxidant production, a previously described oscillator, and pericellular proteolysis were shown to oscillate during cell locomotion (5). We propose that NADPH oxidase phosphorylation may "tune" the oxidase's K_d to intercept the "signal" NADPH oscillations, which are then decoded into a series of superoxide bursts. Kinetic studies have shown that when NADPH is near its peak concentration, the production of superoxide by the NADPH oxidase is also near its maximal rate. Furthermore, the amount of superoxide production parallels NAD(P)H amplitude and frequency changes (6). Superoxide oscillations may, in turn, explain the multiple, periodic cytolytic events observed during cell-mediated cytotoxicity (7). Pericellular proteolytic oscillations are out-of-phase with NAD(P)H oscillations, suggesting that they are driven by another metabolite.

Several experiments support or are consistent with the proposed model of signaling. NAD(P)H oscillations with 10 or 20 sec. periods are found during cell locomotion, phagocytosis, and spreading. Other workers have suggested that neutrophils possess an internal clock to support cell migration; metabolic clocks may represent this time-keeping mechanism. Indeed, we have recently shown that neutrophils compare FMLP concentrations at 10 sec. intervals to initiate changes in cell polarization (8). Cells are also capable of detecting mechanical stimulation via applied electric fields when frequency and phase matched with intracellular metabolic oscillators (9); neutrophils respond to these periodic forces by increasing the NAD(P)H amplitude 3-fold and extending to ~40-50 μm in length. Anti-inflammatory compounds, such as indomethacin can reduce the amplitude and frequency of NAD(P)H oscillations. Neutrophils from pediatric-onset pyoderma gangrenosum patients display aberrations in intracellular oscillators and motility or shape that can be ameliorated by pharmacologic or physical perturbations which restore sinusoidal oscillations (10, 11).

We have also tested the role of intracellular oscillators in cytokine signaling in RAW264 macrophages. IFN-γ treatment for 4 hr led to increased NAD(P)H amplitude 3-fold. IL-6, IL-2, TNF-α and IL-1β alone had no effect on cells. However, addition of IL-6 or IL-2, but not TNF-α or IL-1β, to IFN-γ treated cells doubled NAD(P)H oscillation frequency. NO production, which incrementally increased in phase with the NAD(P)H oscillations, was doubled after IL-6 or IL-2 treatment of IFN-γ primed cells in comparison to cells treated with only IFN-γ. Amplitude modulation appears to be a key in frequency doubling since electric field exposure restores IL-6 and IL-2 frequency doubling in the absence of IFN-γ. Thus, amplitude and frequency modulation may contribute to synergistic cytokine singaling and entrain NO production, which utilizes NADPH as an electron donor.

We suggest that cell oscillators may be linked to cellular functions such as kinase/phosphatase pathways, oxidant production, pericellular proteolysis, cytolysis and microfilament extension (6). This may also account for previously reported neutrophil oscillations in actin assembly, shape change, oxidant release, membrane potential, and calcium. Hence, leukocyte metabolic clocks may contribute to signaling at a time-scale of seconds and drive certain functions.

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Title: "Polarization of adhesion molecules and its interaction with ERM actin-binding proteins in chemoattractant-stimulated neutrophils".

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Abstract

Chemoattractants are able to switch the neutrophil shape from a spherical to a polarized motile morphology with the formation of a uropod at the rear of the cell. We have studied here the changes in the redistribution of adhesion molecules triggered by different chemoattractants as well as the neutrophil-membrane interactions with proteins of the Ezrin/Radixin/Moesin (ERM) family. Immunofluorescence analyses showed that the PSGL-1, ICAM-3, CD43, and CD44 molecules are redistributed to the uropod of neutrophils stimulated with chemokine IL-8, classical chemoattractants C5a or fMLP, and the chemotactic cytokine IL-15. The ERM protein moesin co-localized with PSGL-1 and ICAM-3 in the uropod of the chemoattractant-activated neutrophil. We have investigated the possible interaction of PSGL-1 and ICAM-3 with ERM actin-binding protein. The association of ICAM-3 and moesin was demonstrated by their copurification from neutrophil lysates with an anti-ICAM-3 immunoaffinity chromatography column, followed by Western blot. The interaction of moesin with PSGL-1 and ICAM-3 was also analyzed by precipitation from metabolically labeled HL-60 cells with GST-fusion proteins containing the cytoplasmic domains of PSGL-1 and ICAM-3.The PSGL-1 cytoplasmic domain co-precipitated several bands of 75-78, 81, 90 and 110 kDa while ICAM-3 cytoplasmic domain co-precipitated only the 75-78 kDa bands. Analyses of Western-blots carried out in parallel revealed that the 75-78 kDa bands corresponded to moesin and the 81 kDa band bound to PSGL-1 was ezrin. These data suggest that interactions of cell adhesion molecules and ERM actin-binding proteins could be regulated during neutrophil migration towards gradients of chemoattractants. Moreover, interactions of PSGL-1 with so far unidentified proteins could facilitate the leukocyte rolling on endothelial cells.

Protein and lipid kinases in chemokine-mediated leukocyte activation

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Selective attraction of particular leukocytes to inflammatory sites or to lymphoid organs is mediated by chemokines. The specificity of the response is ensured by distinct ligand receptor interactions. Chemokines comprise a large family of structurally related 7-10 kDa proteins. Today four subgroups, the CXC, CC, CX_3C and C-chemokines have been defined depending on the spacing of their cystein residues that are essential for the formation of functional intramolecular disulfide bridges. In the case of the well characterized CXC chemokines the first two cysteins are separated by an amino acid, whereas they are adjcent in CC-chemokines (1). All known chemokines bind to heptahelical receptors that are coupled to *B. pertussis* toxin sensitive heterotrimeric G_i-proteins. However, several chemokines can bind to the same receptor and one chemokine can bind to several receptors. To this promiscuity some exception do exist, where a chemokine only binds to one receptor. Such receptor ligand pair is the SDF-1 the CXCR4 couple. Target gene disruption of both, SDF-1 or CXCR4 results in a similar phenotype with developmental and B cell lymphopoiesis defects (2,3). The observations indicate that the chemokine system not only functions in inflammation but also fulfills an important role in hemostasis and in embryogenesis. Moreover several chemokine receptors act as co-receptors in HIV infection of $CD4^+$ cells, in particular CCR5 and CXCR4 which are essential for the infection with primary isolates of R5- and X4-tropic HIV strains. respectively.

Signal transduction through G_i proteins leads the rapid elevation of intracellular free calcium via the release of $\beta\gamma$ subunits and the activation of the phospholipase Cβ-isoforms. In addition, chemokine receptors mediate the activation of multiple protein and lipid kinases (4). Early experiments revealed that neutrophil responses, such as the respiratory burst and exocytosis are inhibited by pretreatment of the cells with the fungal metabolite wortmannin (5). Purification of the target of wortmannin from human neutrophils showed that the inhibitor selectively binds PI 3-kinase (6). Expression of a membrane targeted PI 3-kinase (p110CAAX) in hematopoietic cells results in constitutively elevated levels of PIP_3, the basal phosphorylation of the NADPH-oxidase component $p47^{phox}$, and the stimulus independent activation of protein kinase B (Akt/RAC-PK) (7). The latter finding indicated that protein kinase B is a downstream effector of PI 3-kinase. In several cell systems protein kinase B was shown to stimulate cell survival by the activation of anti-apoptotic pathways through the phosphorylation of BAD and caspase-9. We could demonstrate that chemoattractants, such as fMet-Leu-Phe and the CXC-chemokines IL-8 and GROα stimulate the transient activation of protein kinase B in neutrophils (8). Chemokines also trigger the Ras/Raf signaling cascade leading to MAP-kinase activation in a PI 3-kinase-independent fashion. Comparison of the efficiency of different chemokines to activate protein kinase B and MAP-kinase in leukocytes disclosed the induction of distinct levels of protein kinase B and ERK-2 activity. Stimulation with IP-10 (CXCR3 ligand) or LARC (CCR6 ligand) resulted in a borderline kinase activation. Whereas IL-8 (CXCR1 ligand), MCP-1 (CCR2 ligand), ELC (CCR7 ligand) and MIP-1β (CCR5) induce the transient activation of the kinases. Most striking was the effect of SDF-1 (CXCR4 ligand) which stimulates a sustained activation of both kinases, which can last for up to two hours. However, addition of SDF-1 caused the rapid internalization of more than 75% of CXCR4 within 10 min. By contrast, the activity of protein kinase B and ERK-2 required the continuos interaction of SDF-1 with its receptor CXCR4 at the cell surface, since addition of the a specific receptor antagonist instantly abrogated the response. Furthermore, the addition of the antagonist also led to a rapid decrease in the levels of 3-phosphoinositides, indicating that a continuous supply of PI 3-kinase products is necessary to maintain sustained protein kinase B activity. Taken together, the results demonstrate that a

fraction of cell surface expressed CXCR4 is required and sufficient to propagate sustained SDF-1-induced signal transduction.

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Session 3: Signaling processes in neutrophil functions

Chair: C. Wayne Smith

The Role of Lipids Signaling in the Regulation of Polymorphonuclear Leukocytes (PMN) Phagocytosis.

