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The role of granulocyte antibodies in monocyte and granulocyte activation

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To my family

Når man føler hvor lidet
man nåer med sin flid
er det nyttigt at mindes at
Ting Tar Tid.

Piet Hein

ABSTRACT

Granulocytes and monocytes are important cells during the first phase of inflammation. They are activated and recruited from the blood vessels to inflammatory sites via the regulation of receptors involved in adhesion to endothelium. Through phagocytosis and the production of reactive oxygen metabolites and cytokines, they are crucial for the initiation of an immune response. Antibodies against surface and intracellularly expressed granulocyte antigens are important in a number of clinical conditions. Detection of anti neutrophil cytoplasmic antibodies (ANCA) is a diagnostic marker for systemic vasculitis and there is also increasing evidence for a pathophysiological role of the antibody. The objective of this thesis was to analyse the functional responses of granulocytes and monocytes to activation, with special attention paid to the relevance of granulocyte antibodies.

In the first study the expression of receptors associated to adhesion to endothelium (CD62L and CD11b), binding of complement and immunoglobulin (CD35 and CD16) and the production of reactive oxygen metabolites were analysed on granulocytes after storage for different periods of time. Next we evaluated a flow cytometric method for the detection of granulocyte antibodies against surface and intracellular antigens. In the third study we determined the *in vitro* expression of CD62L and CD11b and the production of reactive oxygen metabolites in monocytes in response to incubation with ANCA. Finally, in a prospective clinical study the monocyte activation and the concentration of soluble inflammation markers and soluble adhesion molecules were analysed in patients with acute anti-proteinase 3 (PR3) positive systemic vasculitis.

In our first study we demonstrated a marked decrease in granulocyte CD62L expression concomitant with CD11b up-regulation at 24 hours storage. The expression of receptors associated with phagocytosis and the capacity to produce oxygen metabolites remained more stable. In the next study we showed that antibodies against granulocyte antigens can be detected by flow cytometry and by using both un-permeabilized and permebilized target cells, antibodies against surface and intracellularly expressed antigens can be distinguished. We found a decreased CD62L expression and enhanced production of oxygen radicals in monocytes upon *in vitro* ANCA stimulation. Compared to ANCA positive IgG-fractions, ANCA positive sera induced a more pronounced CD62L down-regulation, which supports the contribution of other soluble serum factors beside IgG. In contrast with our *in vitro* finding, monocytes from patients with acute ANCA-positive vasculitis showed reduced capacity to produce oxygen radicals. This may be a consequence of a prolonged period of immune activation and a more dynamic situation *in vivo*. High concentration of anti-PR3 correlated to decreased CD62L and increased CD11b expression on monocytes. Soluble inflammation markers (sCD14, IL-6, sTNFR1, IL-8, IL-10) as well as soluble adhesion molecules (sVCAM-1, sICAM-1) were enhanced compared to in healthy controls and similar compared to in a control group with acute ANCA-negative infection. The regulation of adhesion receptors is crucial in the initial attachment to the endothelium in an inflammatory response and a dysregulated CD62L/CD11b expression on monocytes may have pathophysiological implications for the endothelial damage seen in vasculitis.

LIST OF PUBLICATIONS

I. **Wikman A**, Lundahl J, Fernvik E, Shanwell A.
Altered expression of adhesion molecules (L-selectin and Mac-1) on granulocytes during storage.
Transfusion 1994; 34: 167-171.

II. **Wikman A**, Olsson I, Shanwell A, Lundahl J.
Detection by flow cytometry of antibodies against surface and intracellular granulocyte antigens.
Scand J Clin Invest 2001; 61: 307-316.

III. **Wikman A**, Fagergren A, Forslid J, Jacobson SH, Johansson SGO, Lundahl J.
ANCA induce decreased CD62L expression and enhanced metabolic activity in monocytes.
Scand J Immunol, In press.

IV. **Wikman A**, Fagergren A, Johansson SGO, Lundahl J, Jacobson SH.
Monocyte activation and relation to anti-proteinase 3 in acute vasculitis.
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CONTENTS

LIST OF ABBREVIATIONS

ANCA	Anti neutrophil cytoplasmic antibodies
BVAS	Birmingham vasculitis activity score
C3b	Complement factor 3b
C5a	Complement factor 5a
C-ANCA	Cytoplasmic-ANCA
CD	Cluster of differentiation
CFDA	Carboxyfluorescein diacetate
CRP	C-reactive protein
DCFH	Dichlorofluorescein diacetate
EDTA	Ethylene diaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FITC	Fluorescein isothiocyanate
FMLP	N-formyl-methionyl-leucyl-phenylalanine
FOG	Fixation and permeabilization with OG
GAT	Granulocyte agglutination test
GIFT	Granulocyte immunofluorescence test
HNA	Human neutrophil antigen
ICAM	Intercellular cell adhesion molecule
Ig	Immunoglobulin
IIF	Indirect immunofluorescence
IL	Interleukin
INF	Interferon
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAIGA	Monoclonal antibody-specific immobilization of granulocyte antigen
MBL	Mannan binding lectin
MCP	Monocyte chemoattractant protein
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MPA	Microscopic polyangiitis
MPO	Myeloperoxidase
NK	Natural Killer
OG	n-octyl-β-D-glucopyranoside
P-ANCA	Perinuclear-ANCA
PBS	Phosphate buffered saline
PE	Phycoerythrin
PFA	Paraformaldehyde
PMA	Phorbol 12-myristate 7-acetate
PR3	Proteinase 3
RPMI	Rosewell Park Memorial Institute
TGF	Transforming growth factor
Th	T helper
TNF	Tumor necrosis factor
VCAM	Vascular cell adhesion molecules
WG	Wegener´s granulomatosis

1 INTRODUCTION

The inflammatory response is essential in the defense against microorganisms and for tissue repair. The immune defense system is activated by a complicated sequence of events that produces vasodilatation, vascular leakage of fluid and proteins and local infiltration of inflammatory cells resulting in heat, pain, redness, swelling and decreased function (calor, dolor, rubor, tumor, functio laesa). Co-operation between cellular and humoral factors is required to obtain an optimal inflammatory response. Granulocytes and monocytes are important cells during the first phase of inflammation. They are immediately available and recruited from the blood vessels to inflammatory sites via the regulation of receptors involved in adhesion to endothelium and receptors for complement and common bacterial constituents. Through phagocytosis and the release of enzymes and other substances toxic to microorganisms and through the production of cytokines and chemokines, granulocytes and monocytes are crucial to the initiation of an immune response. An inappropriate or redundant immune response can contribute to chronic inflammation and tissue injury.

Antibodies against surface and intracellularly expressed granulocyte antigens are important in a number of clinical conditions. For instance, alloimmune neonatal neutropenia, autoimmune neutropenia and transfusion complications are associated with antibodies against antigens expressed on the cell surface. Systemic vasculitis is strongly associated with antibodies against antigens localized in granules in granulocytes and in monocytes, anti-neutrophil cytoplasmic antibodies (ANCA). The detection of ANCA is a diagnostic marker for systemic vasculitis, an autoimmune disease, and there is also increasing evidence for a pathophysiological role of the antibody.

This thesis is based on studies focusing on functional inflammatory parameters of granulocytes and monocytes in the initial phase of activation, with special attention paid to the relevance of granulocyte antibodies.

1.1 General aspects of inflammation

Inflammatory processes involve innate and adaptive immune responses and are critical for host defense against invading microorganisms and for wound healing. The innate immune response largely involves granulocytes and monocytes/macrophages. These cells are immediately available, they provide an early defense and play a crucial part in the initiation and subsequent direction of inflammatory cells. The adaptive immune response depends upon lymphocytes, divided into T-lymphocytes, which express specific receptors, and B-lymphocytes, which can be stimulated to the production of specific antibodies. Some of the lymphocytes persist as memory cells and form the basis of immunological memory, which ensures a more rapid and effective response on a second encounter with an antigen and thereby provide lasting protective immunity. Activated monocytes/macrophages and lymphocytes secrete cytokines, that is proteins released by cells that affect the behavior of other cells that express corresponding receptors. Monocytes/macrophages also release proteins known as chemokines that attract cells with chemokine receptors - such as neutrophils and monocytes - from the blood stream. One important effect of cytokines is the initiation of the acute-phase response, which involves a shift in the proteins produced in the liver. The acute phase proteins, such as C-reactive protein (CRP) and mannan-binding lectin (MBL), are important triggers of complement activation, a system of plasma proteins that activates a cascade of proteolytic reactions.

An exaggerated or inappropriate immune response may result in autoimmune inflammation or hypersensitivity reactions; on the other hand an insufficient immune response may result in increased susceptibility to infections or tumors.

1.2 Inflammatory cells

1.2.1 Granulocytes

The inflammatory cells arise from pluripotent stem cells through two main lines of differentiation. The myeloid progenitor is the precursor of granulocytes, macrophages, dendritic cells and mastcells and the lymphoid progenitor is the origin of the lymphocytes, *Fig. 1*. The differentiation is regulated by cytokines. There are three types of granulocytes: the neutrophil which is the most numerous and important cell in the innate immune response, the eosinophil important in parasitic infections and allergic inflammation and the basophil, expressing the high affinity receptor for IgE play a role in allergic and anaphylactic reactions. The granulocytes contain densely staining granules in their cytoplasm. Four well-defined types of granules have been defined, azurophil (primary) granules, specific (secondary) granules, gelatinase (tertiary) granules and secretory vesicles *[Kuijpers TW, 1991, Borregard N, 1993, Sengelov H, 1994]*. The azurophilic granules fuse with phagocytotic vesicles and generally contain antimicrobial compounds such as myeloperoxidase (MPO), a protein that catalyzes the production of oxygen radicals, and defensin, a protein that kills a variety of bacteria, fungi and viruses. These granules also contain the serine proteinases elastase, cathepsin G and proteinase 3 (PR3). The specific granules are largely destined for release into the extracellular space. Their content is lactoferrin, plasminogen activator, lyzosomes and collagenase, probably important for degrading and remodeling tissue. Specific granules also contain a number of membrane-bound molecules that are also expressed on the cell surface. These include CD11, CD18, CD66a, CD66b, NB1, N-formyl-methionyl-leucyl-phenylalanine (fMLP)-receptors and C5a receptors. Like specific granules, tertiary granules contain many membrane proteins that are up-regulated to the cell surface with activation. The secretory vesicles appear to be formed by endocytosis; they contain membrane proteins such as CD35, CD11b, CD14 and CD16 and are up-regulated to the surface upon weak stimulation *[Detmers PA, 1995]*. In contrast to specific and tertiary granules they can be up-regulated to the cell surface in the absence of extra-cellular calcium.

Normally the production and elimination of neutrophils is balanced, with a fairly constant concentration of neutrophils, 1.5-6x10^9/L, in peripheral blood. The neutrophils are short-lived cells, T1/2 in peripheral blood is 6-7 hours. In the initial phase of inflammation, neutrophils are recruited into the inflamed tissue in large numbers concomitantly with increased production and release from the bone marrow. Neutrophils are recruited to the inflammatory site by the regulation of adhesion molecules. They have surface receptors for common bacterial constituents and complement and are together with monocytes the principal cells that engulf and destroy the invading microorganisms. Enzymes and proteins involved in microbial killing are released from granules in activated neutrophils and oxygen radicals toxic to microorganisms are produced. Both the enzymes released from granules and the oxygen radicals when these are ocasionally released in the extracellular space, may cause tissue damage.

The neutrophils are endstage cells and undergo apoptosis soon after they have accomplished phagocytosis.

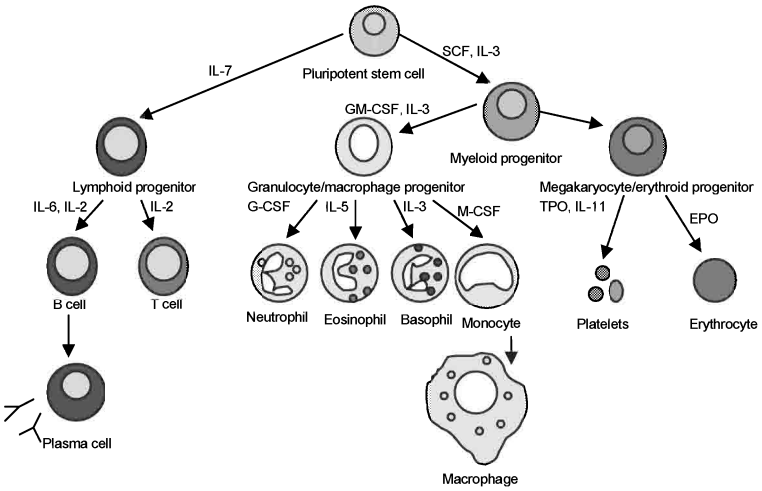


Fig. 1 Differentiation of the hematopoietic cells.

1.2.2 Monocytes

The mononuclear phagocyte system (MPS) is composed of monocytes and macrophages. Monocytes circulate in the blood and differentiate continuosly into macrophages upon migration into the tissues. The concentration of monocytes in peripheral blood is 0.1-0.8 x10^9/L with T1/2 4.5-10 hours (mean 8.4 hours). The marginal monocyte pool is about three to four times the size of the circulating pool. In response to various stimuli, macrophages differentiate and take on characteristic morphologic and functional qualities in different tissues, e.g. osteoclasts, microglial cells, Kupffer cells, alveolar cells, Langerhans cells.

Monocytes are the next cell type after neutrophils to be recruited to an inflammatory site, after 18-48 hours. Like the neutrophils the monocytes/macrophages contain granules and express surface receptors for bacterial peptides and complement- and immunoglobulin-receptors. Upon activation they release toxic enzymes and proteins and produce oxygen radicals.

Monocytes/macrophages produce pro- and anti-inflammatory cytokines and are thereby important in orchestrating the inflammatory response and activating the adaptive immunity. The released cytokines can stimulate proliferation of T- and B-lymphocytes, cause hyperthermia by action on hypothalamic cells, enhance fibroblast proliferation, enhance catabolic activities and cause synthesis of acute phase reactants *[Dinarello CA, 1996]*. Monocytes/macrophages also play important roles in angiogenesis *[Sunderkotter C, 1994]*.

CD14 expressed on monocytes is the receptor for bacterial lipopolysaccharide (LPS). LPS binding to CD14 initiates transmembrane signaling and changes in cellular function *[Ziegler-Heitbrock HWL, 1993]*.

Monocytes/macrophages are important as antigen-presenting cells. The antigens are processed by the monocyte/macrophage and are then presented via either class I or class II MHC molecules to the T-cells.

1.3 Adaptive immune response

1.3.1 T-lymphocytes

The T-lymphocytes, or T-cells, are responsible for cell-mediated immune responses. The antigen recognition molecules on T-cells are the T-cell receptor (TCR) consisting of either a pair of α:β chains or a pair of γ:δ chains.
Cytotoxic T-cells, expressing CD8, act by direct killing of the target cell. CD8 T-cells recognize peptide fragments originating in the cytosol (e.g. those generated from an intracellular infection) displayed with MHC class I molecules on the cell surface.
CD4 expressing T-cells are specialized to activate macrophages and B-cells. CD4 T-cells recognize exogenous antigens internalized by phagocytosis or endocytosis and presented together with MHC class II on the surface of the antigen presenting cells.

1.3.2 B-lymphocytes and immunoglobulins

The activation of B-cells and their differentiation into antibody-secreting cells is triggered by antigen and requires stimulation from CD4 T-cells. The antigen recognition molecules of B-cells are immunoglobulin and are made both as a membrane-bound receptor for antigen and as secreted antibodies that bind antigen. The antigen binding region varies extensively between antibody molecules and is called the variable region. The region of the antibody molecule that engages effector functions in the immune system is called the constant region, *Fig. 2*. This constant region, Fc portion of the antibody, bind to Fc-receptors on phagocytes, NK-cells and eosinophils. There are five major classes of immunoglobulins with different functional activity, IgM, IgD, IgG, IgA and IgE. IgG is by far the most abundant immunoglobulin and has four subclasses (IgG1, IgG2, IgG3 and IgG4), which also have different characteristics, *Table 1*.

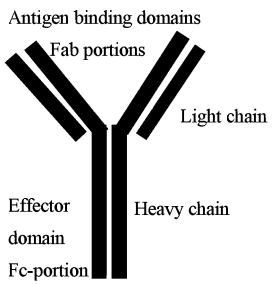


Fig. 2 Structure of immunoglobulin

Functional activity	IgG1	IgG2	IgG3	IgG4
Concentration mg/mL (mean)	9	3	1	0.5
Neutralization	++	++	++	++
Opsonization	+++	(+)	++	+
Activates complement system	++	+	+++	-

Table 1 Characteristics of IgG subclasses.

1.4 Cytokines, acute phase proteins and complement

Cytokines are small proteins, around 25 kDa, that are released by various cells. They can act in an autocrine manner, affecting the behavior of the cell that releases the cytokine, in a paracrine manner, affecting the behavior of the adjacent cell, or in an endocrine manner, affecting the behavior of more remote cells. Chemokines constitute a class of cytokines that have chemoattractant properties, inducing cells with appropriate receptors to migrate to the source of the chemokine. The cytokines secreted by monocytes/macrophages include interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-12 (IL-12), tumor necrosis factor-α (TNF- α) and the chemokine interleukin-8 (IL-8). These cytokines are secreted early in immune response and are often referred to the proinflammatory cytokines. Biological activities of TNFα are mediated by two distinct TNF receptors, type 1 (TNFR1) and type 2 (TNFR2). These receptors, expressed on target cells and soluble (sTNFR) in plasma, regulate the effect of TNFα .

The cytokines produced by T-lymphocytes are usually classified in Th1- and Th2-response. Th1 cells typically secrete interferon-γ (INF-γ), TNF-α and interleukin-2 (IL-2) which activate macrophages and induce a cellular response whereas Th2-cells secrete interleukin-4 (IL-4), interleukin-5 (IL-5) which activates B-cells and interleukin-10 (IL-10) which inhibits macrophage activation. Transforming growth factor-β (TGF-β) secreted by T-lymphocytes is a suppressor of Th1-response.

The proinflammatory cytokines, TNF- α, IL-1 and IL-6, induce an acute-phase response, which implies production of acute-phase proteins. The functions of these proteins are to opsonize pathogens and activate complement. One of these acute-phase proteins is CRP that increases rapidly in early immune response.

Complement can be activated through any of the three pathways, the classical pathway, the mannan-binding lectin pathway or the alternative pathway. The classical pathway is activated by binding of C1q, either directly to a pathogen surface or to antibody:antigen complexes. The mannan-binding lectin pathway and the alternative pathway are activated through binding to bacteria or viruses and all three pathways lead to the generation of C3 convertase, followed by a cascade of proteolytic reactions. One of the major functions of the cleavage products is opsonization of pathogens and thereby stimulating phagocytosis. The complement fragments C3b and iC3b bind to complement receptors CR1 (CD35) and CR3 (CD11b/CD18) expressed on granulocytes and monocytes. The terminal complement components interact to form a membrane-attack-complex, which creates a pore in the cell membrane and leads to cell death. The proteolytic derivatives e.g. C5a, are mediators of inflammation with several different activities, such as increasing vascular permeability, inducing the expression of adhesion molecules and acting as chemoattractants for neutrophils and monocytes.

1.5 Autoimmune inflammation

1.5.1 General aspects of autoimmunity

Autoimmune disease is caused by an adaptive immune response to self antigens and can be mediated by autoantibodies and/or by autoreactive T-cells. The normal consequence of an adaptive immune response against a foreign antigen is the clearance of the antigen from the body, but when an adaptive immune response develops against self antigens, it is usually impossible for immune effector mechanisms to eliminate the antigen completely and the consequence is a sustained response. Tissue damage can result from direct attack on the cells bearing the antigen, from immune-complex

formation, from local inflammation or from a combination of these. It is not known which triggers are responsible for autoimmunity but both environmental and genetic factors, especially major histocompatibility complex (MHC) genotype, are clearly important. An association to MHC genotype has been shown for e.g. ankylosing spondylitis, type I insulin-dependent diabetes mellitus and Goodpasture´s syndrome *[Arnett FC, 1997, Baisch JM, 1990, Rees AJ, 1978]*. Some diseases show a significant bias in sex ratio, e.g. systemic lupus erythematosus with a female dominance and ankylosing spondylitis with a male dominance, indicating that sex hormones are involved in the pathogenesis *[Ansar Ahmed S, 1985]*. There is evidence that some autoimmune disorders are triggered by infectious agents *[von Herrath MG, 1996]*. There is, however, also evidence that many autoimmune disorders occur through internal dysregulation of the immune system without the participation of infectious agents. Autoimmune disorders are usually classified as organ-specific autoimmune diseases, in which autoimmunity is restricted to one specific organ, or systemic autoimmune diseases, in which many tissues of the body are affected.

1.5.2 Self tolerance

The diverse repertoire of B-cell receptors and T-cell receptors is generated during the development of B-cells and T-cells, respectively, from their uncommitted precursors. Immunological tolerance to self antigens is established through negative and positive selection. Lymphocytes whose receptors interact weakly with self antigens, or bind self antigens in a particular way receive a signal that enables them to survive and differentiate, i.e. positive selection, while lymphocytes whose receptors have no avidity to self antigen or those whose receptors bind strongly to self antigens receive signals that lead to apoptosis, i.e. negative selection *[Ohashi PS, 1996, Goodnow CC, 1996]*. The activation of lymphocytes requires interaction with cells expressing both the appropriate peptide:MHC complex and co-stimulatory molecules. In the absence of full co-stimulation the antigen recognition leads to anergy or deletion. Anergic T-cells could serve to prevent activation of autoreactive T-cells, by competively binding to antigens without responding, thus contributing to tolerance.
One group of autoantigens is those that do not trigger clonal deletion in the thymus either because they are not abundant enough or because they are tissue-specific and not expressed in the thymus. Autoimmunity to these antigens causes organ-specific autoimmune diseases. There is evidence that infection can trigger autoimmune diseases in genetically susceptible individuals. One possible mechanism is that infectious agents induce co-stimulatory activity on antigen-presenting cells expressing low levels of autoantigen, thus activating autoreactive T-cells. Another proposed mechanism is molecular mimicry, in which antibodies or T-cells generated in response to an infectious agent, cross-react with self-antigens *[Oldstone BA, 1987]*.

1.5.3 Cytokines and autoimmunity

Release of inflammatory cytokines probably plays a pivotal role in the pathogenic activation of autoreactive cells. A predominant Th1 cytokine response is implicated in certain autoimmune diseases such as rheumatoid arthritis *[Liblau RS, 1995]*, whereas a bias towards Th2 type is likely to be involved in certain systemic autoimmune diseases characterized by excessive autoantibody production, such as systemic lupus erythematosus *[Tokano YS, 1999]*. A major effort has been made to determine which cytokines and inflammatory mediators are produced at the site of disease. In Wegener´s

granulomatosis (WG) both a Th2 dominance with increased expression of IL-4 in nasal mucosa *[Balding CEJ, 2001]* and a Th1 dominance with increased expression of INF-γ in T-cells have been suggested *[Csernok E, 1999]*. The understanding of the biologic mechanisms governing cytokine action in autoimmune diseases may be of importance in therapeutic approach.

1.6 Functional parameters of inflammatory cells

1.6.1 Adhesion molecules

Recruitment of phagocytes to the site of infection is mediated by cell-adhesion molecules expressed on the leukocytes and on the blood vessel endothelium, *Fig. 3*. Three families of adhesion molecules are important for leukocyte recruitment. The selectins are membrane glycoproteins with a distal lectinlike domain that binds specific carbohydrate groups. L-selectin expressed on leukocytes is down-regulated upon activation, and the leukocytes decrease their velocity and start rolling and tethering along the endothelium *[Spertini O, 1991]*. Leukocyte activation is induced by leukotriene B4, complement fragment C5a, bacterial peptides such as LPS and cytokines. The next step requires tighter adhesion, which is achieved through integrins, heterodimeric proteins on leukocytes, consisting of an α- and a β-chain, binding to intercellular adhesion molecules (ICAMs) on the endothelium. One important integrin expressed on granulocytes and monocytes is complement receptor 3 (CR3) (CD11b/CD18), which also functions as a complement receptor. CD11b/CD18 stored in secondary granules is up-regulated upon activation and binds to ICAM-1, up-regulated on activated endothelium *[Gahmberg CG, 1997]*. Increased expression and a conformational change with increased adhesive properties in CD11b/CD18 are induced by a variety of cytokines, such as TNFα, and chemokines, such as IL-8. ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) belong to the immunoglobulin superfamily. When endothelial cells become activated ICAM-1 and VCAM-1 are released and may be detected in soluble form, sICAM-1 and sVCAM-1 *[Leeuwenberg J, 1992]*. Elevated levels of soluble adhesion molecules have been detected in disorders where leukocyte-endothelial cell interactions play a significant role, such as infections, chronic inflammatory diseases and vasculitis *[Janssen RA, 1994, Ara J, 2001]*.
In the next phase the leukocytes extravasate, and cross the endothelial wall. This step also involves CD11b/CD18 as well as further adhesive interaction involving other immunoglobulin-related molecules and proteolytic enzymes which break down proteins of the basement membrane. These interactions enable the phagocyte to squeeze between the endothelial cells. The final step in extravasation is the migration of leukocytes through the tissues under the influence of chemokines. IL-8 is released by macrophages that first encounter pathogens and it recruits neutrophils in large numbers in the early part of the induced response. Their influx usually peaks within the first six hours of an inflammatory response, while monocytes can be recruited later through the action of chemokines such as monocyte chemoattractant protein-1 (MCP-1) *[Baggiolini M, 1998]*.