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The stimulation of human PMN with EIgG occurs via binding of FcγRII. In turn FcγRII-mediated engagement is association with signaled transduction steps leading to ERK I and ERK II phosphorylation and activation. We found that EIgG is a potent stimulus for the translocation of PKCδ, a calcium-independent isoenzyme of PKC, to the plasma membrane of PMN during phagocytosis of EIgG. Coincident with PKCδ translocation to the plasma membrane during phagocytosis, Raf-I is translocated. The appearance of PKCδ and Raf-I in the plasma membrane by three minutes during phagocytosis of EIgG correlated with the maximal rise of diradylglycerol (DRG) generated from phospholipase D activation. We also found that translocation of Raf-1 to the plasma membrane led to activation of ERK through the ability of Raf-1 to activate MEK, a kinase that directly activates ERK. Following the activation of ERK, we found that ERK activation led to phosphorylation of myosin like chain kinase (MLCK) thereby linking the MAP kinase pathway to activation of cytoskeletal components which are required for pseudopod formation. The kinetics of MLCK activation indicated that MLCK activity targeted ingestion with peak MLCK activity during phagocytosis occurring at 4-6 min. The activity of MLCK increased 3-to-5 fold as measured by the phosphorylation of the MLCK substrate peptide sequence derived from the primary structure of myosin light chain. The MLCK inhibitor, ML-7 (10 μM) inhibited both phagocytosis of EIgG and MLCK activity to basal values thereby supporting linkage between the functional response and the requirement for MLC activation. In the presence of the MEK inhibitor, PD098059 phagocytosis and MLCK activity was reduced by approximately 50% while ERK activity was reduced by 85%. To directly link ERK activation to MLCK activation, ERK was immunoprecipitated from PMN which had ingested EIgG. The isolated ERK in increasing concentrations was incubated with PMN cytosol to which the MLCK peptide was added. Under these conditions phosphorylation of MLCK substrate occurred in a concentration dependent fashion. Since MLCK activates myosin, we evaluated the effect of directly inhibiting myosin ATPase. When PMN challenged with EIgG were incubated with 2, 3-butanedione monoxime, an inhibitor of myosin ATPase, phagocytosis was inhibited by greater than 90%, but MLCK activity remained unaffected. These results are consistence with the notion that MEK activates ERK and ERK, in turn activates MLCK and then MLCK activates myosin. The latter being a critical component of the cytoskeleton that is involved in pseudopod formation.

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Not only is PLD activated during phagocytosis to generate DRG to activate PKCδ, but during phagocytosis of EIgG the sphingomyelin pathway is activated. We found that generation of ceramide and as well as its metabolite sphingosine are capable of inhibiting phagocytosis through their ability to inhibit ERK activation. We found that we are able to restore phagocytosis by treating PMN with exogenous diglyceride. The addition of diglyceride leads to activation and translocation of PKCδ and Raf-I to the plasma membrane which in turn allows activation of ERK, thereby substantiating the role of this critical pathway in the phagocytic response. We have found that ceramide and sphingosine activation peaks at 30 minutes, a point at which phagocytosis is terminated; thereby supporting the role of these lipids in regulating the phagocytic response

Mechanisms of phagosome formation and maturation.

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Nascent phagosomes mature by fusing with endomembrane compartments which include endosomes and lysosomes. These endomembranes fuse actively and preferentially with the phagosomal membrane. The mechanisms responsible for the vectorial fusion of endosomes with the phagosomal membrane are not known. In other systems, the selectivity of defined fusion events is dictated by the unique combination of SNARE proteins present in the vesicular and target membranes (1). Because early and recycling endosomes have been reported to express VAMP-3 (cellubrevin), we studied the role of this SNARE in the early steps of phagosomal maturation. Treatment of cells with toxins that cleave VAMP-3 greatly reduced the efficiency of phagocytosis (2). Moreover, VAMP-3 was found to incorporate into the membrane of the nascent phagosome. This was readily apparent in cells transfected with constructs of VAMP-3 tagged with enhanced green fluorescent protein (EGFP). The fluorescent chimeras of VAMP-3 were found to accumulate in the area of the forming phagosome, suggesting that localized fusion of early endosomes precedes completion of phagocytosis and may in fact be an essential component of this response. Experiments using antibodies to the chimera are in progress to verify the occurrence of exocytosis prior to phagocytosis. Jointly, these experiments suggest that focal secretion of endomembrane vesicles, including endosomes bearing VAMP-3, is an important component of phagosome formation.

The processes of phagosomal and endosomal maturation bear a strong resemblance. Like nascent phagosomes, endocytic vesicles fuse sequentially with early endosomes, which in turn merge with late endosomes and eventually with lysosomes. Biochemical and genetic studies have shown that coatomer (COP-I) is essential for budding of early carrier vesicles from early endosomes destined to the late endosomes. We therefore studied whether COP-I assembly is also required for phagosomal maturation. We used Chinese hamster ovary *ldlF* cells, which bear a temperature-sensitive mutation in ε-COP (3). At the non-permissive temperature, delivery of endosomal contents to the lysosome is impaired in *ldlF* cells. These cells were made phagocytosis-competent by stable transfection of the FcγRIIa receptor. At the permissive temperature (34°C) these cells internalized IgG-coated particles into phagosomes, that fused sequentially with early/recycling endosomes and with late endosomes/lysosomes, as revealed by immunofluorescence of transferrin receptors and LAMP-1, respectively. Incubation of these cells for 6-8 h at the restrictive temperature (39°C) resulted in the disappearance of immunoreactive ε-COP. Disappearance of ε-COP-under these conditions was verified functionally: in such cells fluid-phase markers taken up by endocytosis never reached the acidic lumen of the lysosomes. Nevertheless, phagocytosis proceeded under these conditions. More importantly, fusion of endosomes and lysosomes with the phagosome was also observed. The kinetics of phagosomal maturation was similar in cells with and without ε-COP. Moreover, phagosomal acidification was also indistinguishable, reaching pH 4.1-4.3 in both cases. These findings provide evidence that phagosome maturation is essentially independent from COP-I assembly and is therefore not truly parallel to the process of endosomal maturation.

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Molecular Dissection of Fcγ Receptor Mediated Phagocytosis

Zena K. Indik, Paul Chien, Sergio Grinstein and Alan D. Schreiber

The macrophage Fcγ receptors provide a major mechanism for the clearance of IgG coated cells. The family of Fcγ receptor proteins includes the macrophage high affinity receptor FcγRI as well as receptors of the FcγRII and FcγRIII classes. In order to dissect on a molecular level which Fcγ receptors transmit a phagocytic signal and the receptor sequences involved, we have established model systems in which Fcγ receptors are transfected into cells which do not express endogenous Fc receptors.

We have determined that each of the three classes of Fcγ receptors contains a gene product which can mediate phagocytosis. These are FcγRI, FcγRIIA, and FcγRIIIA. FcγRIIA mediates a phagocytic signal in the absence of an accessory subunit, whereas FcγRI and FcγRIIIA require an associated γ subunit. The mechanism of the FcγRI/γ and FcγRIIIA/γ phagocytic signal differs from that of FcγRIIA in several respects, most notably in the interaction(s) with the critical tyrosine kinase Syk in the initial stages of receptor signal transduction. Studies with human monocytes/macrophages using antisense Syk kinase which specifically ablates Syk kinase expression have determined the requirement of Syk for phagocytosis by FcγRI and FcγRIIA.

Of note, FcγRI, in contrast to FcγRIIA, does not contain tyrosines in its cytoplasmic domain. We have determined that the cytoplasmic domain of FcγRI is not required for the FcγRI/γ mediated phagocytic signal. Nevertheless, we have observed that the FcγRI cytoplasmic domain can transmit a Ca^{2+} signal in the absence of the γ chain. The FcγRI cytoplasmic domain is unusual in that it does not contain tyrosines but rather serines in a sequence suggesting a signaling recognition site. The FcγRI cytoplasmic domain may also interact with the FcγRI γ subunit to facilitate activation of other gene programs.

Epithelial cells which do not express endogenous Fc receptors can be induced to mediate phagocytosis of IgG coated cells following transfection with Fcγ receptors. Epithelial cells in the liver transfected with Fcγ receptors mediate the clearance of IgG containing immune complexes. Following Fcγ receptor transfection, the resulting phagosomes closely resemble those of macrophages. The advent of fluorescent proteins such as green fluorescent protein (GFP) has revolutionized our ability to monitor the behavior of proteins in situ and has provided us with a non-invasive means to study FcγRI/γ distribution and protein-protein-interactions during internalization.

Session 4: NADPH oxidase: molecular constituents and clinical disorders

Chair: Harry L. Malech

Components of the NADPH oxidase of phagocytic cells and their abnormality in the molecular pathology of Chronic Granulomatous Disease (CGD).

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Phagocytic cells of the immune system contain an oxidase that is important for the killing and digestion of engulfed microbes. This oxidase is an electron transport chain that transfers electrons from NADPH in the cytosol to oxygen to form superoxide and hydrogen peroxide in the phagocytic vacuole. Absence or abnormality of this oxidase results in the syndrome of CGD, characterised by a profound predisposition to infection. This electron transport chain consists of a flavocytochrome b located in the plasma membrane and membrane of the specific granules. It is composed of α and β subunits, with apparent molecular masses of 23kDa (p22*phox*) and 76-92kDa (gp91*phox*) respectively. The subunit is a member of the FNR family of reductases with FAD and NADPH binding sites in the C terminal half. We have been able to construct a model of this FNR like region of the β subunit, based upon the crystal structure of FNR, which acts as a guide to the organisation of the molecule, and provides a template on which to map mutations in CGD. The N terminal half has a number of transmembranes helices that contain the binding sites for the two haems.

Three specialised proteins, p40*phox*, p47*phox* and p67*phox*, all of which contain SH3 domains, and the small GTP binding protein, p21rac, in the cytosol are required for activation of electron transport. Oxidase activity can be reconstituted in a "cell-free system" from these proteins, although p40*phox* , which normally forms a complex with p67*phox*, and might be important for its natural regulation, is not required in this *in vitro* system. The cytosolic *phox* proteins are associated with the cytoskeleton through an actin binding protein, coronin, which was first described in amoebae, and might be important in the spacial and temporal regulation of the activity of the oxidase. p47*phox* binds, and is phosphorylated by, the catalytic fragment of PKC for which it acts as a regulator.