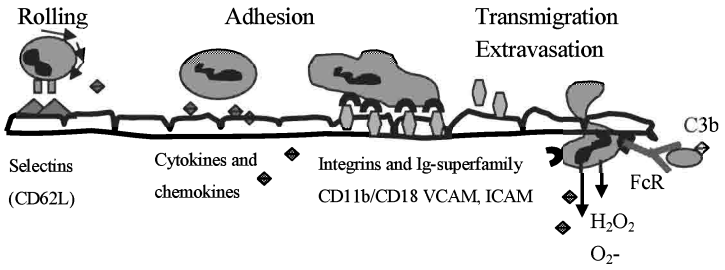


Fig. 3 Leukocyte adhesion, migration and effector mechanisms.

1.6.2 Phagocytosis

At the inflammatory site neutrophils and monocytes/macrophages are able to eliminate many pathogens by phagocytosis. Monocytes/macrophages and neutrophils recognize pathogens by means of cell surface receptors such as complement receptors, Fc-receptors and CD14, *Fig. 3*. Phagocytosis is an active process, in which the bound antigen is first surrounded by the phagocyte membrane and then internalized in a membrane bounded vesicle known as phagosome, which becomes acidified. In addition to being phagocytic, the inflammatory cells have granules that contain enzymes, proteins and peptides that can mediate an intracellular antimicrobial response. Complement enhances phagocytosis by opsonizing targets and the bound complement components are recognized by complement receptors on phagocytes. Phagocytes can interact with IgG antibodies, especially IgG1 and IgG3 that bind to specific Fcγ receptors on the phagocyte surface. Aggregated IgG, bound to an antigen, binds to Fcγ receptors with higher avidity than immunoglobulin monomers. FcγRI (CD64) and FcγRII (CD32) bind preferentially aggregated to IgG, whereas FcγRIII (CD16) binds monomeric IgG.

1.6.3 Production of oxygen metabolites

Upon phagocytosis, monocytes/macrophages and neutrophils produce a variety of toxic oxygen metabolites that help kill the engulfed microorganism. This process is known as the respiratory burst, *Fig. 4*. There is an increase in oxygen consumption and by activation of NADPH oxidase the superoxide anion (O_2-) and hydrogen peroxide (H_2O_2) are produced. Myeloperoxidase present in high concentration catalyzes the reaction from H_2O_2 to other potent oxidizing radicals such as oxidized halogens.

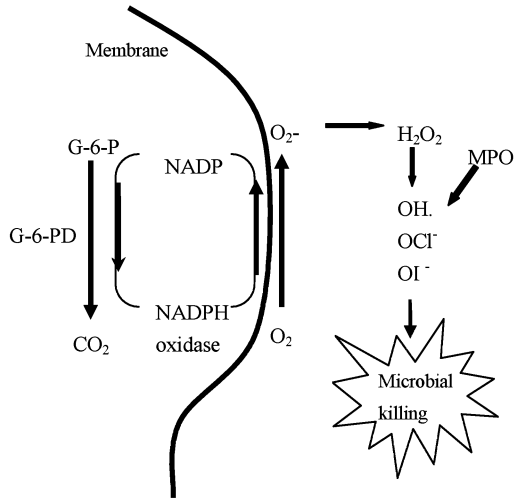


Fig. 4 Respiratory burst

1.7 Effect of storage on granulocyte functional parameters

1.7.1 Clinical background

The clinical usefulness of granulocyte transfusions remains controversial, but with indications limited to selected cases with severe persistent neutropenia and antibiotic-resistant infection, and with higher numbers of granulocytes in each granulocyte concentrate, there is increasing evidence of the efficacy *[Strauss RG, 1995, Hubel K 2001]*. These cases are most often patients who have undergone high-dose chemotherapy and hematopoietic stem cell or bone marrow transplantation *[Hubel K, 2002]*. It is important that the granulocyte concentrate quality is optimal regarding adequate numbers of granulocytes, normally functioning granulocytes, compatibility with recipient, storage condition, and that it is unable to transmit disease and will not be toxic on administration *[Price TH, 1995]*. Granulocyte concentrates are prepared from one donor by continous-flow centrifugation leukapheresis. The minimum recommended transfusion dose is 10^{10} granulocytes *[Council of Europe, 2002]*. There have been problems collecting sufficient numbers of granulocytes from a single donor. The administration of granulocyte colony-stimulating factor (G-CSF) to healthy donors has now enhanced the leukapheresis yields 3-5 times *[Joos K, 2002, Hubel K 2000]*. Adverse effects of granulocyte transfusions are alloimmunization to HLA-antigens, transmission of latent viruses (cytomegalovirus, Epstein-Barr virus, etc.) and other pathogens, non-hemolytic transfusion reactions and transfusion-related acute lung injury (TRALI) *[Council of Europe, 2002, Herzig RH, 1989]*.

1.7.2 Granulocyte concentrates

The storage of granulocytes results in progressive functional impairment and cell loss *[Glasser L, 1983, Lane TA, 1979]*, and it is therefore recommended that granulocytes be transfused as soon as possible after collection. If unavoidable, storage should be limited to 24 hours at +20°C to +24°C *[Council of Europe, 2001]*. Immediately after collection granulocyte functions are reported to be normal or near normal with regard to posttransfusion recovery and to blood disappearance time *[McCullogh J, 1976, Price*

TH, 1978]. The functions that are most rapidly and severely affected during storage are reported to be migration and chemotaxis, which are abnormal after 24 hours of storage *[Lane TA, 1981, 1988]*. *In vitro* tests of adherence and chemotaxis generally employ two techniques, the Boyden chamber filter-migration technique *[Boyden SV, 1962]* and agarose gel technique *[Nelson RD, 1988]*. Granulocyte microbicidal activity can be measured by the production of oxygen radicals by chemiluminiescence assay or by flow cytometry, and have been reported to be normal for at least 24 hours of storage *[Glasser L, 1983]*. Phagocytosis may be measured by a variety of techniques, visual analysis of particle uptake, spectrophotometric, isotopic and flow cytometric techniques *[Hampton MB, 1999, van Eeden SF, 1999]* and are unaffected up to 48 hours *[Lane TA, 1990]*. These data indicate that the early granulocyte functions that are affected during storage are the capacity to adhere to endothelium and chemotaxis, which is a prerequisite for the granulocyte migration to sites of infection.

1.8 Granulocyte antibodies

1.8.1 Clinical background

Antibodies to granulocyte antigens can cause neonatal alloimmune neutropenia, autoimmune neutropenia, febrile transfusion reactions and TRALI. Neonatal alloimmune neutropenia is a maternal alloimmunization against fetal granulocytes and the incidence is reported to be 2/1000 *[Madyathsa PR, 1989, Levine DH, 1982]*. Autoimmune neutropenia includes primary autoimmune neutropenia which occurs predominantly in infancy with a prevalence of 1/100000 *[Lyall EGH, 1992]*, and secondary neutropenia associated with other diseases such as systemic lupus erythematosus. Primary autoimmune neutropenia is associated with minor infections and destruction of neutrophils is caused by auto-reactive neutrophil antibodies. Spontaneous remission is shown in 95% of the patients within 2 years *[Bux J, 1998]*. Granulocyte antibodies not only cause neutropenia but can also activate neutrophils, affect phagocytosis and inhibit neutrophil adhesion to endothelial cells *[Bux J, 1993, Shastri KA, 1993]*. Febrile transfusion reactions are caused by antibodies (HLA- or granulocyte specific-) in the recipient against leukocytes in the blood component. TRALI is a rare and probably underdiagnosed complication of transfusion. Leukocyte antibodies in the blood component react with the granulocytes in the recipient and cause an acute, sometimes fatal, pulmonary reaction characterized by dyspnea, hypoxia and infiltrates on pulmonary x-ray *[Popovsky MA, 2000]*.

Antigens found on granulocytes can either be granulocyte-specific antigens whose tissue distribution is restricted to granulocytes, or granulocyte antigens which can be detected not only on granulocytes but also on other cell lines. In 1998 a new nomenclature of the granulocyte-specific antigens was introduced: human neutrophil alloantigen (HNA) based on the antigen´s glycoprotein location, *Table 2 [Bux J, 2001]*. The clinically most important granulocyte antibodies, in neonatal alloimmune neutropenia as well as in autoimmune neutropenia and TRALI, are directed against the NA1 and NA2 antigens (HNA1a/b), localized on FcRγIII (CD16). CD16 is stored in secretory vesicles in granulocytes, expressed on the cell surface and released in a soluble form upon activation *[Moldovan I, 1999]*. The second most clinically relevant granulocyte-specific antigen is the NB1 glycoprotein which is now called HNA-2. Anti-NB1 and anti-Mart antibodies are reported to give a cytoplasmic immunofluorescence pattern in indirect immunofluorescence (IIF)-ANCA testing, and can cause false positive ANCA results *[Stroncek DF, 1993]*. Antibodies against

antigens co-expressed on granulocytes as well as other leukocyte populations are most often anti-HLA class I antibodies.

Antigen system	Polymorphism	Old terminology	Location	Antigen frequency in Europeans (%)
HNA-1	HNA-1a	NA1	FcγRIIIb (CD16)	54-58
	HNA-1b	NA2	FcγRIIIb (CD16)	87-88
	HNA-1c	SH	FcγRIIIb (CD16)	5-7
HNA-2	HNA-2a	NB1	GP50	87-94
HNA-3	HNA-3a	5b	GP70-95	99
HNA-4	HNA-4a	MART	CD11b	96
HNA-5	HNA-5a	OND	CD11a	96

Table 2 Nomenclature and localization of granulocyte antigens.

1.8.2 Detection and characterization

No single technique is available that detects all clinically relevant granulocyte antibodies. Granulocytes are short-lived cells and easy to activate, and thus, working with granulocytes requires technical skill and experience. Granulocytes can not be stored and must therefore be freshly isolated for testing. A combination of the granulocyte immunofluorescence test (GIFT) and the granulocyte agglutination test (GAT) are most effective in granulocyte antibody detection *[Lucas GF, 1990, Bux J, 1997]*. GIFT is considered to be the most sensitive method, but on the other hand some autoantibodies and the anti-5a, -5b, -9a and -NB2 are more readily detectable using GAT. The immunofluorescence pattern is staining of the cell membrane, *Fig. 5*. Immune complexes and aggregates in patient´s sera can cause false-positive results in GIFT.

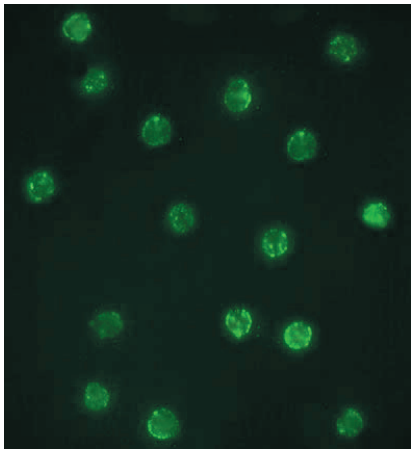


Fig.5 Immunofluorescence pattern, anti-NA2.

For antibody identification an antigen capture assay based on monoclonal antibody-specific immobilization of granulocyte antigens (MAIGA) *[Bux J 1993]* or immunoblot and immunoprecipitation assays can be used. Antigen typing of granulocytes has for a long time been restricted by the availability of human sera. Most of the available sera

are derived from alloimmunized mothers who gave birth to neonates with alloimmune neonatal neutropenia. However human sera often contain additional HLA antibodies that can cause mistypings. Now monoclonal antibodies against the antigens NA1, NA2 and NB1 are available. Phenotyping by GIFT requires large number of granulocytes and many of these patients have very low counts of neutrophils. With the recognition of the molecular basis of granulocyte antigens, genotyping by DNA-techniques has become feasible *[Bux J, 2001]*.

1.9 Anti neutrophil cytoplasmic antibodies (ANCA)

1.9.1 Detection and characterization

In 1985 autoantibodies were detected in the sera of patients with Wegener´s granulomatosis (WG) that reacted with cytoplasmic constituents of neutrophils *[van der Woude, 1985]*. The target antigen of these cytoplasmic anti-neutrophil cytoplasmic antibodies (C-ANCA) proved to be proteinase-3 (PR3), a serine protease stored in azurophilic granules of granulocytes and monocytes. PR3 has several physiological, and possibly pathophysiological, functions. It plays a role in growth and differentiation of granulocytes and monocytes, microbicidal activity, degradation of tissue and modulation of inflammatory mediators *[van der Geld YM, 2001]*. Anti-PR3 antibodies produce a diffuse staining of the cytoplasm by indirect immunofluorescence on ethanol-fixed neutrophils, *Fig. 6a*. Later it became apparent that some sera produced a perinuclear fluorescence pattern (P-ANCA) clearly different from the C-ANCA pattern, *Fig. 6b*. P-ANCA are usually directed against myeloperoxidase (MPO) *[Falk RJ, 1988]*, an enzyme critical for oxidative killing *[Hampton MB, 1998]*. The P-ANCA fluorescence pattern represents an artefact of ethanol fixation. Positively charged granule constituents rearrange around and upon the negatively charged nuclear membrane. Anti-MPO antibodies are associated with crescentic glomerulonephritis (GN) and microscopic polyangitiis (MPA).

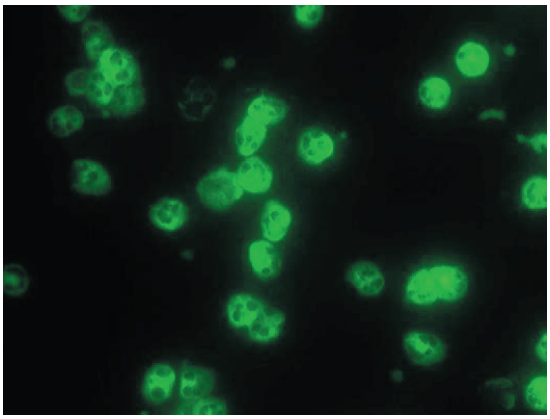


Fig. 6a Indirect immunofluorescence, C-ANCA.

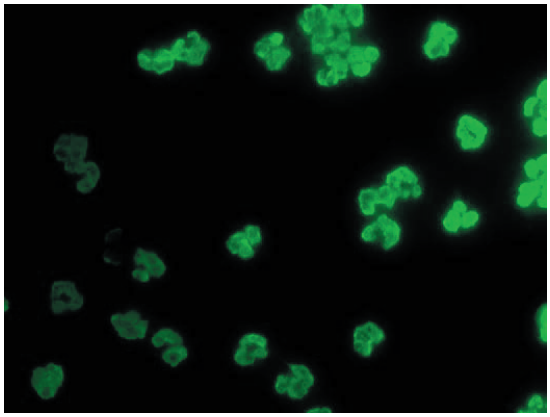


Fig. 6b Indirect immunofluorescence, P-ANCA.

ANCA detected by indirect immunofluorescence are usually confirmed by enzyme-linked immunoassays (ELISA) coated with the specific antigen. The identification of anti-MPO antibodies is particularly important since P-ANCA, directed against elastase, cathepsin G, lactoferrin or lysozyme may occur in a variety of non-vasculitic disorders. Testing for ANCA using both indirect immunofluorescence and antigen-specific ELISA provides high sensitivity and specificity *[Savige J, 2000]*. ELISA is a direct quantitative technique. The theory that increasing ANCA-levels predict disease relapse is controversial. ANCA has been reported to closely correlate to disease activity and treatment initiated based on increased ANCA-levels has been reported to prevent relapses *[Cohen-Tervaert JW, 1990]*. However, others have not found such a relation *[Nowack R, 2001]*. Since ANCA interacts with Fc-receptors and different subclasses have various activating capacity, the subclass distribution of ANCA has been investigated. The ANCA seem to consist mainly of IgG1 and IgG4 *[Segelmark M, 1993]* with a relative increase of the IgG3 subclass in active disease *[Mulder AH, 1995]*. However, a direct relation between IgG3 levels and clinical disease has not been confirmed. ANCA do not occur incidentially in the normal older population, unlike anti nuclear antibodies (ANA) and rheumatoid factor *[Maillerfert JF, 1997]*

1.9.2 Systemic vasculitis

The pathology of vasculitis involves inflammation of blood-vessel walls. The vasculitis syndromes are usually classified according to the site, type and size of vessels involved *[Jennette JG, 1994]*, and the diagnosis is based on clinical, histological and serological data. Inflammation in the large vessels includes giant cell arteritis and Takayasu´s disease and is not associated with ANCA whereas inflammation in the small vessels in syndromes such as WG, microscopic polyangiitis (MPA), idiopathic (pauci-immune) necrotizing glomerulonephritis and Churg-Strauss syndrome, are strongly associated with PR3-ANCA and MPO-ANCA. The etiology of the diseases is mostly unknown. In some forms of medium vessel vasculitis such as polyarteritis nodosa and Kawasaki´s disease infective agents have been identified as the cause or suspected cause of the disease *[Lie JT, 1996]*. Also in the small vessel syndromes associated with ANCA there have been reports of an infection preceding the onset and relaps of vasculitis *[Stegeman CA, 1994]* but convincing correlations to a specific infectious agent have not been demonstrated. Inherited determinants can increase a person´s risk of

developing WG. Defiency of α1-antitrypsin, the main inhibitor of PR3, is overrepresented in PR3-ANCA positive patients *[Esnault VLM, 1993]*. A polymorphism in FcγRIIIb, has been suggested to be critical in binding of ANCA and to have implications for disease susceptibility *[Tse WY, 2000]*. More than 95 percent of the patients with active WG are ANCA-positive and 90 percent have antibodies specific for PR3. About half of the patients with WG are aged over 60 years.
The usual clinical presentation of a patient with Wegener´s is an upper respiratory tract disease including rhinorrhea, sinusitis or otitis media. More than 70 percent of the patients have pulmonary infiltrates and other affected sites can include eyes, skin, joints, kidneys and nervous system *[Hubitz M, 1998]*. Often it is a long time before the diagnosis is established. The histopathology shows granuloma formation *[Bajema IM, 1996]*. The treatment in WG is initially a combination of prednisone 1-2 mg/kg daily and cyclophosphamide 1-2 mg/kg daily, and then as maintenance therapy, a combination of prednisone and azathioprine or cyclophosphamide *[Jayne DRW, 1997]*.The long-term outcome for patients with WG has improved dramatically with this treatment. Untreated patients have a 90 percent mortality rate within two years in comparison to 20-28 percent five-year mortality in cyclophophamide-treated patients *[Matteson EL, 1996]*. The major causes of death are complications from underlying disease and complications to therapy. Seventy-five percent of the patients reach complete remission within 1-2 years; however 30-50 percent will have at least one relaps *[Hoffman GS, 1992]*.
The clinical symptom of MPA and necrotizing GN have similarities with those of WG, the histopathology shows focal fibrinoid necrosis of the capillaries and venules, but does not include granuloma formation, and ear-nose-throat organs are generally spared. The characteristic glomerular lesion is a focal segmental necrotizing glomerulonephritis usually with crescents. Uninvolved glomerular segments are often almost normal *[Bajema IM, 1996]*. The treatment recommendations in MPA and necrotizing GN are the same as for WG. The five-year survival is approximately 80 percent in MPA *[Gayraud M, 2001]*. The response to treatment can be measured using disease activity score, Birmingham vasculitis activity score (BVAS) *[Luqmani RA, 1994]*.
Churg-Strauss syndrome is rare. It is associated with asthma, peripheral blood eosinophilia and a positive ANCA and the treatment regimen is the same as that for WG and MPA.

1.9.3 Pathophysiologic role of ANCA

ANCA as a diagnostic and prognostic marker for systemic vasculitis is well established, and there is also evidence supporting a pathogenic role. *In vitro* studies have demonstrated that ANCA have the capacity to activate cytokine-primed neutrophils to degranulate and to producte oxygen free radicals *[Falk RJ, 1990, Charles LA, 1991]*. The hypothesis is that cytokines, e.g. TNFα, induce expression of PR3 and MPO on the surface of neutrophils which make them available for interaction with ANCA. How these antigens are expressed on the cell surface is unclear. One explanation may be that the cationic proteins PR3 and MPO bind to the negatively charged cell membrane via charge interactions. Recently, other mechanisms for binding of ANCA antigens to the cell membrane have been suggested. Binding of MPO may be mediated by β2 integrin, especially the CD11b subunit *[Johansson MW, 1997]* whereas binding of lactoferrin which shares 54% aminoacid sequence homology with PR3, appears to be mediated by β2 integrin CD11a unit *[Zimmerman F, 1998]*. ANCA

antigen has been demonstrated on the surface of apoptotic granulocytes, as a result of cytoplasmic granular translocation during apoptosis *[Gilligan HM, 1996]*. Since the onset of the diseases often is preceded by an infection it has been hypothesized that proinflammatory cytokines such as TNFα and IL-1 released *in vivo*, prime neutrophils to express PR3 and MPO on their cell surface. Upon binding of ANCA to these antigens, an activating signal has to be transduced into the cell. Possible candidates for signal transduction are β2 integrins and Fcγ receptors. Although some studies have demonstrated that incubation of primed neutrophils with IgG F(ab)2 ANCA results in neutrophil activation *[Falk RJ, 1990, Kettritz R, 1997]* other studies suggest that both antigen binding and Fc receptor interactions are involved *[Mulder AH, 1994, Porges AJ, 1994, Reumaux D 1995]*. In these *in vitro* experiments ANCA-induced neutrophil activation did not occur when F(ab)2 fragments of ANCA IgG were used, and was strongly inhibited when blocking monoclonal antibodies against FcγRII were used. Monocytes/macrophages play a pivotal role in lesion development in vasculitis. An important histopathological feature in patients with WG is granulomatous inflammation which consists of accumulated activated monocytes/macrophages. In addition the proliferative lesions and crescents in necrotizing glomerulonephritis are characterized by the accumulation of activated monocytes *[Ferrario F, 1999]*. Upon *in vitro* ANCA stimulation monocytes can be triggered to produce oxygen radicals *[Weidner S, 2001]* and chemokines *[Ralston DR, 1997, Casselman BL, 1995]*. In these studies increased production of reactive oxygen species was measured after ANCA IgG stimulation for 30 min up to 240 min. Increased production of IL-8 after ANCA IgG and monoclonal anti-PR3 stimulation could be seen for 5 hours to 25 hours and increased production of MCP-1 was observed with a maximum after 24 hours of incubation. Furthermore, after ANCA stimulation for 18 hours moncytes up-regulated CD14 and CD 18 expression *[Nowack R, 2000]*. In patients with WG, PR3 expression on circulating neutrophils is increased compared to PR3 expression on neutrophils from healthy controls, and correlates with disease activity *[Muller-Kobold A, 1998]*. The expression of PR3 and MPO on circulating monocytes have not been shown to be increased in WG, compared to in healthy controls. In WG circulating neutrophils are activated in terms of down-regulated CD62L *[Riecken B, 1994]* and up-regulated CD11b *[Haller H, 1996]*. In systemic vasculitis the endothelial cells are activated with increased expression of adhesion molecules *[Cohen Tervaert JW, 1997, Dupuy E, 1998]* and increased levels of soluble adhesion molecules during active disease *[Stegeman CA, 1994, Mayet WJ, 1996]*.

As in many other autoimmune diseases, it is still unknown what initiates the process in ANCA-positive vasculitis. Based on experimental and clinical data cited above the following hypothesis has been generated, *Fig. 7 [Muller Kobold AC, 1999]*. An infection often preceds the onset of the disease and as a result proinflammatory cytokines such as TNFα and IL-1 are released which prime neutrophils and monocytes. These circulating leukocytes will then express the target antigens for ANCA, PR3 and MPO. ANCA can bind to the leukocytes which become activated and bind to the endothelium via the adhesion receptors. A sequential and integrated regulation of CD62L and CD11b is a key step in the recruitment of monocytes and neutrophils into the extravascular space, and a dysregulation may result in cell entrapment in the endothelial cell lining. Upon stimulation by ANCA these activated neutrophils and monocytes release toxic products such as oxygen radicals and lytic enzymes, leading to endothelial cell injury. Circulating neutrophils and monocytes are attracted and

accumulate at the inflammatory site. These cells release chemoattractants and cytokines such as MCP-1 and IL-8, attracting other inflammatory cells. The inflammatory reaction is intensified.

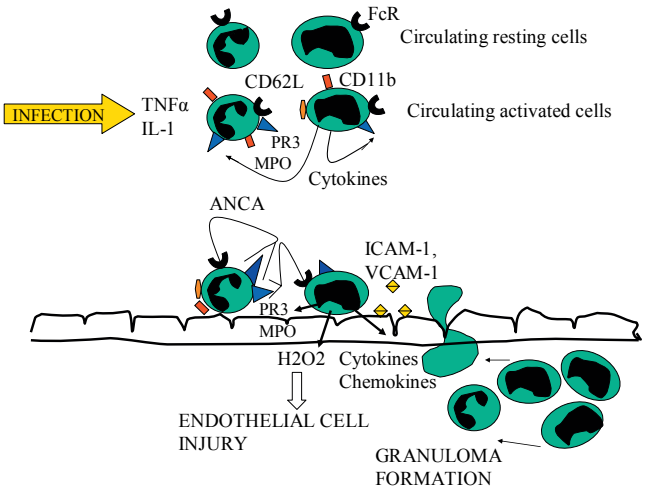


Fig. 7 Hypothesis: Pathophysiologic mechanisms in ANCA associated vasculitis. The figure is modified from Muller-Kobold AC, 1999.

2 AIMS OF THE STUDY

The objective of this thesis was to analyse the functional responses of inflammatory cells, granulocytes and monocytes, to activation, with special attention paid to the relevance of granulocyte antibodies.

The specific aims were:

I. To analyse the effect of storage for different time periods on granulocyte receptors associated with adhesion to endothelium (CD62L, CD11b), binding of complement and immunoglobulin (CD35, CD16) and on the capacity to produce reactive oxygen metabolites.

II. To apply a flow cytometric technique to detect granulocyte antibodies, with special attention on the distinction between antibodies directed against surface and intracellular-stored antigens.

III. To study the initial events in the interaction between ANCA and monocytes *in vitro* with special attention paid to adhesion molecule expression and the production of oxygen metabolites.