Defects of the components of the flavocytochrome, and of p47*phox* and p67*phox*, account for almost all cases of Chronic Granulomatous Disease (CGD), in which this electron transport chain is defective. This oxidase system is important for the killing and digestion of bacteria and fungi. This might be accomplished in a number of ways. The oxidase produces superoxide and hydrogen which might themselves be toxic. The hydrogen peroxide can act as substrate for myeloperoxidase which can oxidise chloride and iodide to chlorine and iodine and their hypohalous acids.

The proteins contained within the cytoplasmic granules are also very important in the killing process. These are neutral proteinases that require a neutral or slightly alkaline pH for optimal activity. The oxidase transports electrons, unaccompanied by protons, across the wall of the phagocytic vacuole, resulting in an elevation of the vacuolar pH, thereby optimising conditions for killing and digestion of engulfed organisms by these neutral proteinases. The retention of undigested bacterial and autologous debris within the phagocytic vacuoles is probably responsible for the formation of the granulomata within the tissues that are so characteristic of CGD.

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Structure-function relationships of cytochrome b_{558}

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Neutrophils and other professional phagocytes (monocytes/macrophages, eosinophils) destroy microorganisms by phagocytosis and intracellular killing. The killing process is mediated by the joint action of proteins released from the granules and reactive oxygen products generated by the NADPH oxidase enzyme. This enzyme consists of several subunits, each encoded by a separate gene. Mutations in any of these genes may cause Chronic Granulomatous Disease (CGD). This disease is a rare (1:250,000) immunodeficiency, characterized by recurrent, life-threatening infections of the subcutaneous tissues, airways, lymph nodes, liver and bones with bacteria, fungi and yeasts. The majority of the CGD patients (about 70%) suffers from the X-linked form of the disease, caused by mutation in *CYBB*, the gene that encodes the beta subunit of cytochrome b_{558}, also called gp91^{phox}. This protein is the actual enzymatic unit of the NADPH oxidase. It contains an FAD moiety and two hemes, binds NADPH and reduces molecular oxygen to superoxide. Mutations in three other subunits cause autosomal forms of CGD. This concerns the alpha subunit of cytochrome b_{558} (p22phox), needed for stabilization of the cytochrome in the plasma membrane of the phagocytes, and the cytoplasmic proteins p47^{phox} and p67^{phox} that translocate to the cytochrome during cell activation, a process needed for induction of enzymatic activity after phagocytosis of micro-organisms.

Over the past five years, the mutations in several hundreds of X-CGD families have been identified. The spectrum of these mutations proved to be very heterogeneous: deletions ranging from several megabases to one single nucleotide, insertions from 2 kilobases to one nucleotide, splice site mutations, missense mutations and nonsense mutations, everything except inversions were found. Most of these mutations are specific for one X-CGD family, proving the absence of a founder effect. Only a few hot spots exist, mostly in CpG sequences. The mutations are scattered over all exons and exon-intron boundaries and usually lead to instability of mRNA and/or gp91^{phox} protein. Only a few mutations lead to partial expression of gp91^{phox}, causing a concomitant decrease in NADPH oxidase activity. Even more rare, a dozen mutations have been identified that cause a normal expression of a completely inactive gp91^{phox} protein.

Sofar, X-CGD mutation analysis has been mainly used for two purposes: prenatal diagnosis in families with previously identified X-CGD patients and structure-function analysis of gp91^{phox}. In our own laboratory, we have performed prenatal diagnosis in ten pregnancies at risk in families from six different European countries. Such investigations are always performed in cooperation with a clinical-genetic center that checks the fetal origin of the DNA, often performs additional marker gene analysis, and presents the results to the families. Concerning structure-function analysis, especially the mutations that lead to expression of inactive gp91^{phox} have proven to be of great value. Further evaluation of such mutated proteins have led to the recognition of NADPH, heme and FAD binding regions in the protein as well as to sites of interaction with other NADPH oxidase components. Similar mutations in other oxidase components have provided insight in the mechanism of enzyme activation. Mutations in the promoter region of CYBB are used for the identification of transcription factors involved in gene expression.

Mutations in the autosomal CGD genes fall into two categories: those in *CYBA* (for p22^{phox}) and *NCF2* (for p67^{phox}) are very rare (accounting for about 5% of all CGD cases) and are as heterogeneous as those causing X-CGD. However, the mutations in *NCF1* (for

p47^{phox}) are more common (about 25% of all CGD cases) and almost always the same, i.e. a GT dinucleotide deletion at the start of exon 2. This last phenomenon is caused by recombination events during meiosis or DNA repair between *NCF1* and two pseudogenes that both lack the GT sequence.

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Session 5: Gene therapy and clinical studies

Chair: Anthony W. Segal

Gene Therapy for Chronic Granulomatous Disease

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Chronic granulomatous disease (CGD) is an inherited immune deficiency associated with recurrent infections and affecting 1 in 200,000 people. Phagocytic cells (neutrophils, monocytes and eosinophils) fail to produce microbicidal hydrogen peroxide because there is a defect in the superoxide-generating phagocyte NADPH oxidase. There are four genetic forms of CGD with a common phenotype, each genetic form resulting from mutations in any one of the 4 genes encoding subunits of the phagocyte oxidase (phox). The most common and severe form of CGD is X-linked, gp91phox deficiency affecting two-thirds of patients. The next most common form is autosomal recessive, p47phox deficiency affecting about 30% of patients. In a first trial of gene therapy 5 patients with p47phox deficient CGD were treated with a single cycle of gene therapy using amphotropic MFGS-p47phox retrovirus vector for ex vivo transduction of G-CSF mobilized peripheral blood CD34+ cells (Malech et al, PNAS 94:12133, 1997). In that study serum free medium was used for the culture and transduction and the Nexell Therapeutics, Isolex® 300 stem cell selection system was used to purify CD34+ cells which were cultured in the PL2417 gas permeable flexible plastic containers. Patients were not subjected to any marrow conditioning. Following gene therapy peak numbers of functionally corrected neutrophils in the peripheral blood approached 1 in 2000 in one of the patients with corrected neutrophils detected for 2 to 5 months in these patients. In a second ongoing study a very high titer amphotropic MFGS-gp91phox retrovirus vector (>10^7 infectious units/ml) was used in a similar ex vivo approach for gene therapy of four patients with the severe X-linked form of CGD (Malech et al, Blood 92:690a, 1998). Additional features of this ongoing study include mobilization of CD34+ cells by treatment of patients with the combination of Flt3-ligand and GM-CSF (Immunex Corp.); the inclusion of Flt3-ligand in the ex vivo culture; and the use of the fibronectin fragment CH-296 (RetroNectin®, Takara Shuzo Corp.) to enhance transduction. Ex vivo tranduction of CD34+ cells averaged 70% and in two of the patients gene therapy resulted in peak levels of functionally corrected neutrophils in the peripheral blood of 0.1 to 0.2% with lower levels of corrected neutrophils detected for over 10 months indicating durability of the effect. These results represent important milestones in the development of an ex vivo approach to gene therapy of CGD, an inherited immune deficiency. This approach is generally applicable to other inherited defects of immunity. Achieving high level permanent correction of immune cell function will require mild suppression (conditioning) of the patient's marrow to enhance engraftment of gene corrected stem cells.

Dennis D. Hickstein, M.D.

Retroviral-Mediated Gene Transfer Studies In Leukocyte Adhesion Deficiency

Leukocyte adhesion deficiency or LAD is a congenital immunodeficiency disease characterized by recurrent bacterial infections in which the leukocytes from affected children fail to adhere to endothelial cells and migrate to the site of infection. Heterogeneous molecular defects in the leukocyte integrin CD18 subunit are responsible for the failure to synthesize a CD18 subunit which is capable of forming heterodimers with the leukocyte integrin CD11 subunits and becoming inserted into the surface membrane. LAD is candidate disease for human gene therapy in that: 1) the disease is life-threatening in the severe deficiency form, 2) transfer of CD18 into LAD EBV B-cells reconstitutes a functional CD11/CD18 heterodimer on the cell surface, 3) observations from children with the moderate deficiency form of LAD indicate that even low levels of CD11/CD18 surface expression are likely to result in considerable improvement in the clinical phenotype, 4) since the defect in LAD involves a membrane receptor the results of therapy can be quantitated using peripheral blood and flow cytometry.

To assess the feasibility of human gene therapy for LAD, we have transduced G-CSF- mobilized, CD34+ peripheral blood stem cells from two patients with the severe deficiency form of LAD using supernatant from the retroviral vector PG13/LgCD18. The highest transduction frequencies (20-30%) were found after exposure of the cells to retroviral vector on a recombinant fibronectin substrate (CH296) in the presence of growth factors IL-3, IL-6, and stem cell factor. When the transduced cells were differentiated in vitro they demonstrated adhesion to endothelial cells that was approximately twice that of non-transduced cells. These experiments demonstrate that retrovirus-mediated gene transfer of the leukocyte integrin CD18 subunit complements the defect in LAD CD34+ cells resulting in CD11/CD18 surface expression, and that the differentiated myelomonocytic cells derived from the transduced LAD CD34+ cells display CD11/CD18-mediated adhesion function. These results indicate that ex vivo gene transfer of CD18 into LAD CD34+ cells, followed by re-infusion of the transduced cells, may represent a therapeutic approach to LAD.

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An immunoregulatory function for neutrophils during the induction of a Th2 response in mice infected with *Leishmania major*

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Resistance and susceptibility to infection with *L. major* have been correlated with the appearance of parasite-specific CD4+ Th1 or Th2 cells respectively. The role of specific cytokines secreted by Th1 or Th2 cells has been shown to play a predominant role in directing the functional differentiation of CD4+ T cell precursors. We have shown that an early production of IL-4 by CD4+ T cells during the first 48 hours following infection of mice with *L. major* is responsible for the subsequent development of a Th2 response.
Recently several studies have demonstrated that PMNs transcribe actively many genes coding for cytokines including IL-12, IL-10 and TGF-β suggesting that these cells could also play an immunomodulatory role early during infections with *L. major.*
We have first analysed the recruitment of PMNs within the site of injection of *L. major* (footpad) using the myeloperoxidase method. PMNs were already present within 1 hour after injection of the parasite . Their number remained elevated at least during 8 days following infection in susceptible BALB/c mice whereas the number of PMNs decreased dramatically 72 hours post infection in mice of the resistant C57BL/6 strain. To assess a putative role for PMNs in Th1/Th2 differentiation in the murine model of *L. major* infection, PMNs have been selectively depleted by the use of the NIMP-R14 monoclonal antibody. A single injection i. p. of 1 mg of this mAb is sufficient to deplete circulating PMNs for 3 to 6 days. Six hours post injection of the monoclonal antibody, susceptible and resistant mice depleted of PMNs were injected with *L. major*. Cytokine expression was analysed at 16 hours, 10, 40, and 60 days post injection as markers of Th1/Th2 polarisation. In susceptible BALB/c mice, a single injection of the mAb against PMNs was sufficient to change the default Th2 response to a Th1 pattern. The impairment of Th2 development was already suggested by the absence of the early IL-4 mRNA burst in the draining lymph node of treated mice 16 hours post injection with *L. major.* Accordingly, BALB/c mice treated with anti-PMNs partially resolved their lesions and controlled the multiplications of parasites. We further showed that the protective effect of PMN depletion was dependent on IL-12 as injection of an antibody against IL-12 at the same time as PMN depletion resulted in maintenance of the Th2 phenotype in these mice. Intersestingly, PMN depletion had no incidence on the development of the resistant Th1 phenotype normally observed in C57BL/6 mice. These results suggest a role for PMNs in the establisment of susceptibility to *L. major* infections.
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Session 6: Neutrophil granules and exocytosis

Chair: Hal E. Broxmeyer

Protein Targeting and Structure/Function Relationship in Human Neutrophils.

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The import role of the human neutrophil in innate immunity relies on the ability of the neutrophil to localize rapidly and efficiently to sites of microbial invasion and to expose the microorganisms to the highest possible concentration of bactericidal substances without damaging the surrounding tissue.
The ability of neutrophils to kill microorganisms intracellularly i.e. after taking these up into a phagocytic vacuole serves two purposes: to expose the microorganism to the highest possible concentration of microbicidal substances and to minimize exposure to surrounding cells.
The circulating human neutrophil is a quiescent cell both in terms of metabolism and in terms of responsiveness to stimuli. One reason is that most receptors that mediate the various forms of activation e.g. chemotaxis receptors, complement receptors are localized to intracellular stores in neutrophils. These receptors must be mobilized to the surface to allow the neutrophil to be activated, yet, not all intracellular stores should be mobilized at the same time. Receptors for chemotaxis should be mobilized before bactericidal proteins are exocytosed to minimize damage to endothelial cells during diapedesis.

The neutrophil contains several different types of mobilizable intracellular stores: The granules proper which are further subdivided into azurophil, specific and gelatinase granules with overlapping forms existing, and the secretory vesicles which are formed by endocytosis but behave like regulated storage granules in that they require a stimulus for mobilization. These different stores each have their characteristic protein profile. In addition, these have different requirements for mobilization. Two main reasons can be given for this seemingly complex organization. One is that different proteins are needed at different stages during the functional life of a neutrophil. The other is that some proteins cannot co-exist in the same granule - but are need in a high concentration in the phagocytic vacuole and thus have to be stored in granules.

Targeting can be explained solely on the basis of timing of biosynthesis during myelopoiesis, where azurophil granule proteins are synthesized at the promyelocyte stage and specific granule proteins at the myelocyte/metamyelocyte stage of maturation, while gelatinase granule proteins are synthesized during the band cell stage of myeloid maturation. The functionally important neutrophil granule heterogeneity can be explained by the fact that differences exist in the timing of biosynthesis of the granule proteins which are found in different granule subsets. No specific targeting signals are needed. In accordance, when mRNA levels of a wide variety of granule proteins are investigated there seems to be a highly individual profile which corresponds to the known localization of the corresponding proteins. This indicates but does not prove that regulation of gene transcription of granule proteins is highly individualized.

The human neutrophil is capable of delivering a wide variety of antibiotic molecules to bacteria. Some of these molecules are short lived reactive species that are generated in response to stimulation such as reactive oxygen species and nitric oxide. Others are antibiotic peptides, molecules that have been identified during the last 15 years. Most of these are localized to azurophil granules, but one of the most potent, hCAP-18 is localized to specific granules. Like most other cathelicidins, hCAP-18 is stored in an inactive form. This may be advantageous since the activated form, the C-terminal part, is toxic to eukaryotic cells. The activation of hCAP-18 is mediated by a protease in azurophil granules. Thus, activation occurs only when azurophil and specific granule contents meet.

The targeting of the azurophil granule protease and hCAP-18 to distinct granules is a prerequisite for normal function of this activatable antibiotic system. Most granule proteins are efficiently retained in myeloid cells during biosynthesis. Consequently, only little free granule protein is liberated to the circulation. hCAP-18 is efficiently retained in cells during biosynthesis, yet, a very high concentration of the protein is found in plasma and serum compared to other neutrophil granule proteins with the notable exception of lysozyme. The basis for this is that hCAP-18 binds to circulating lipoproteins which serve as a carrier and can store hCAP-18 whenever it is released to the circulation. hCAP-18, like many other proteins which are constitutively present in granules of human neutrophils, is induced in epithelial cells when these are engaged in inflammatory reactions. This indicates that these granule proteins are important for host defense and under complex regulation of expression.

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Characterization of secretory gelatinase-containing granules and SNARE proteins in human neutrophils

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Mature peripheral blood neutrophils show a low capacity of macromolecule synthesis, despite expressing constitutively very high mRNA levels for c-*jun*, *jun*B, *jun*D, and c-*fos* proto-oncogenes, main components of the transcription factor AP-1. Once in the blood stream, mature neutrophils are fully equipped and programmed to perform efficiently their major role in the surveillance system of host organism against foreign invaders and to undergo a rapid constitutive and spontaneous apoptotic process, having the shortest half-life span of all circulating leukocytes. An early event occurring in neutrophil activation is exocytosis of cytoplasmic granules. The neutrophil granule constituents, secreted or translocated into the cell surface upon cell activation, seem to affect both the interaction of neutrophils with endothelial cells and the migration of neutrophils from the vascular lumen to the extracellular tissue, a critical and early event in neutrophil function. In neutrophils, the membrane of intracellular granules serves as a major reservoir of several proteins whose activity in the intact cell is dependent on plasma membrane localization. Upon cell stimulation, the cytoplasmic organelles degranulate and incorporate their membrane components into the plasma membrane, facilitating the replenishment of some cell surface molecules and conforming a regulatory mechanism of the activity of certain proteins. This degranulation was mediated by cytoplasmic microtubules. Four distinct granule populations have been found in human neutrophils, namely: azurophilic or primary granules; specific or secondary granules; gelatinase-containing granules, also named tertiary granules; and alkaline phosphatase-rich granules, also named phosphasomes or secretory vesicles. Fusion of the different granule populations with the plasma membrane may occur independently. Thus, mobilization of gelatinase-containing tertiary granules can be induced readily under conditions that mobilize slightly the specific granules and hardly mobilize the azurophilic granules. We have postulated that the rapid mobilization of these gelatinase-containing granules can modulate early neutrophil responses upon cell activation. This is supported by the fact that we have found that these granules contain a wide number proteins playing a key role in several physiological actions, including: a) adhesion proteins (CD11b/CD18, CD11c/CD18); b) extracellular-matrix degradative enzymes (gelatinase, heparanase); c) signaling proteins (CD45, diacylglycerol lipase, Rap1A, Rap2B); d) activation antigens (p19); e) proteins involved in superoxide anion generation (cytochrome b_{558}); f) proteins involved in vacuolar acidification (H^+-ATPase); g) proteins involved in synthesis of lipid mediators, such as platelet-activating factor (acetylCoA: lyso-PAF acetyltransferase); h) calcium-binding proteins. Following immunoblotting and immunoprecipitation analysis in subcellular fractionation analysis, confocal analysis and secretion assays we found a major co-localization of gelatinase, heparanase and CD11b/CD18 in resting neutrophils in the gelatinase-containing granules, and a parallel secretion of the matrix degrading enzymes and translocation of CD11b/CD18 to the cell surface, suggesting that mobilization of this organelle can regulate extravasation of human neutrophils. Nevertheless, little is known about the underlying mechanism that regulates the exocytic process in human neutrophils. In contrast, a wide number of proteins have been identified in the neuronal system that play a role in the docking and fusion of synaptic vesicles at the plasma membrane, leading to postulate a model for synaptic vesicle exocytosis, known as SNARE hypothesis. The SNARE hypothesis predicts that a combination of vesicular (v-SNARE) and target (t-SNARE) membrane

proteins interact to form the SNARE (SNAP receptor) complex which serves as the target for the binding and action of soluble fusion components NSF and SNAP. The specificity of vesicle targeting is thought to be mediated by the correct association of v- and t-SNARE isoforms. In the nervous system the vesicle-associated membrane protein VAMP (also named synaptobrevin) acts as a v-SNARE and binds to the t-SNARE membrane proteins syntaxin and SNAP-25 (synaptosome-associated protein of 25 kDa) to form a stable tripartite complex. In an attempt to elucidate the molecular mechanisms of exocytosis in human neutrophils we focused on identifying SNARE proteins in these cells. To this aim, we have used purified human neutrophils as well as the human AML HL-60 cell line, a homogeneous cell culture model for neutrophils. We identified and cloned two isoforms of SNAP-23, named A and B, and syntaxin 6 in human neutrophils and HL-60 cells. Preparation of antibodies against SNAP-23, indicated a subcellular localization for SNAP-23 in both plasma membrane and intracellular granules, as well as translocation towards the cell surface upon cell activation. Syntaxin 6 was located in the plasma membrane, and *in vitro* interaction experiments with GST-SNAP-23 fusion proteins indicated that both SNAP-23A and B could interact with syntaxin 6. Using RT-PCR, cloning, and sequencing techniques, we have also found that human neutrophils and neutrophil-differentiated HL-60 cells express syntaxins 1A, 3A, 3B, 4, 5, 6, 7, 9, 11, and 16, as well as VAMP-1 and VAMP-2. Syntaxin 1 and SNAP-25, previously considered to be specific to neural tissues, were found to be present in human neutrophils. Expression of syntaxin 1A in human neutrophils was evidenced by genetic and immunological approaches. In addition, syntaxin 1A was mainly located to neutrophil granule membranes by confocal microscopy and by immunoblotting of subcellular fractions. Also, with the use of highly specific antibodies against SNAP-25 and the specifically cleavage by botulinal neurotoxin A, we identified SNAP-25 in the granule membranes of human neutrophils. These results demonstrate that human neutrophils contain all the components required to constitute the high affinity SNARE complex, suggesting that this SNARE mechanism can be active in regulating exocytosis in human neutrophils. The expression of a wide number of SNARE proteins involved in vesicle-membrane fusion processes in human neutrophils could be related to their high secretory capacity and to the presence of distinct neutrophil cytoplasmic granules with different exocytic capabilities.