IV. To test the hypothesis that the nature and magnitude of *in vivo* monocyte activation differs between ANCA-positive and ANCA-negative acute inflammation, in terms of the expression of adhesion molecules, the production of oxygen metabolites as well as generation of cytokines and soluble adhesion molecules and CD14.

3 MATERIALS AND METHODS

3.1 Subjects and study design [I-IV]

3.1.1 Preparation and storage of granulocyte concentrates [I]

Single-donor granulocyte concentrates were prepared by apheresis technique from voluntary blood donors. Five granulocyte concentrates were collected, two on cell separator V50 (Haemonatics Corporation Braintree, MA) and three on CS 3000 (Baxter R&D, Deerfield IL). Two donors were used twice, once on each cell separator. The donors were given prednisone prior to donation (20 mg 12 hours before the procedure and 20 mg 2 hours before). The cell separator manufacturer´s methods for granulocyte preparation were used. Hydroxyethylstarch (6%; Plasmasteril, Fresenius AG, Bad Homburg Germany) was used as a sedimenting agent. The granulocyte concentrates were stored in storage containers (PL-146 Baxter) at room temperature without agitation. Blood was collected from the donors before the granulocyte apheresis procedure (base line values), samples were then drawn from the storage container after 1 to 2, 4, 24 and 48 hours. The study was approved by the Ethics Committee of Karolinska Institutet.

3.1.2 Detection of granulocyte antibodies in serum samples [II]

A flow cytometric method for the detection of granulocyte antibodies was evaluated. Sera from 20 healthy non-transfused male blood donors were used as negative controls. As a positive control we used a serum sample obtained from a patient immunized during pregnancy. The specificity (anti-NA2) was identified by P Metcalfe and AH Waters, Department of Haematology, St. Bartholonew´s Hospital and Medical College, London.

Patient sera were selected as follows; 20 samples, 10 with positive and 10 with negative results by GIFT, were analysed by flow cytometry. Another 10 samples tested positives by IIF with different immunofluorescence patterns were analysed against permeabilized and unpermeabilized cells by flow cytometry. The study was approved by the Ethics Committee of Karolinska Institutet.

3.2 Patient and control sera in an *in vitro* study [III]

ANCA positive sera were obtained from patients admitted to the Karolinska Hospital. The sera were either positive for anti-PR3 (n=7) or anti-MPO (n=7). All patient samples were positive in indirect immunofluorescence test with either cytoplasmic-ANCA or perinuclear-ANCA staining pattern. The specificity was further confirmed by ELISA (Wieslab AB, Ideon, Lund, Sweden). Patient characteristics at the time of sampling were documented, *Table 3*. The patient sera and the IgG-fractions from patient sera were incubated with leukocytes from healthy blood donors, and monocyte and granulocyte activation were analysed. Sera from healthy blood donors served as controls. The study was approved by the Ethics committee of Karolinska Institutet.

	Anti-PR3 (n=7)	**Anti-MPO** (n=7)
Age (years) median [range]	62 [17-80]	70 [59-79]
Antibody conc (units) median [range]	208 [113-808]	>320[100->320]
Main organ involvement	Joints 5, lungs 3, muscles 1	Kidneys 4, joints 2, lungs 1, muscles 1
Other organ involvement	[1]ENT 4, kidneys 5, skin 2, lungs 1, eyes 1	Kidneys 2, ENT 1, polyneuropathy 1
CRP mg/mL median [range]	19 [<10-362]	<10[<10-119]
Leukocyte count $x10^9$/L median [range]	8.2 [4.7-18.2]	10.1 [5.2-24.9]
S-creatinine μmol/L median [range]	83 [154-168]	257 [86-791]
Immunosuppression	steroids 2, [2]imm.supp 1, imm.supp + steroids 3	steroids 3, imm.supp + steroids 2

[1]ENT=ear nose throat, [2]imm.supp= immunosuppressive drugs

Table 3 Patient characteristics, study III.

3.2.2 Patients and control groups in a prospective clinical study [IV]

During the period November 1999-December 2001, all patients with a new positive PR3-ANCA were asked if they agreed to participate in the study. Twelve consecutive patients gave informed consent and were prospectively included in the study. Peripheral blood was collected at the time of inclusion. All patients had a diagnosis of systemic vasculitis based on clinical, serological and histological criteria. All patients had signs of kidney involvement including hematuria and proteinuria, and 10 had renal-biopsy-proven crescentic GN. The clinical characteristics and laboratory test results of the patients at the time of inclusion were documented. All samples were ANCA-positive as shown by indirect immunofluorescence on ethanol-fixed granulocytes with C-ANCA. The specificity, anti-PR3, was confirmed by an ELISA (Wieslab AB, Ideon, Lund, Sweden).

As control, peripheral blood was obtained from a group of patients prospectively included among patients acutely admitted to the Infectious Disease Clinic at Karolinska Hospital, because of acute clinical and laboratory signs of infection. The inclusion criteria for these patients were CRP>50 mg/L, fever 38,5°C and normal urine sediment. A second control group consisted of voluntary healthy blood donors. All samples in the control groups were ANCA-negative. Characteristics of the patients and the two control groups are summarized in *Table 4*.

The study was approved by the Ethics Committee of Karolinska Institutet.

Group	**Acute anti-PR3 positive vasculitis (n=12)**	**Acute infection (n=12)**	**Healthy controls (n=12)**
Age (years)	45 [13-89]	46 [16-88]	58 [22-67]
Sex M/F	5/7	3/9	6/6
CRP mg/mL	141 [10-362]	205 [92-542]	<7 [<7-40]
Leukocyte count $x10^9$ /L	13.7 [3.9-25.4]	13.5 [3.3-28]	5.1 [3.9-7.2]
Monocyte count $x10^9$ /L	0.4 [0.1-0.7]	0.4 [0.2-2.0]	ND
S-creatinine μmol/L	134 [54-862]	70 [50-111]	74 [64-112]
Anti-PR3 (units)	335 [30-636]	0[0-0]	0[0-0]

Table 4 Characteristics of patients and control groups, study IV.

3.2 Preparation of leukocytes [I-IV]

Peripheral blood from the subjects was collected into glass tubes containing citrate (Vacutainer, 5 mL, with 1 mL 0.129 M 9NC, Becton Dickinson) **[III, IV]** or into tubes containing EDTA (Vacutainer 5 mL with 50 μL of 21 % EDTA, Terumo) **[I, II]**. The blood was hemolysed in 100-150 μL portions by addition of 2-3 mL +4°C isotonic NH_4Cl-EDTA lysing solution (154 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2) and incubated for 5 min at +15 °C. After centrifugation at 300 g for 5-6 min at +4°C, the supernatant was aspirated. The leukocyte suspensions were then washed and resuspended in +4°C phosphate buffered saline (PBS).

When pools of leukocytes from three different donors were used **[II]**, 150 μl blood from each of the three donors was hemolysed in 3 ml lysing solution. After one wash the cells were resuspended in 150 μl PBS-EDTA. Fifty microliters from each of the three blood donors were pooled in one test tube, washed a second time and finally resuspended in 150 μl PBS-EDTA.

All leukocyte preparations were stored on ice until further treatment.

3.3 Preparation of IgG fractions [III]

IgG fractions were prepared from the serum samples using a protein G column (Hi Trap G affinity column, 1 mL; Pharmacia Biotech, Uppsala, Sweden), according to the manufacturer's instructions. The IgG-recovery was > 80%.

3.4 Viability testing [I]

To measure cell viability leukocytes were suspended in 200 μL buffer (RPMI-1640, Northumbria Biologicals, Crankington, UK) supplemented with carboxyfluorescein diacetate (CFDA) (10 mg/mL) (Becton Dickinson Immunocytometry System, Mountain View, CA) incubated for 15 min at 20°C and washed once in PBS. CFDA is hydrolysed by intracellular esterases and retained in cells with intact membranes, yielding green fluorescence. The degree of hydrolysed CFDA was analyzed by flow cytometry. Results were expressed as the percentage of cells with positive fluorescence.

3.5 Cell membrane permeabilization [II]

The leukocytes were treated according to a cell membrane permeabilization technique, the FOG-method *[Halldén G 1989]*. The leukocytes were suspended in 200 μl 4% (w/v) paraformaldehyde (PFA) (Sigma, St Louis, MO, USA) in PBS for 10 minutes at 22°C and then washed in 3 ml PBS-EDTA by centrifugation at 400g for 8 minutes. After fixation the leukocytes were permeabilized by an incubation in 200 μL 0.74% n-octyl-β-D-glucopyranoside (OG) (Sigma, St Louis, MO, USA) dissolved in PBS, for 6 minutes at 22°C and finally washed in 3 ml +4°C PBS-EDTA.

3.6 Lymphocytotoxicity test [II]

Lymphocytotoxic antibodies were demonstrated using the two-step complement dependent test against a panel of lymphocytes from 12 selected donors carrying the most frequently occurring class I HLA-antigens *[Staff, NIH]*. Briefly, 1 μL patient serum and 1 μL lymphocyte suspension were incubated in wells on microtiter plates for 30 min at 22°C. After an additional incubation for 60 min at 22°C with 5 μL rabbit

complement (Biotest, Germany) eosin dye was added and the wells were read in a microscope. The result was judged as positive when >20 percent of the lymphocytes were stained.

3.7 Immunostaining [I-IV]

3.7.1 Monoclonal antibodies [I-IV]

Cell surface antigens were immunostained by adding the respective fluorescent or unconjugated monoclonal antibodies (mAb) to leukocytes. The following antibodies to surface antigens were used: Fluorescein isothiocyanate (FITC)-conjugated anti-CD62L (Becton Dickinson, San Jose, CA, USA), phycoerythrin-conjugated anti-CD11b (Dako A/S, Glostrup, Denmark), FITC-conjugated anti-CD16 (Becton Dickinson, San Jose, CA, USA), FITC-conjugated monoclonal anti-CD14, clone RMO52 (Immunotech, Marseille, France), mouse monoclonal anti-CR1 (CD35) (Dako A/S, Glostrup, Denmark), mouse monoclonal anti-human PR3, clone 4A5 (Wieslab, Lund, Sweden) and mouse monoclonal anti-human MPO clone MPO-7 (Dako A/S, Glostrup, Denmark). Leukocytes stained with unconjugated mAb were secondary immunostained with FITC-conjugated rabbit anti mouse immunoglobulin, $F(ab')_2$ (Dako A/S, Glostrup, Denmark), for 30 min at +4°C and washed with PBS. Isotype matched control antibodies, phycoerythrin-conjugated IgG2, FITC-conjugated IgG1 (Becton Dickinson) and mouse IgG1 (Coulter, Hialeah) in corresponding concentrations were used as backgrounds to define the value for positive fluorescence. All cell suspensions containing mAb were incubated for 30 min at +4°C and then washed once in PBS, before flow cytometric analysis.

3.7.2 Human sera [II]

Serum samples were added to the leukocyte suspension in the test tube (100 µL to single donor leukocytes, 150 µL to pooled leukocytes and 50 µL to permeabilized leukocytes), and incubated for 30 min at room temperature. Thereafter the cells were washed three times in PBS-EDTA and 100 µL FITC-labeled rabbit anti human IgG $(Fab')_2$ class specific for IgG (Dako A/S, Glostrup, Denmark) diluted 1:20 was added and the leukocyte pellets were incubated for an additional 30 min at +4°C. Finally the leukocytes were washed once in 3 mL PBS-EDTA and resuspended in 0.5 mL PBS-EDTA before analysis. If analysis could not be performed immediately cells were resuspended in 0.5 mL 1 % PFA stored at +4°C and analyzed within 4 h.

GIFT was performed according to the method originally described by *Verheught et al, 1977* and modified as an assay on microtiter plates *[Press C, 1985]*. Briefly, leukocytes were prepared from EDTA-blood by a single step separation of mononuclear leukocytes, granulocytes and erythrocytes using a discontinuous Ficoll-Hypaque gradient (Pharmacia LKB, Biotechnology, Uppsala, Sweden). Fifty microliters of each patient or control serum was added to 50 µL of the granulocyte suspensions on microtiter plates, and incubated 45 minutes at 22°C. The microtiter plates were washed three times in PBS supplemented with 0.2 % bovine serum albumin by centrifugation at 600 g in 10 min, and then incubated with FITC-conjugated (F(ab')2) goat anti human immunoglobulins, anti-IgG or anti-IgM (Kallestad, Diagnostics, South Austin TX, USA) diluted 1:400 at +4°C. After one wash one drop of glycerine-PBS was added before the granulocyte suspensions were added on glass slides. The reactions were considered positive according to judgement in fluorescence

microscope. The positive pattern was scored from weak positive, + reaction, to strong positive, +++ reaction.