ARF-regulated phospholipase D as a regulator of exocytosis in neutrophils

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Phospholipase D is an enzyme that hydrolyses phosphatidylcholine to phosphatidic acid. In the presence of ethanol, the production of PA is diverted to phosphatidylethanol and this reagent is the only available tool for studying PLD function in cell physiology. The basal activity of phospholipase D in most cell types is very low, yet the enzyme can be activated in most cell types, rapidly and transiently, by a wide variety of stimuli including hormones, neurotransmitters, growth factors and cytokines. Two mammalian PLDs have been cloned, PLD1 and PLD2. Both proteins can be regulated by ARF. In addition Rho family of GTPases and protein kinase C can regulate PLD1 [1].

HL60 cells is a cell-line which has been extensively used as a model for neutrophils. FMetLeuPhe stimulated secretion of azurophilic granules from "primed" HL60 cells and human neutrophils is inhibited by ethanol implying a role for PLD activity in this process [2]. Removal of cytosolic proteins from these cells by permeabilisation leads to loss of secretory function [3]. This is accompanied by the inability of cells to activate PLD activity. Restoration of both functions can be achieved by re-addition of ARF protein [3].

ARF proteins have been found to have a number of disparate functions including the formation of COPI-coated vesicles and delivery of paxillin to focal adhesions. To identify how ARF reconstitutes secretion, we have mapped the residues of ARF1 that interact with PLD1 [4]. These regions are the α2-helix, part of the β-strand and N-terminal helix and its ensuing loop. ARF mutants with increased or decreased ability to activate PLD have been utilised to examine which of the many functions of ARF are mediated by phospholipase D activation. Mutational analysis confirm that the ability of ARF to stimulate PLD activity is essential for restoration of secretory function but not for vesicle formation.

ARF restored secretory function by its ability to indirectly regulate phosphoinositide production [3]. ARF by regulating the activity of phospholipase D stimulates the production of PA that functions as a second messenger. PA directly regulates the activity of Type I PIP 5kinase, an enzyme that converts PI 4-P to PI (4,5) P_2. The function of PIP_2 in exocytosis is not defined but is most likely due to its ability to recruit lipid binding proteins at the membrane.

To identify the site of ARF regulated phospholipase D and hence PIP_2 production, we have used subcellular fractionation [5,6]. In human neutrophils, ARF-regulated PLD activity was found on secretory vesicles, which translocated to the plasma membrane upon priming with FMLP [3]. Since "priming" is required for both secretion of azurophilic granules and the activation of phospholipase D in intact cells, phospholipase D activation at the plasma membrane is a pre-requisite for secretion.

Finally, FMLP also regulates the translocation of ARF and Rho proteins to membranes [7]. ARF translocation is only observed in primed neutrophils. We conclude that the

ARF-phospholipase D pathway is regulated by cell-surface receptors and is part of the signalling cascade that leads to exocytosis in human neutrophils.

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Bactericidal/permeability-increasing protein: Structural insights on antibacterial functions. J. Weiss, Departments of Internal Medicine and Microbiology and The Inflammation Program, The University of Iowa College of Medicine, Iowa City, Iowa, USA.

Proteins within the granules of neutrophils (PMN) play a major role in destruction of ingested bacteria and may also, after secretion, promote extracellular bacterial killing and regulation of the inflammatory response[1]. Granule proteins contribute substantially to both O_2-dependent and –independent bactericidal mechanisms of neutrophils. Prominent among the O_2-independent systems is the bactericidal/permeability-increasing protein (BPI). BPI is remarkable for its highly potent and selective cytotoxic activity toward Gram-negative bacteria (GNB) reflecting avid and selective binding to lipopolysaccharides (LPS) on the surface of GNB[2]. BPI binding to LPS also potently inhibits pro-inflammatory host cell activation by LPS (endotoxin).

Early stages of the action of PMN against ingested BPI-sensitive GNB closely resemble the action of BPI including the selective activation of the antibacterial action of Group IIA phospholipase A2 (PLA2) and the inhibition of specialized type III secretion needed for escape of virulent Shigellae from phagocytic vacuoles[1,3]. Inflammatory fluids from PMN-rich exudates are potently bactericidal against encapsulated, phagocytosis-resistant strains of GNB[1,4]. Extracellular bacterial killing is fully dependent on BPI via its collaborative interactions with sublethal assemblies of MAC, PLA2 and other granule-derived PMN antibacterial proteins[4,5].

BPI is most closely related to the plasma-derived lipopolysaccharide-binding protein (LBP). The primary structures of BPI and LBP are nearly 50% identical and both proteins appear to form highly extended 2-domain molecules in which the N-terminal domain confers LPS recognition and the C-terminal domain mediates transfer of endotoxin-containing particles to specific host acceptors[2,6-8]. However, the physical and functional consequences of the interaction of GNB and cell-free LPS with BPI and LBP are remarkably different[1,2,7,9]. LBP greatly increases host pro-inflammatory responses to LPS and can promote delivery of GNB to phagocytes without inflicting independent cytotoxic effects. In contrast, BPI is potently cytotoxic toward GNB and can promote delivery of intact GNB to PMN and cell-free LPS to monocytes without stimulating pro-inflammatory reactions but rather increases destruction and detoxification of GNB and endotoxin[7,10,11]. Studies are ongoing to further define the structural basis of the functional differences between these closely related LPS-binding proteins. Together, LBP and BPI comprise important arms of the endotoxin-recognizing machinery of the host mediating, respectively, mobilization of host defenses against invading GNB and subsequent destruction, clearance, and detoxification of the bacteria and released endotoxin.

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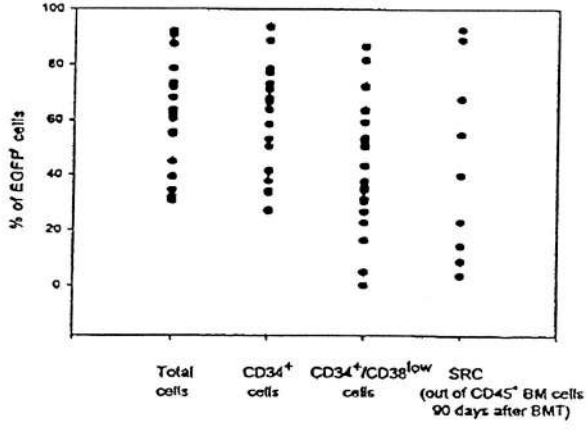
POSTERS

EFFICIENT TRANSDUCTION OF HUMAN NOD-SCID REPOPULATING CELLS USING A PSEUDOTYPED RETROVIRAL VECTOR. Barquinero J, Segovia JC*, Ramirez M*, Guenechea G*, Limón A, Puig T, García J, Bueren JA*. Department of Cell Therapy, Institut de Recerca Oncològica, Hospitalet, Barcelona, Spain; and *Department of Molecular and Cell Biology, CIEMAT, Madrid, Spain. Sponsored by a grant from CICYT (SAF96-0130) and "Fundación Ramón Areces".

With the aim of stably transduce human repopulating cells, retroviral vectors packaged with the *env* protein of the gibbon ape leukemia virus (GALV) were generated. PG13 cells were infected with supernatants of the amphotropic PA317/EGFP1 cell line (Limon *et al.* Blood 1997; 90: 3316-3321). More than 600 clones were isolated, expanded and screened on the basis of green fluorescence intensity and titer on HeLa cells. The clone PG13/EGFP7 with a titer of 10^6 was finally selected for further studies of gene transfer into human hematopoietic cells. Purified $CD34^+$ cells from cord blood (n=22, median purity 92%) were pre-stimulated for 48 hours with recombinant human stem cell factor (rhSCF) (100 ng/mL), MGDF (10 ng/mL) and flt-3 ligand (50 ng/mL). Thereafter, cultures were fed daily with infective supernatants for 48 hours in recombinant CH-296 fibronectin fragment-coated dishes (*Takara Shuzo Co.*, Japan). At day +5, 30.5 to 92.1% (mean 59%) of the total nucleated cells expressed EGFP as assessed by flow cytometry. Transduction efficiency was also analysed in the CD34+ subpopulation and in the more immature $CD34^+$ $CD38^{low}$ subset (see figure 1) by triple staining (CD34-PE, CD38-APC and EGFP) using a dual laser cytometer. To evaluate the efficacy of this procedure to transduce human repopulating cells, infected samples were transplanted into irradiated NOD-SCID mice. In some experiments GFP positive cells were sorted out and transplanted into further groups of NOD-SCID mice. Preliminary results obtained in 10 engrafted mice indicate that the transduction levels of the NOD/SCID-repopulating cells (SRC) is also high (mean engraftment level 42,8%, mean transduction efficiency 42,3 % in human $CD45^+$ cells, analysed in the bone marrow 90 days after transplantation). We conclude that the described infection procedure is highly efficient for transducing very primitive human hematopoietic precursors capable of extensive *in vivo* repopulation.