IIF of ethanol-fixed polymorphonuclear cells was used for the detection of antibodies against intracellularly stored antigens in granulocytes *[van der Woude FJ, 1985]*. Granulocytes were prepared from EDTA blood from healthy blood donors using gradient separation Percoll^{TM} (Amersham, Pharmacia, Biotech AB, Uppsala, Sweden) and diluted to a concentration of 20 x 10^5 granulocytes/ mL. Cytocentrifuge slides were prepared and after ethanol fixation, the glass slides were stored at -70°C until analysis. Thirty microliters of patient serum diluted 1:20, was added to the glass slides and incubated for 30 min in 22°C and washed. Thereafter FITC conjugated (F(ab´)2) rabbit anti human IgG (Dako A/S, Glostrup, Denmark) diluted 1:40 was added to the glasses and further incubated for 30 min at room temperature. The slides were stored in the dark at +4°C until microscopic reading. The staining pattern was judged as either cytoplasmic (C-ANCA), perinuclear (P-ANCA) or atypical.

3.8 Production of hydrogen peroxide [I, III, IV]

3.8.1 Production of hydrogen peroxide in resting cells [I, III, IV]

When dichlorofluorescein diacetate (DCFH) (Eastman Kodak Company, Rochester, NY) permeates the leukocyte membrane it is oxidized by hydrogen peroxide to highly fluorescent 2´7´-dichlorofluorescein. The amount of hydrogen peroxide produced can be quantified by measuring the fluorescence intensity by flow cytometry.
After leukocyte preparation leukocyte pellets were incubated for 15 min at 37°C in 5 μM DCFH suspended in 200 μL PBS-glucose. The tubes were stirred several times during the incubation to obtain an optimal penetration of DCFH. Thereafter 1 mL +4°C PBS-glucose was added, before analysis.

3.8.2 Production of hydrogen peroxide after incubation with ANCA [III]

After the incubation with DCFH for 15 min at 37°C, as described above, the leukocytes were incubated with TNFα diluted in PBS-glucose to a final concentration 4 ng/mL and either with 100 μL ANCA positive serum, or ANCA positive IgG-fraction diluted in 100 μL RPMI or monoclonal anti-PR3 or anti-MPO diluted in 100 μL RPMI, respectively, for 30 min at 37°C. After one wash in PBS, the cells were resuspended in PBS-glucose before analysis. ANCA negative sera, ANCA negative IgG fractions and isotype matched IgG1 (Dako A/S), respectively, were analysed in parallel as negative controls.

3.9 In vitro stimulation [I, III, IV]

3.9.1 *In vitro* TNFα priming [III]

Leukocytes were incubated for 5, 15 and 30 min at +37°C in tubes, with either serum, IgG-fraction or monoclonal antibodies in the presence of TNFα (Pepro Tech EC Ltd, London, UK) diluted in 100 μL RPMI-medium to a final concentration of 4 ng/mL. One houndred microliters of ANCA positive serum or ANCA negative AB-serum diluted 1:2 was added to the leukocytes together with 100 μL TNFα diluted in RPMI. In the experiments with IgG-fractions, 100 μL of corresponding IgG-fractions from ANCA-positive sera and AB-sera, diluted 1:2 in RPMI–medium, were incubated with leukocytes and TNFα diluted in RPMI. When monoclonal antibodies were used 10 μL monoclonal anti- MPO or 10 μL monoclonal anti-PR3 diluted 1:5 in PBS,

were added to 100 µL leukocytes suspended in RPMI-medium and TNFα diluted in RPMI. The cells were thereafter washed two times with RPMI.

3.9.2 Expression of CD11b and CD35 after fMLP stimulation *in vitro* [I, III]
The leukocytes were incubated for 15 min at 37°C in 200 µL RPMI alone or RPMI supplemented with $5x10^{-7}$ M fMLP (Sigma Chemical, St Louis, MO) to measure the degree of mobilization of the receptors CD11b/18 and CD35 to the cell surface. The cells were washed once after the stimulation and resuspended in 100 µL of PBS-EDTA before immunostaining.

3.9.3 Production of hydrogen peroxide after fMLP and PMA stimulation *in vitro* [III, IV]
Hydrogen peroxide production in monocytes after *in vitro* stimulation was determined by adding a receptor-dependent stimulus, $5x10^{-7}$M fMLP (Sigma Chemical, St Louis, MO., USA) or a receptor-independent stimulus, $2.5x10^{-7}$M phorbol 12-myristate 7-acetate (PMA) (Sigma Chemical) suspended in PBS-glucose, for 15 min at +37°C. The cells were suspended in 1 mL +4°C PBS-glucose before analysis by flow cytometry.

3.10 Analysis by flow cytometry [I-IV]
The cells were analysed in an EPICS Profile 1 **(I and II)** or EPICS XL **(II, III and IV)** flow cytometer (Coulter Inc., Hialeah, FL, USA). In the flow cytometer, cells are distinguished by their light scattering properties. Gates were set around the granulocyte, monocyte and lymphocyte clusters in a two-parameter histogram with linear amplification, *Fig. 8.* A fixed number of cells were counted, for granulocytes 5000 and for monocytes 500. The instrument was calibrated daily with standardized 10 mm fluorospheres, Flow-Check (Beckman Coulter). Flow-set (Beckman Coulter), another fluorosphere with controlled fluorescence intensity, was used to standardize the mean fluorescence intensity (MFI) before each experiment. The percentage and absolute number of positively immunostained leukocytes was determined and a quantification of antigen was obtained by measuring the MFI-units of the positive cell population.

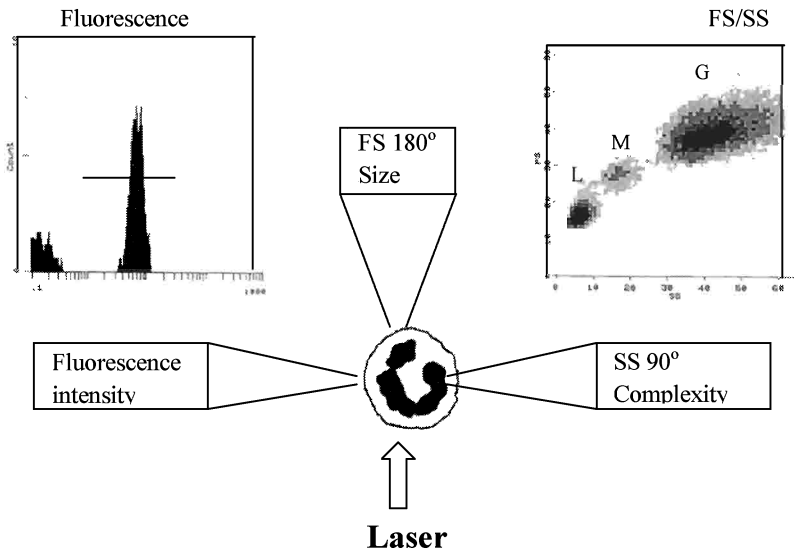


Fig. 8 The principle for flow cytometry. Leukocyte populations are distinguished by their scattering properties and are detected in a two-parameter scatter plot histogram. SS=side scatter, FS=forward scatter, L=lymphocytes, M=monocytes, G=granulocytes.

3.11 Analysis of soluble inflammation markers [IV]

The serum concentration of soluble adhesion molecules sICAM-1, sVCAM-1 and of cytokines and chemokines IL-6, IL-10, TGFβ, IL-8, MCP1, soluble TNFR1 and soluble CD14 were determined by ELISA (R&D systems, Europe, Ltd, OX, UK). The detection limit was less than 2.0 ng/mL for sVCAM, 0.35 ng/mL for sICAM, 0.70 pg/mL for IL-6, 3.9 pg/mL for IL-10, 7 pg/mL for TGFβ, 10 pg/mL for IL-8, 5 pg/mL for MCP-1, 3.0 pg/mL for sTNFR1 and 125 pg/mL for sCD14. Analyses were performed according to manufacturer´s instruction. Serum was freezed within 4 hours of collection and stored at -70°C until analysis.

3.12 Statistical analysis [I-IV]

Results were expressed as mean±SD [I] or as mean±SE [II]. We analysed differences between groups by using a non-parametric method, Mann-Whitney U test and considered them significant at $p<0.05$.

In paper III and IV data were presented as medians, interquartile range and range. Statistical comparisons were made by using the non-parametrical Kruskal-Wallis ANOVA and Mann-Whitney U-test. Differences were considered statistically significant at $p< 0.05$. Multiple linear regressions were used for correlation analysis.

4 RESULTS AND DISCUSSION

4.1 PAPER I

4.1.1 Cell recovery and cell viability

The characteristics of the five granulocyte preparations are described in *Table 5*. Cell viability measured by CFDA was >95 % in the granulocyte population both immediately after collection and after 48 hours´ storage. However after 48 hours of storage the absolute cell number was reduced by approximately 30 percent. This may be due to the adhesion of granulocytes to the surface of the plastic bag.

Granulocyte concentrate	Cell separator	Volume (mL)	WBC (x 10^8)	Granulocytes (%)
1	CS-3000	210	315	77
2	V50	548	568	68
3	CS-3000	202	226	60
4	CS-3000	213	141	54
5	V50	520	190	70

Table 5 Characteristics of granulocyte concentrates

4.1.2 Expression of granulocyte adhesion molecules, receptors associated to phagocytosis and production of hydrogen peroxide during storage

In our study we analysed the expression of receptors associated with adhesion to endothelium (CD62L and CD11b), receptors associated with phagocytosis (CD35 and CD16) and the production of hydrogen peroxide in granulocytes during storage. Neither the expression of functional receptors nor the capacity to produce hydrogen peroxide was affected by the apheresis procedure. CD11b expression gradually increased during storage while CD62L expression decreased. At 24 and 48 hours´ storage CD11b was significantly ($p<0.01$) up-regulated and CD62L was significantly ($p<0.01$) down-regulated compared to baseline values, *Fig. 9*.

The surface expression of CD35 gradually increased in a pattern similar to that of CD11b, while the expression of CD16 remained unchanged during 48 hours storage. FMLP-induced up-regulation of CD11b and CD35 was significantly decreased ($p<0.01$ and $p<0.05$, respectively) at 48 hours of storage.

The intracellular production of hydrogen peroxide in resting granulocytes gradually increased during storage and at 24 and 48 hours of storage was significantly ($p<0.01$) higher than baseline values. The production of hydrogen peroxide in response to *in vitro* PMA stimulation was unaltered after 48 hours of storage.

The observed change in adhesion phenotype during storage ($CD62L^{high}/CD11b^{low}$ to $CD62L^{low}/CD11b^{high}$) may contribute to impaired recruitment of the stored granulocytes, as their ability to roll will be reduced. The impaired recruitment is supported by studies showing that intravenous administration of IL-8 impairs the transmigration of granulocytes to an inflammatory site *[Hechtman DH, 1991]*. A plausible explanation for this finding is that if granulocytes shed CD62L in the circulation their ability to roll is abolished *[Luscinskas FW, 1991]*. Phagocytosis involves binding of the opsonin receptors to their respective ligands. We found that the

expression of CD35 increased gradually, but that CD16 expression was unaltered. Previous studies have demonstrated that phagocytic activity remains unchanged during 48 hours´ storage *[Lane TA, 1990]*. Both CD35 and CD11b are formed and stored in intracellular pools *[O`Shea JJ, 1985]*, and are mobilized upon stimulation with chemotactic factors *[Miller LJ, 1987]*. In this study we demonstrate impaired fMLP-induced up-regulation of CD11b and CD35 at 48 hours´ storage, which indicates a reduced sensitivity to chemotactic factors. This phenomenon could correspond to the decreased chemotactic activity in granulocytes during storage, reported by others *[Lane TA, 1981]*. Production of hydrogen peroxide in granulocytes in response to PMA stimulation was unaltered, despite the increased production in resting granulocytes. Previously, others have reported that the capacity for intracellular killing is unaffected by storage *[Glasser L, 1983]*.
In conclusion, the most prominent finding in our study was a marked decrease in CD62L expression in parallel with up-regulated CD11b at 24 hours´ storage. Since the regulation of these receptors is crucial in the initial phase of adhesion to endothelium, altered expression can contribute to impaired granulocyte function.

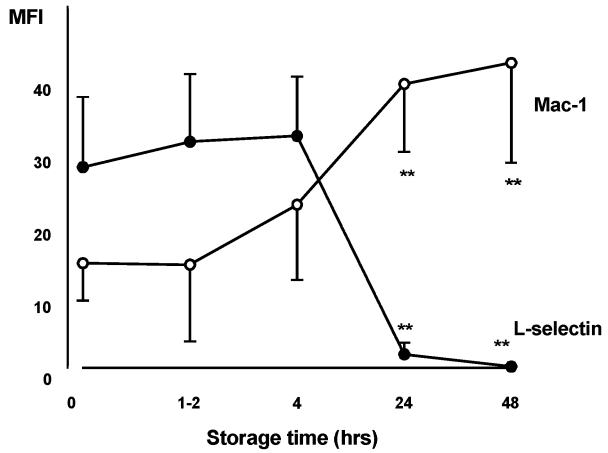


Fig.9 The expression of L-selectin (CD62L) and Mac 1(CD11b) on granulocytes at storage.