Figure 1. Gene transfer efficiency in human hematopoietic progenitors. Dots represent transduction efficiency (percentage) in individual cord blood samples. Each column represents a specific subset of cells (total nucleated cells, $CD34^+$ cells, $CD34^+/CD38^{low}$ cells and SRC).



Interferon gamma induces the expression of $p21^{waf-1}$ and arrests macrophage cell cycle that prevents induction of apoptosis

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Summary

Incubation of bone marrow macrophages with lipopolyssacharide (LPS) or interferon gamma (IFNγ) blocks Macrophage-Colony Stimulating Factor (M-CSF)-dependent proliferation. LPS treatment or withdrawal of M-CSF arrest the cell cycle at early G_1, and induce apoptosis. Treatment of macrophages with IFNγ stops the cell cycle latter, at G_1/S boundary, induces $p21^{Waf1}$, and does not induce apoptosis. Moreover, pretreatment of macrophages with IFNγ protects from apoptosis induced by LPS, dexamethasone or growth factor deprivation. Inhibition of $p21^{Waf1}$ by antisense oligonucleotides or using KO mice, shows that the induction of $p21^{Waf1}$ by IFNγ mediates the arrest of cell cycling and the protection from apoptosis. Therefore, IFNγ makes macrophages unresponsible to apoptotic stimuli by inducing $p21^{Waf1}$ and arresting the cell cycle at the G_1/S boundary.

ACTIVATION OF HUMAN POLYMORPHONUCLEAR NEUTROPHILS (PMN) BY SILICA PARTICLES

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Silicosis is a pathological process characterised by the development of fibrotic nodules in the lung, whose primary origin is the exposure to crystalline silica (1). Ingestion of silica particles by phagocytic cells is known to have an important role in the first steps of silicosis. We have studied the *in vitro* activation of macrophage cell lines by silica particles and we have found that the cell line RAW264.7 of murine macrophages becomes activated by silica to produce TNFα without the production of NO (2). We also have isolated nine gene sequences in activated macrophages termed SIG (silica induced genes) (3).

Little is known about the activation of the PMN by silica. Release of *reactive oxygen species* (ROS) is an important mechanism by which PMN can cause tissue damage, but the PMN also produce enzymes and signalling molecules (4). Even though the amounts of cytokines produced by one PMN cell are observed to be low, their contribution may be significant due to their large amount at the wound sites (5).

We have observed that when silica particles are instilled into the trachea of rats, the first cells to be recruited into the alveolar space are polymorphonuclear neutrophils (PMN). These are then followed by monocytes probably in response to specific monocyte chemoattractants. Once in the tissue, the monocytes are progressively activated and become macrophages. Since the PMN are the first cells present at a site of silica accumulation, it is likely that PMN have a function in the first steps of development of silicosis. Thus, we have initiated a study on the activation of the PMN by silica particles in vitro. We have found that human PMN are activated by silica particles to produce small amounts of ROS. Serum slows down the activation rate. We have studied the influence of the particle size in the activation process. Using three different sizes of particles, we found no significant difference on the ROS production. We have initiated studies on gene expression by activated PMN. Using purified PMN and RT-PCR methods we have determined that TNFα is transcriptionally activated in LPS-treated PMN. The expression of TNFα and other cytokines is being determined in silica-activated PMN.

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THE PROTEIN TYROSINE KINASE SYK,BUT NOT FGR AND HCK IS INVOLVED IN IgG-MEDIATED PHAGOCYTOSIS AND PHAGOSOME-LYSOSOME FUSION IN MURINE MACROPHAGES.

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In this study we have studied the role of certain members (Fgr, Hck,Lyn,Src) of the Src-family of protein tyrosine kinases, the protein kinase Syk, phosphorylated proteins (PPs) and filamentous actin (F-actin) in phagocytosis and phagosome-lysosome fusion (PLF) of IgG-coated particles. Using immunofluorescence staining and confocal imaging system, we have analyzed the subcellular distributions of Lyn, Src, Syk and PPs in bone marrow-drived macrophages (BMDM) from both wild type and Fgr^-/Hck^- mice. In BMDM not challenged with IgG-coated particles these kinases were evenly distributed. However, when the cells were allowed to ingest IgG-yeasts, these kinases were intimately associated within the phagocytic caps and colocalized with both F-actin and PPs. In phagocytic cells treated with PP1, a drug known as a specific Src-family kinase inhibitor, we found a significant decrease in the translocations of these kinases, but not in the translocation of PPs or F-actin. PP1 has only a partial inhibitory effect (10-20%) on phagocytosis and PLF as determined by antibody against the lysosomal marker, LAMP-1. Moreover, Piceatannol (Pi), a drug known to specifically inhibit the activity of Syk, inhibited phagocytosis of IgG-coated erythrocytes, despite it has no effect on IgG-mediated phagocytosis of yeasts particles. Importantly, Pi inhibited (> 75%) PLF of IgG-coated yeasts. Our results indicate that the signals for the translocations of protein kinases within the phagocytic caps and those accompany phagocytosis are not linked and controlled by the same mechanisms. Furthermore, PPs and Syk have a selective role in PLF.

A FUNCTIONAL INVESTIGATION OF THE MURINE S100 PROTEIN, MRP-14, *IN VITRO* AND *IN VIVO*.

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MRP-14 is a member of the S100 family of EF-hand containing calcium binding proteins. MRP-14 has a restricted tissue distribution; it is highly expressed in the cytosol of neutrophils and monocytes and it is also expressed to a lesser extent in certain specialised epithelia. MRP-14 is commonly coexpressed with the closely related molecule, MRP-8 (CP-10). Functional data is sparse although human MRP-8/14 complex has been found close to marginating neutrophils on the surface of vascular endothelium (Hogg *et al*, EJI, (1989) 19:1053-1061). Furthermore, human MRP-14 has been shown to stimulate human neutrophil binding to fibrinogen via Mac-1 (Newton & Hogg, J.Immunol. (1998) 160:1427-35). Taken together, these data suggest that MRP-14 may have a role in inflammation.

We have cloned and expressed murine MRP-14 protein in *E.Coli*. We have then used purified protein to investigate the function of MRP-14 in various *in vitro* and *in vivo* systems.

In vitro profiling was performed using thioglycollate-elicited neutrophils. In a static adhesion assay, MRP-14 and fMLP were found to stimulate the Mac-1 dependent adhesion of cells to fibrinogen. However, fMLP but not MRP-14 caused calcium flux, shape change and chemotaxis. Therefore, murine MRP-14 shares the characteristics of human MRP-14 in promoting integrin mediated adhesion but not other functions associated with other bioactive molecules, such as the chemokines.

In vivo investigations utilised an air pouch model of inflammation. This model consists of generating a pocket of sterile underneath the skin on the back of a mouse, followed by the injection of a stimulus into the enclosed space. Recruited cells are then retrieved from the air pouch at various times by washing. Unexpectedly, this model showed that MRP-14 caused specific and profound neutrophil and monocyte recruitment with a maximal effect at 6 hours. This was in contrast to LPS which recruited mainly neutrophils. We hypothesise that the effect of MRP-14 *in vivo* is indirect, as MRP-14 does not induce chemotaxis *in vitro*.

These findings suggest that MRP-14 is a novel proinflammatory agent for myeloid cells.

Altered FPR ligand interactions in CML PMNL.

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Chronic myeloid leukemia (CML) is a progressive stem cell disorder characterized by occurence of Philadelphia chromosome which is formed as a result of t(9;22) (q34.1; q11) translocation, leukocytosis and accumulation of immature and mature myeloid cells in the peripheral blood. One of the mature myeloid lineage cells- polymorphonuclear leukocytes(PMNL) act as the first line of defense during microbial infections. PMNL from CML patients are morphologically indistinguishable from the normal PMNL. However, our earlier studies have shown that CML PMNL show altered biochemical and functional responses. Stimulation of CML PMNL with a classical chemoattaractant- fMLP showed defective chemotaxis, pinocytosis, actin and tubulin polymerization, degranulation, etc.

Binding of fMLP to its receptors (FPR) is the key event in stimulation of PMNL. Therefore, to understand the mechanism of these defects we have studied interactions between chemoattractant formyl peptide and FPR in normal and CML PMNL. These interactions were studied by standard radioligand binding assays as well as by flow cytometry and laser confocal microscopy, using fluorescinated ligand and antibodies to the FPR. Our results show that formyl peptide FPR interactions are altered in CML PMNL as compared to that in normal PMNL. These altered interactions could be one of the possible mechanisms behind altered stimulation of CML PMNL.

NEUTROPHIL FUNCTION IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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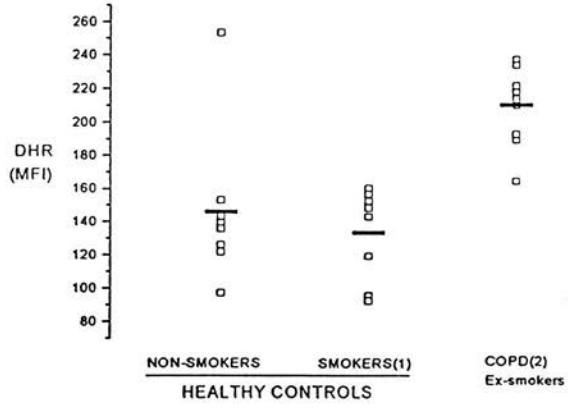
Cigarette smoking is the major risk factor for clronic obstructive pulmonary disease (COPD). However, only 15-20% of cigarette smokers develop the disease. This indicates that there are additional genetic factors that may contribute to the development of COPD. Neutrophils are considered to be an important effector cell in the inflammation and lung injury which occur in air spaces in COPD. Recruitment and activation of neutrophils is an important event in its pathogenesis and expression of several adhesion molecules and production of oxygen metabolites are important steps in this process.
In this study we have determined reactive oxygen species (ROS) production by neutrophils in 8 non-smokers, and in smokers, 10 with (susceptibles) and 8 without COPD (non-susceptibles).