4.2 PAPER II

4.2.1 Validation of the flow cytometric method

GIFT is claimed to be the best method for the detection of granulocyte specific antibodies *[Bux J, 1997]*. Yet, the GIFT has some drawbacks; it is a time-consuming method and it is not possible to distinguish positive reactions due to granulocyte-specific antibodies from those due to HLA-specific antibodies. Recently, flow cytometric methods for the detection of granulocyte antibodies have been described *[Veys PA, 1989, Robinson JP, 1987]*. In this study a flow cytometric method for the detection of granulocyte antibodies was evaluated. To establish negative and positive cut off values, the negative control sera and the positive control serum were analyzed against granulocytes from randomly selected blood donors. These analyses yielded MFI values of 6.8±0.5 (mean±SEM) for negative controls and a MFI value of 47.7±3.0 (mean±SEM) for the positive control. In compliance with the above results a sample

was judged as positive when >90% of the granulocytes expressed >16 MFI-units, based on the mean values + 2 SD for negative sera.
The intra-assay variation (a positive sample run against cells from the same donor ten times) was 3%, while the inter-assay variability (a positive sample run against cells from ten different donors positive for respective antigen) was 33%, probably mirroring the biological variation in antigen density between individuals. For quantification, titration of five sera positive for granulocyte antibodies was performed by GIFT and by flow cytometry. The titer obtained by flow cytometry was equal or higher compared to the titer obtained by GIFT. Results obtained by flow cytometry are expressed as numerical values, giving clear positive and negative results based on cut off values, whereas if the assay is read in a microscope, the results are based on the judgement of the reader. In analysis by flow cytometry both the antigen density expressed as MFI and the percentage of positive cells are taken into consideration.

4.2.2 Analysis of granulocyte-specific and HLA-specific antibodies

Analysis of mixed leukocytes by flow cytometry allows distinction to be made between antibodies directed towards antigens expressed exclusively on granulocytes and those co-expressed on other leukocytes, e.g. HLA-antigens. The different patterns of fluorescence given by a positive serum containing exclusively anti-NA2 and a serum containing multispecific anti-HLA-antibodies are presented in *Fig. 10 and 11*.
Detection of granulocyte antibodies by flow cytometry allows the use of pooled leukocytes from different donors, *Fig. 12*. We screened a material of sera from female blood donors with one or more previous pregnancy, for leukocyte antibodies (not included in the published paper). The screening procedure included GIFT (n=419) performed towards leukocytes from 2-4 single blood donors, flow cytometry (n=68) performed towards pools of leukocytes from three donors and lymphocytotoxicity test (n=525). Positive results were found in 4.4% by flow cytometry, 3.3% by GIFT and in 2.1% by lymphocytotoxicity test. Leukocyte antibodies in transfused blood components may cause TRALI, a serious and underdiagnosed transfusion reaction *[Williamson L, 2000]*. A study from Linköping demonstrated that transfusion of plasma from multiparous donors was associated with lower oxygen saturation in intensive care patients *[Palfi M, 2001]*. Flow cytometric analysis for leukocyte antibodies can be carried out against a pool of leukocytes, which is an advantage for screening purposes.

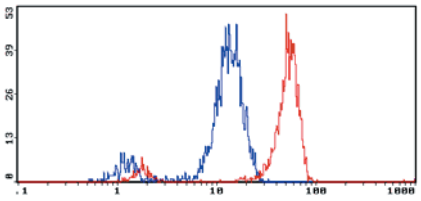


Fig. 10 Anti NA2 binding to granulocytes (anti NA2 in red, negative control in blue).

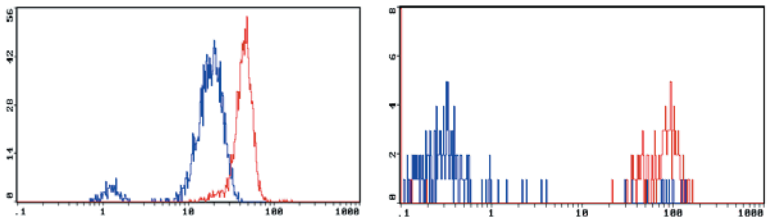


Fig.11 Anti HLA antibodies binding to granulocytes and lymhocytes, respectively. (Anti-HLA in red, negative control in blue).

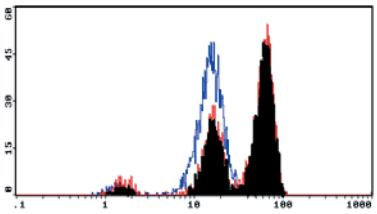


Fig.12 Anti NA2 analysed against a pool of leukocytes from three donors, two of the donors expressing NA2, one negative for NA2. (Anti-NA2 in black, negative control in blue).

4.2.3 Analysis of antibodies directed against granulocyte surface antigens and intracellularly expressed antigens

Ten sera previously tested positive by GIFT were re-tested by flow cytometry. Nine samples gave equivalent results. In serum sample 10 the result in GIFT was positive, displaying a combination of intracellular and surface binding fluorescence pattern, while it was not detectable by flow cytometry. However when the serum was tested against permeabilized cells a positive binding was obtained. The actual antibody detected was directed against PR3, verified by ELISA (Wieslab AB, Ideon, Lund, Sweden).

Ten sera previously tested negative by GIFT, were analyzed by flow cytometry. In seven sera, antibodies could not be detected, in one serum sample anti-HLA antibodies were detected by flow cytometry and verified by a lymphocytotoxicity test, and in two sera granulocyte antibodies were detected by flow cytometry.

Ten sera tested positive for ANCA by IIF were tested against both non-permeabilized and permeabilized leukocytes by flow cytometry to distinguish between surface and intracellular binding. All ten sera showed positive intracellular staining while one serum showed an additional positive surface staining, *Table 6*.

The binding of antibodies both to the cell surface and to intracellular antigens can be explained by binding to antigens co-localized intracellularly and on the cell membrane *[Tosi FM, 1992, Stroncek DF, 1990, Stroncek DF, 1993]* or to antigens mainly stored intracellularly which can be expressed on the surface of activated cells *[Csernok 1994]*. Flow cytometry has previously been reported to detect ANCA with high sensitivity *[Yang YH, 1994]*. However false positive reactions were noted among patients with high ANA titers and the specificity should be further defined by ELISA. In the study by

Yang et al Tween 20 and saponin were used for cell permeabilization, while we used OG permeabilization in our study *[Halldén G, 1989]*.
There are several technical advantages with the flow cytometric technique. The method is easy, objective (results are expressed as numerical values), and allows analysis against different leukocyte populations. By using both un-permeabilized and permeabilized target cells, antigens directed against intracellular- and surface-expressed antigens can be distinguished.

Case	Age/Sex	Diagnosis	IIF	FC surface/MFI	FC intracellular/MFI
1	16/F	Ulcerative colitis	C-ANCA	Pos 20.9	Pos 110.0
2	86/F	Vasculitis	P-ANCA	Neg	Pos 44.3
3	61/F	Hepatitis C	P-ANCA	Neg	Pos 150.3
4	50/F	Hepatitis C	C-ANCA	Neg	Pos 82.5
5	75/F	Vasculitis	C-ANCA	Neg	Pos 93.1
6	48/M	Vasculitis	C-ANCA	Neg	Pos 111.9
7	70/F	Vasculitis	C-ANCA	Neg	Pos 119.5
8	80/F	?	P-ANCA	Neg	Pos 40.8
9	70/F	?	P-ANCA	Neg	Pos 76.7
10	41/F	Vasculitis	C-ANCA	Neg	Pos 112.9

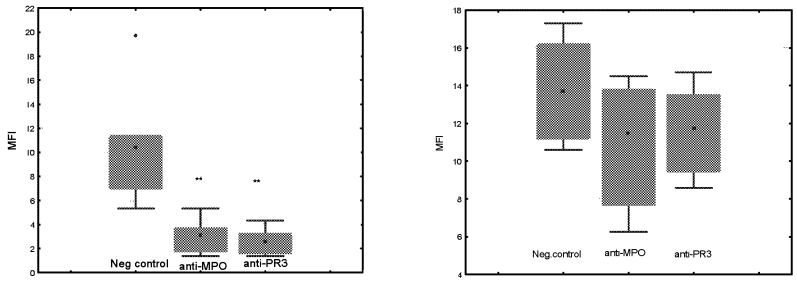
FC=flow cytometry, MFI= mean fluorescence intensity
Table 6 Characterisation of sera ANCA positive by IIF.

4.3 PAPER III

4.3.1 Expression of adhesion molecules in monocytes after ANCA incubation *in vitro*

The aim of this *in vitro* study was to assess the ability of ANCA to affect functional parameters in monocytes and granulocytes. The expression of CD62L on TNFα primed monocytes was reduced ($p<0.01$) after five min incubation with anti-MPO and anti-PR3 positive sera when compared to ANCA-negative AB-sera. The CD62L down-regulation was even more pronounced on granulocytes. ANCA-positive IgG fractions, as opposed to ANCA-negative IgG-fractions, decreased the CD62L expression on granulocytes whereas the reduction on monocytes was not statistically significant, *Fig. 13*. Monoclonal anti-MPO and anti-PR3 did not significantly reduce the CD62L expression on either monocytes or granulocytes.
The expression of CD11b determined after 30 min was not altered following incubation with ANCA-positive sera, ANCA-positive IgG-fractions or monoclonal anti-PR3 or anti-MPO, compared to negative controls. Others have reported on an up-regulation of CD11b after *in vitro* incubation with ANCA-positive sera and IgG-fractions for one hour *[Johnson PA, 1997]* as well as for six hours *[Nowack R, 2000]*. However in our study we focused on early changes and we therefore can not rule out the possibility of ANCA-induced CD11b up-regulation when the incubation time is extended.
The mechanisms by which ANCA activate monocytes and neutrophils are not fully elucidated. There are indications both for a direct activation by ANCA binding to target antigens exposed on the cell surface *[Brouwer E, 1994]* as well as for activation via Fc

receptors *[Kettriz R, 1997, Remaux D, 1995]*. Activated neutrophils have been shown to express PR3 on their surface, both *in vitro* and *in vivo [Csernok E, 1994, Harper L, 2001]*. Regarding the monocytes it is more unclear, in experimental studies the monocytes may be activated by ANCA even without prestimulation *[Novack R, 2000]* and in active disease circulating monocytes have not been shown to express increased levels of PR3 on their surface *[Muller Kobold AC, 1998, paper IV]*. In our study 6.2±0.5 % of the moncytes and 23.7±7.5 % of the neutrophils expressed MPO and PR3 on their cell surface after TNFα *in vitro* stimulation. The potential involvement of complement and other soluble serum factors is not clear *[Matthiesen PW, 1993, Sundy JS, 2002]*. The results from our experiments with a more prominent effect in CD62L down-regulation after incubation with ANCA positive sera compared to ANCA positive IgG-fractions, support the contribution of additional serum factors besides IgG.



Serum IgG-fraction

Fig. 13 Expression of CD62L on monocytes from healthy blood donors after incubation with negative controls (ANCA-negative sera and corresponding IgG-fractions) and anti-MPO positive and anti-PR3 positive sera and IgG-fractions, respectively.

4.3.2 Production of hydrogen peroxide in monocytes after ANCA incubation *in vitro*

The production of hydrogen peroxide was significantly increased in monocytes after 30 min incubation with ANCA positive IgG-fractions ($p<0.01$) and monoclonal anti-MPO and anti-PR3 ($p<0.05$), compared to ANCA-negative IgG fractions and isotype IgG1 control. This enhanced hydrogen production was noted also after stimulation with receptor dependent stimuli (fMLP) and for anti-PR3 also after receptor independent stimuli (PMA). After incubation with ANCA-positive sera the production of hydrogen peroxide was not increased, compared to ANCA-negative sera. Our data confirm and extend previous reports of an ANCA mediated increase in oxygen radical production in monocytes *[Weidner S, 2001]*. There are some important differences between the latter study and ours. The monocytes studied by Weidner et al were isolated by gradient separation and magnetic-activated cell sorting; we used a mixed leukocyte preparation which mimics the physiologic condition in peripherial blood and permits interaction between different leukocyte populations.

Our data in this study support the concept that the pathophysiological effect of ANCA is partly mediated through the action on crucial early events in monocyte activation such as CD62L/CD11b alteration and oxygen radical production.

4.4 PAPER IV

4.4.1 Expression of adhesion molecules on monocytes from patients with acute ANCA-positive vasculitis

In a prospective study we included 12 patients with anti-PR3 positive acute vasculitis during a period of two years. *Fig. 14*. The characteristics of the patients were documented. As controls, blood samples from a group of patients with acute ANCA negative infection and from healthy controls were obtained. The expression of CD62L on monocytes was significantly lower in the group with acute anti-PR3 positive vasculitis compared to the group with acute infection. The expression of CD11b was up-regulated in both groups with acute inflammation compared to in healthy controls. Furthermore the anti-PR3 concentration in the group with vasculitis correlated to a decreased CD62L expression and an increased CD11b expression, *Fig 15*.

There is an on-going debate on the relevance of ANCA titers as a predictor of disease activity. It has previously been reported that relapses are frequently preceded by a rise in ANCA titers and can be prevented by treatment interventions based on changes in those titers *[Cohen Tervaert JW, 1990]*. Others have found changes in titers, even determination of subclasses, of limited prognostic value *[Nowack R, 2001]* and still others have found a weak correlation between reappearing and rising ANCA titers and disease activity *[Girard T, 2001]*.