Methods. Neutrophils were isolated from venous blood by dextran sedimentation of erythrocytes followed by centrifugation on Fycoll-Paque gradient. Neutrophils suspensions (500µl) were adjusted at 2 X 10^6 cells/ml incubated with dihidrorhodamine (DHR 123, Molecular Probes Inc.))(10 µl) 10 min at 37°C with 5% CO_2 followed by another 15 min incubation period with phorbol-myristate-acetate(Sigma Chemicals) (20 µl) before analysis by flow cytometry (Becton-Dickinson FACScan).

Results. We observed that spontaneous ROS production by neutrophils of COPD patients was significantly higher than in non-smokers controls ($p<0.05$) and smokers without COPD ($p<0.01$). Also, PMA stimulated ROS production by neutrophils (Figure) was higher in COPD patients than in non-smokers ($p<0.01$) and smokers without COPD ($p<0.001$).



1- Non susceptibles
2- Susceptibles

These results indicates that tobacco susceptibility is related to higher ROS production by neutrophils. We suggest that this enhanced oxidative capacity can be related to a genetic factor in neutrophils of subjects with tobacco susceptibility, and that would favour the development of COPD.

Generation, characterization and *in vitro* functional correction of EBV-transformed B cell lines derived from chronic granulomatous disease patients using retroviral vectors.

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Chronic granulomatous disease (CGD) is a rare inherited immunodeficiency characterized by a defective leukocyte bactericidal function. As a result, CGD patients suffer diffuse granulomas, anemia and severe recurrent infections, leading in some cases to early death. The disease is caused by defects in NADPH oxidase, a multicomponent enzyme responsible for the reduction of molecular oxygen to superoxide (O_2^-). The congenital defect lies in mutations in any one of the at least four components of this enzyme: gp91phox (causing a X-linked recessive form that affects 75% of all CGD patients), p47phox, p67phox and p22phox.

The aim of this work was to perform a preclinical gene therapy protocol for this disease using retroviral vectors. For this purpose, firstly we generated EBV-transformed B cell lines derived from CGD patients with mutations in the gp91phox protein and from normal controls. These CGD-derived cell lines maintained the functional defects described in their primary cells. Secondly, we infected EBV-B cell lines with retroviral vectors containing the coding sequences of gp91phox and a cytoplasmically truncated version of the human low-affinity receptor for nerve growth factor (ΔLNGFR).

After more than 3 months, ΔLNGFR expression in the EBV B-cell lines were greater than 70% without selection. The functional assays showed that transduced CGD-derived B cell lines restored NADPH oxidase activity to 60-80% of normal levels.

These results suggest that the retroviral vectors used in this protocol can be used for *in vitro* functional reconstitution of NADPH oxidase defect in CGD-derived cell lines.

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NADPH oxidase assembling through cytochrome b_{558}

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Flavocytochrome b_{558} of NADPH oxidase complex generates superoxide O_2^- in phagocytic cells and B lymphocytes through a membrane spanning electron transport chain from NADPH to molecular oxygen. It is a heme containing integral membrane protein consisting of two (1/1) α and β subunits, p22-phox and gp91-phox. The activity of oxidase depends on the assembling of cytosolic activating factors (p47-phox, p67-phox, p40-phox and Rac 1/2) with cytochrome b_{558} at the membrane level. Dysfunction of NADPH oxidase is illustrated in familial chronic granulomatous disease where activity is abolished.

O_2^- generating oxidase of Epstein-Barr-virus immortalized B lymphocytes is 100 times less active than that of neutrophils. Once cytochrome b_{558} has been isolated from membrane of either cell and solubilized, *in vitro* reconstituted oxidase activity is similar. This suggests that the weak activity of B lymphocytes might be the results not only of a different stoechiometry of cytosolic factors with respect to the small amount of expressed cytochrome b_{558} but of a defect in the activation process inherant to an unfavorable cytochrome b_{558} membrane environment.

The β subunit , gp-91-phox, of cytochrome b_{558} from EBV-B lymphocytes is more glycosylated than that of neutrophils ; deglycosylation performed with tunicamycine in intact cells or on isolated cytochrome b_{558} had no effect on oxidase function while stability was impaired and binding of the heme ligand butyl-isocyanide increased. The unmasking of new epitopes on deglycosylated gp91-phox let to a better interaction of monoclonal antibodies with cytochrome b_{558}. Topography of native and deglycosylated cytochrome b_{558} incorporated into liposomes was analyzed through Atomic Force Microscopy. The size of cytochome b_{558} liposomes increased with assembling suggesting that, *in vitro*, transition from resting to activated state of oxidase complex might be mediated through conformational regulation process where p67-phox has been shown to be the limiting factor. Transfection experiment carried out with EBV-B lymphocytes of CGD p67-phox(-) mutants should facilitate investigation of p67-phox function in this assembly and that of p40-phox which *in vitro* downregulates the process.

APOPTOSIS INDEPENDENT PHAGOCYTOSIS OF MOUSE GRANULOCYTES EXPRESSING BCL-2

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Extravasation of leukocytes into the site of inflammation results in an accumulation of cells in the respective tissue. In order to restore leukocyte homeostasis during the healing phase of inflammation removal of the cellular infiltrate is essential. An important mechanism of this process is apoptosis of granulocytes and ensuing phagocytosis by macrophages. This interaction of granulocytes and macrophages is thought to be mediated by acidic phospholipids such as phosphatidylserine expressed on the surface of granulocytes during apoptosis.

In this study we investigated the interrelationship between surface expression of acidic phospholipids, apoptosis and subsequent phagocytosis of granulocytes in mouse inflammation model. For this purpose, transgenic mice were used overexpressing the apoptosis inhibitor Bcl-2 under the control of the granulocyte-specific MRP14 promoter. Following thiogycollate-induced peritoneal inflammation, it was found that Bcl-2 overexpression strongly reduced translocation of phosphatidylserine on the surface of granulocytes and apoptosis. Interestingly, however, Bcl-2^+ granulocytes were phagocytosed to a similar extent as wildtype cells. Our data indicate that in peritoneal inflammation *(i)* phagocytosis of infiltrated granulocytes is not mediated by a mechanisms requiring exposure of acidic phospholipids and *(ii)* apoptosis and phagocytosis of infiltrated cells may be independent events.

Expression of apoptosis-related genes in human neutrophils and during neutrophil differentiation of HL-60 cells

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After leaving the bone marrow, neutrophils are programmed to die within about 24 h as a result of constitutive and spontaneous apoptosis. Thus, neutrophils are a useful model to study programmed cell death, having the shortest half-life of all circulating leukocytes. The acute myeloid leukemia (AML) HL-60 cell line has been widely used as a cell culture model of human neutrophils, as it can be induced to differentiate with DMSO or retinoic acid towards cells sharing most of the features of mature neutrophils, including the onset of spontaneous apoptosis. After programmed cell death is triggered, the process of apoptosis is mainly regulated by different members of two growing gene families, namely *bcl-2*-related genes and caspases. Caspases exert an effector function in programmed cell death and the Bcl-2 family regulate the apoptotic response, through inhibiting (Bcl-2, Bcl-x_L, Bcl-w, Bfl-1) or promoting (Bax, Bak, Bik, Bad, Bcl-x_S) cell death.

HL-60 cells die by apoptosis after neutrophil differentiation with DMSO. In order to analyze the molecular basis of neutrophil apoptosis, the expression of a wide number of apoptosis-related genes, including *bcl-2* and caspase family genes, was investigated in human peripheral blood neutrophils and during DMSO-induced HL-60 differentiation by RT-PCR, cloning and sequencing techniques. We found that the onset of apoptosis in DMSO-HL60 cells correlated with the achievement of an apoptosis-related gene expression pattern similar to that of mature neutrophils. HL-60 cells expressed low levels of fas mRNA which were increased upon DMSO treatment. Fas was highly expressed in mature neutrophils. FasL was weakly expressed in HL-60 cells and neutrophils. HL-60 expressed *bak*, *bik*, *bax*, *bad*, *bcl-2*, *bcl-*x_L, *bcl-w*, *bfl-1*, and caspases 1-4, 7-10. Following DMSO treatment, expression of *bak*, *bcl-w*, *bfl-1* and caspases 1 and 9 was increased, whereas *bik* and *bcl-2* expression was blocked, and caspases 2, 3 and 10 were downregulated. Mature neutrophils expressed *bak*, *bad*, *bcl-w*, *bfl-1*, as well as caspases 1, 4, 7-9, but hardly expressed *bcl-2*, *bcl-*x_L, *bik*, *bax*, and caspases 2, 3 and 10. Overexpression of *bcl-2* or caspase inhibition prevented differentiation–induced apoptosis in Hl-60 cells, but not their capacity to differentiate towards neutrophils. These results suggest that the above differential expression of survival and death-promoting genes during neutrophil differentiation of HL-60 cells play a critical role in the acquisition of the neutrophil apoptotic features during neutrophil differentiation.

Clonogenic Progenitors in Cord Blood, Bone Marrow and G-CSF Mobilized Peripheral Blood

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Granulopoiesis is a complex process in which hematopietic progenitor cells (HPCs) proliferate and differentiate to generate mature polymorphonuclear leukocytes (PMNs).