In our study it was an interesting finding that anti-PR3 levels correlated to the CD62L/CD11b ratio. This may have relevance for the attachment of monocytes to the endothelium and it corresponds with *in vitro* findings on the activating capacity of ANCA *[Paper III, Nowack R, 2000]*. On circulating neutrophils from patients with vasculitis the expression of CD62L and CD11b has previously been reported to be equal between patients with vasculitis and healthy controls, but a decreased CD62L expression and an increased CD11b expression was noted in the patients with active disease *[Muller Kobold AC, 1998, Riecken B, 1994]*.

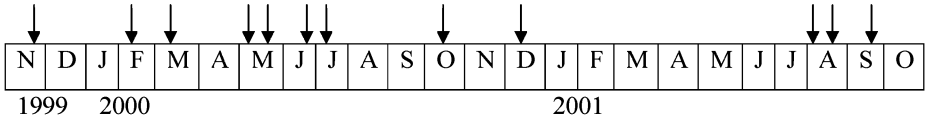


Fig. 14 Time of inclusion in the study (year and month) of 12 prospective patients with anti-PR3 positive vasculitis.

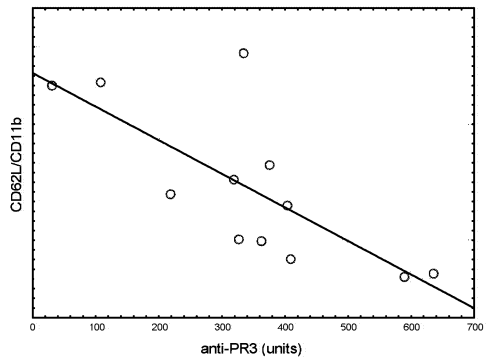


Fig.15 Correlation between anti PR3 concentration and CD62L/CD11b ratio (r=0.74, p=0.006).

4.4.2 Production of hydrogen peroxide in monocytes from patients with acute ANCA-positive vasculitis

Production of hydrogen peroxide in resting monocytes obtained from the peripheral circulation was decreased in the vasculitis patients compared to both control groups. This was also true for receptor-dependent fMLP-induced stimulation. When the receptor-independent stimulus PMA was used hydrogen peroxide production in monocytes was similar in the three groups. A decreased capacity to produce oxygen radicals contrasts with previous reported data *[Weidner S, 2001]* and our own data in paper III, where ANCA *in vitro* induce increased hydrogen peroxide production in monocytes. One explanation for this incongruity may be that vasculitis is a chronic inflammation and in many cases the patient has symptoms for a long period of time before the correct diagnosis is established. This may explain why these patients' circulating monocytes, which have been under continous inflammatory activation for weeks, were unable to respond adequately with production of oxygen free radicals. In our study there was a tendency that patients with acute vasculitis who had had symptoms for a prolonged period of time had a more pronounced reduction in oxygen radical production compared to those with a shorter duration of symptoms, but the difference did not reach statistical significance (r=0.5, p=0.089), *Fig. 16*. Impaired production of oxygen radicals was demonstrated in fMLP stimulated monocytes as well. A down-regulated fMLP response has been identified in other clinical settings and has been attributed to desensitization *[Blackwood RA, 1996]*. Circulating neutrophils from patients with vasculitis have been reported to have impaired *[Riecken B, 1994]* as well as enhanced superoxide production *[Harper L 2001]*. In the former study, as in our study of monocytes, the production of oxygen metabolites was normal after PMA stimulation, suggesting an elevated threshold for activation. It must be emphasized that a dynamic situation exists and the findings noted in circulating cells may not reflect the nature and magnitude of the state of activation at the local inflammatory site.

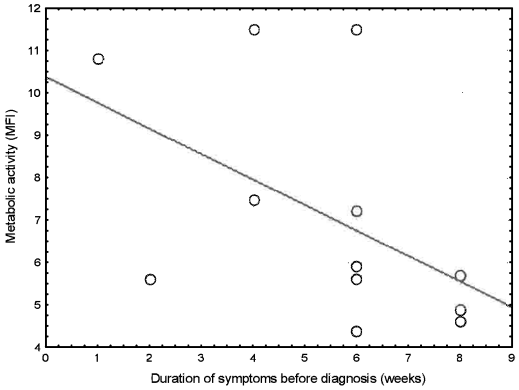
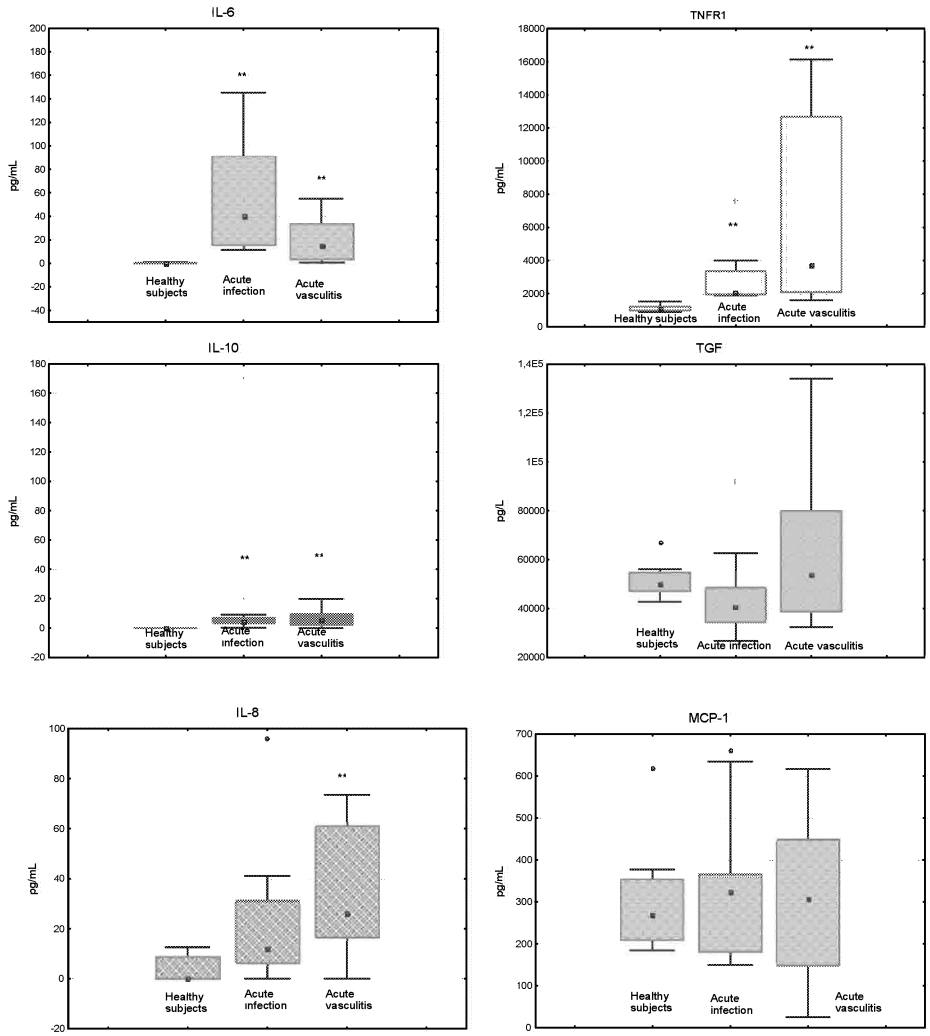


Fig.16 The relation between duration of symptoms before diagnosis and the monocyte capacity to hydrogen peroxide production (r=0.5, p=0.089).

4.4.3 Concentration of soluble inflammation markers in patients with acute ANCA-positive vasculitis

In patients with acute vasculitis the serum concentrations of soluble inflammation markers (sCD14, IL-6, sTNFR1, IL-8, IL-10) as well as soluble adhesion molecules (sVCAM-1, sICAM-1) were increased compared to in healthy controls.The concentration of soluble inflammation markers were elevated also in patients with acute

infection, *Fig. 17*. The CD14 molecule expressed on monocytes is a receptor for lipopolysaccharide. High levels of sCD14 reflect monocyte activation *[Landmann R, 1995, Nockher WA, 1997]* and have previously been reported in Kawasakis disease *[Takeshita S, 2000]*. In our study, the highest concentration of sCD14 was seen in the group with acute infection and negative PR3-ANCA. Also the levels of the acute-phase cytokine IL-6 were highest in this group. These observations support our previous assumption that the patients in the anti-PR3 positive group have had an on-going inflammatory reaction for a longer period. Although there are many reports supporting pathologic activation of the cytokine cascades in patients with vasculitis *[Sundy JS, 2002]*, we did not identify a specific cytokine or soluble adhesion molecule profile that differ between acute vasculitis and acute infection.



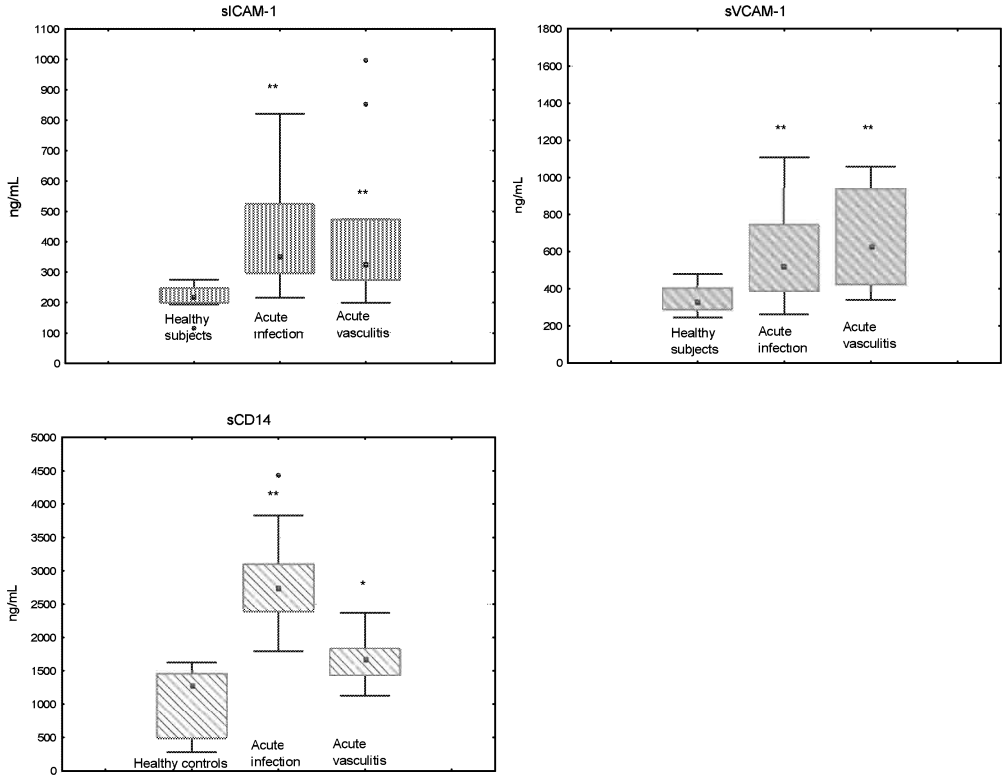


*Fig. 17 Serum concentration of proinflammatory cytokines (IL-6, sTNFR1), antiinflammatory cytokines (TGFß, IL-10), chemokines (IL8, MCP-1), soluble adhesion molecules (sICAM-1, sVCAM-1) and sCD14 in patients with acute anti-PR3 positive vasculitis, acute infection and in healthy subjects. Statistical significance** $p<0.01$, *$p<0.05$ vs healthy subjects.*

CONCLUSIONS

I We demonstrate a marked decrease in CD62L expression accompanied by an up-regulation of CD11b on granulocytes at 24 hours´ storage. The receptors associated with phagocytosis (CD35, CD16) and the capacity to produce reactive oxygen radicals remained more stable. The regulation of adhesion receptors is crucial in the initial attachment to the endothelium in an inflammatory response.

II Detection of antibodies against surface and intracellularly expressed granulocyte antigens can be carried out by flow cytometry. The method is easy, objective (results are expressed as numerical values), and allows analysis against different leukocyte populations. By using both un-permeabilized and permeabilized target cells, antibodies directed against surface- and intracellular-expressed antigens can be distinguished.

III We found a decreased CD62L expression and an enhanced production of oxygen radicals in monocytes upon *in vitro* ANCA stimulation. ANCA positive sera induced a marked monocyte CD62L down-regulation but the down-regulation by ANCA positive IgG-fractions was non-significant, which supports the contribution of other soluble serum factors beside IgG. Increased capacity to produce oxygen radicals was induced by ANCA positive IgG-fractions and monoclonal anti-PR3 and anti-MPO.

IV In our study the main difference between the inflammatory response of monocytes in acute vasculitis and in acute infection was that in patients with acute vasculitis circulating monocytes showed reduced capacity to produce oxygen radicals, which may be a consequence of a prolonged period of immune activation. High concentration of anti-PR3 in the vasculitis group correlated to decreased CD62L and increased CD11b expression, which may have pathophysiological implications for the endothelial damage seen in vasculitis.

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