In the clinical setting HPCs are generally assessed by the enumeration of CD34+ cells. The CD34+ compartment, however, is heterogeneous with differences in the relative frequencies of lineage-committed and pluripotent HPCs. In the present study we have compared the frequencies of clonogenic progenitors in the CD34+ compartment of bone marrow (BM), cord blood (CB) and G-CSF mobilized peripheral blood (mob. PB). For this, lineage-committed progenitors were assessed by the colony-forming cell (CFC) assay, and pluripotent HPCs by scoring of long-term culture initiating cells in the cobblestone area-forming cell (CAFC) assay. The CB derived CD34+ population contained two-fold higher numbers of lineage-committed HPCs (CFU-GM, BFU-E) and six- fold higher numbers of multi and pluripotent HPCs, i.e. CFU-GEMM, week 5 CAFCs and week 8 CAFCs, than the CD34+ population of BM. Except for CFU-GM the CD34+ population of mob. PB included significantly higher frequencies of clonogenic progenitors than BM (BFU-E=2.3×, CFU-GEMM=2.5×, week 5 CAFCs=8.4×, week 8 CAFCs=10.3×). Accordingly, the marrow CD34+ compartment contained 67% of non-clonogenic CD34+ progenitors, whereas mob. PB and CB contained 46% and 31% non-clonogenic CD34+ cells, respectively.

Physiologically, G-CSF application with concomitant induction of hematopoietic growth factors resembles a "stress-situation" for the blood system. This situation calls for the transfer of HPCs to inactive fatty marrow sites for rapid expansion of hematopoietic activity. The candidate cell to transfer would be a pluripotent HPC with high self-renewal capacity rather than a lineage-committed clonogenic or non-clonogenic progenitor cell with relatively limited self-renewal capacity. In fact, this is supported by our data showing that G-CSF preferentially mobilizes CD34+ cells from BM into circulation that are capable of lineage-committed and, in particular, of pluripotent clonogenic expansion.

Interestingly, we found similar frequencies of progenitor subsets within the CD34 compartments of CB and mob. PB. Because of this observation and because circulating HPCs decrease rapidly in the new-born (2 days) one might speculate that the prenatal period and the event of birth reflect a pysiological "stress-situation" for the hematopoietic system maintained by growth factors of placental origin.

DETECTION OF CHRONIC GRANULOMATOUS DISEASE IN AN ADULT PATIENT

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Clinical Features: The patient, a 37 year old man, represents a sporadic case with no similarly affected relatives, either male or female. His motherly grandparents were first cousin. The clinical history showed the following infections:
Recurrent infections since childhood including mouth aphtas, urinary tract infections and perianal abscesses. At age 30, he was diagnosed with kala-azar after spending some weeks in a Spanish endemic area for Leishmania. At age 33, he underwent surgery for drainage of a large penial and scrotal abscess. At age 34, he was diagnosed with brain abscess, with negatives cultures, and treated with antibiotics and surgical drainage and cured. At age 37, he was diagnosed with ecthyma gangrenosum of the right thigh and a pulmonary left lower lobe cavitated pneumonia.. Blood and bronchoalveolar lavage (BAL) bacterial, fungal and viral cultures and blood serologies, including HIV and Legionella, were negatives. He was treated with imipenem and ciprofloxacin with cure
At present, he is healthy and and treated with subcutaneous recombinant human interferon $-\gamma$, 0.05 mg / Mof body surface, three times a week, and trimetroprim-sulphametoxazol, 160 / 800 once a day, and itraconazole, 200mg twice a week were administered as prophylaxis against bacterial and fungal infections.

Immunological study: Serum levels of IgG, IgM, IgA, complement fractions were normal. Granulocytes and lymphocytes were also normal. Phenotypic analysis of peripheral blood mononuclar cells was normal.

Analysis of capacity of phagocytes to activate the NADPH oxidase system involved in the reduction of molecular oxygen to reactive oxygen intermediates (ROI):
Two tests were used:
Reduction of the nitro blue tetrazolium (NBT) dye and Dihidrorhodamine 123 (DHR), a non fluorescence dye which caused to fluorescence by ROI following phagocyte sensitization and can be measured by flow cytometry. (See figure 1)
TABLE I

	PATIENT	MOTHER	FATHER	SISTER
% NBT +	0	~ 100	not done	~ 100
% DHR+	0	88	not done	93
MIF /DHR	13.5	2090	not done	2329
INHERITANCE	AUTOSOMAL			

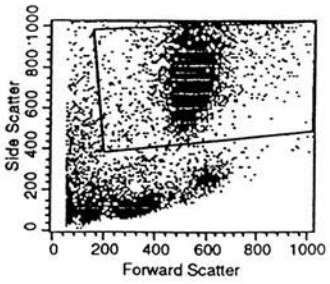
Immunoblotting of cytochrome b558

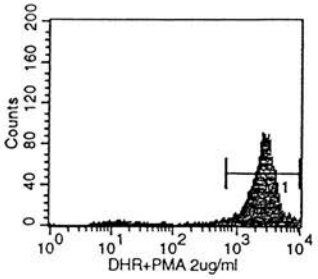
We will report the results of the gp91 ^{phox} protein and p22 ^{phox} protein on Western blots using monoclonal antibodies 48 and 449. Both reagents have been donated by Dr Dirk Roos from the Central laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunoloy, Amsterdam.

Conclusion:

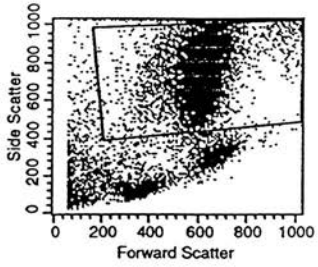
We have identified a sporadic presentation of a Chronic Granulomatous Disease .and it looks like he is an autosomal form. Molecular study will be made at the laboratory above mentioned

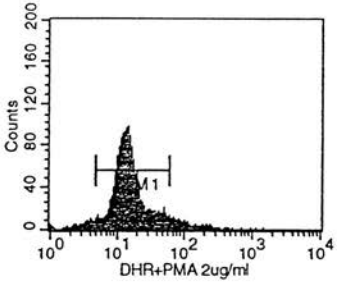
Figure 1





CONTROL
DHR+
PMA 2ug/ml
MFI= 2480





PATIENT
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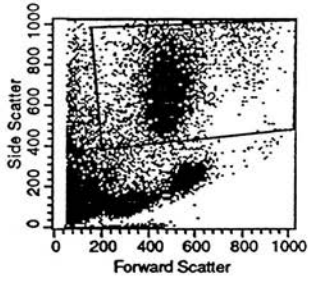
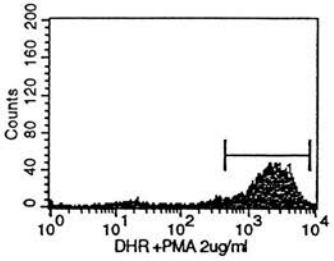
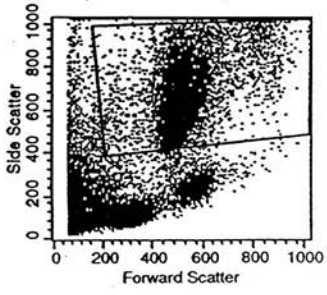
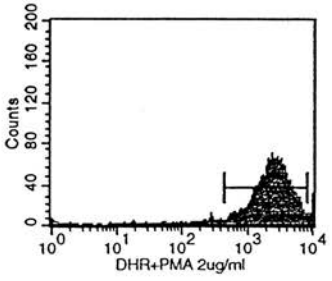
PMA 2ug/ml
MFI= 13.5





MOTHER
DHR+
PMA 2ug/ml
MFI= 2090





SISTER
DHR+
PMA 2ug/ml
MFI= 2329

Role of adhesion in the production of inducible nitric oxide synthase in human neutrophils.

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Nitric oxide (NO) is a potent microbicidal product of neutrophils. Rodent neutrophils release high levels of NO following stimulation with cytokines, produced from the conversion of L-arginine to L-citrulline by inducible nitric oxide synthase (iNOS or NOS II). Production of nitric oxide in human neutrophils remains controversial. We have previously shown iNOS mRNA and protein is produced within neutrophils in human buffy coat preparations stimulated with cytokines (IL-1α, TNFα and IFNγ).

We have continued these investigations with an aim to identify the conditions required for maximal iNOS production and activity. Neutrophils were purified on a plasma-percoll gradient and incubated for 3hours at 37°C with or without cytokines ($0.5ngml^{-1}$ IL-1α, $500Uml^{-1}$ IFNγ and $10ngml^{-1}$ TNFα). Following incubation cell smears were made and iNOS detected by immunocytochemistry using a monoclonal antibody to iNOS. Cytokines had no effect on iNOS production in purified neutrophils, with only 3% of cells in both stimulated and unstimulated suspensions staining positive. This is in contrast to previous results with buffy coat preparations, where 20% of neutrophils made iNOS protein following cytokine stimulation. Cell-free supernatants from stimulated buffy coat cells are unable to induce similar levels of iNOS production in purified neutrophils. This suggests that soluble mediators alone are insufficient to produce maximal iNOS expression. Stimulated buffy coat cells form large aggregates. However, purified neutrophil suspensions do not aggregate to the same degree, suggesting that adhesion may have a role in iNOS induction. When purified neutrophils were allowed to adhere to glass and stimulated with cytokines, the proportion of neutrophils producing iNOS increased to as much as 40%. Unstimulated neutrophils were less able to adhere to glass, although those that did were often positive for iNOS. Coating the glass with human plasma, FCS or fibronectin reduced this non-specific background. Initial results indicate that transmigration of neutrophils through a cytokine stimulated endothelial layer can increase iNOS production in purified neutrophils.

These observations reveal that in addition to cytokine stimulation adhesion is required to induce the production of iNOS protein in human neutrophils, indicating a role for neutrophil adhesion receptors such as integrins in induction of iNOS synthesis.

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