Scientific Report 2004

Sanquin

Blood and Beyond

Scientific Report 2004

Contents

Introduction

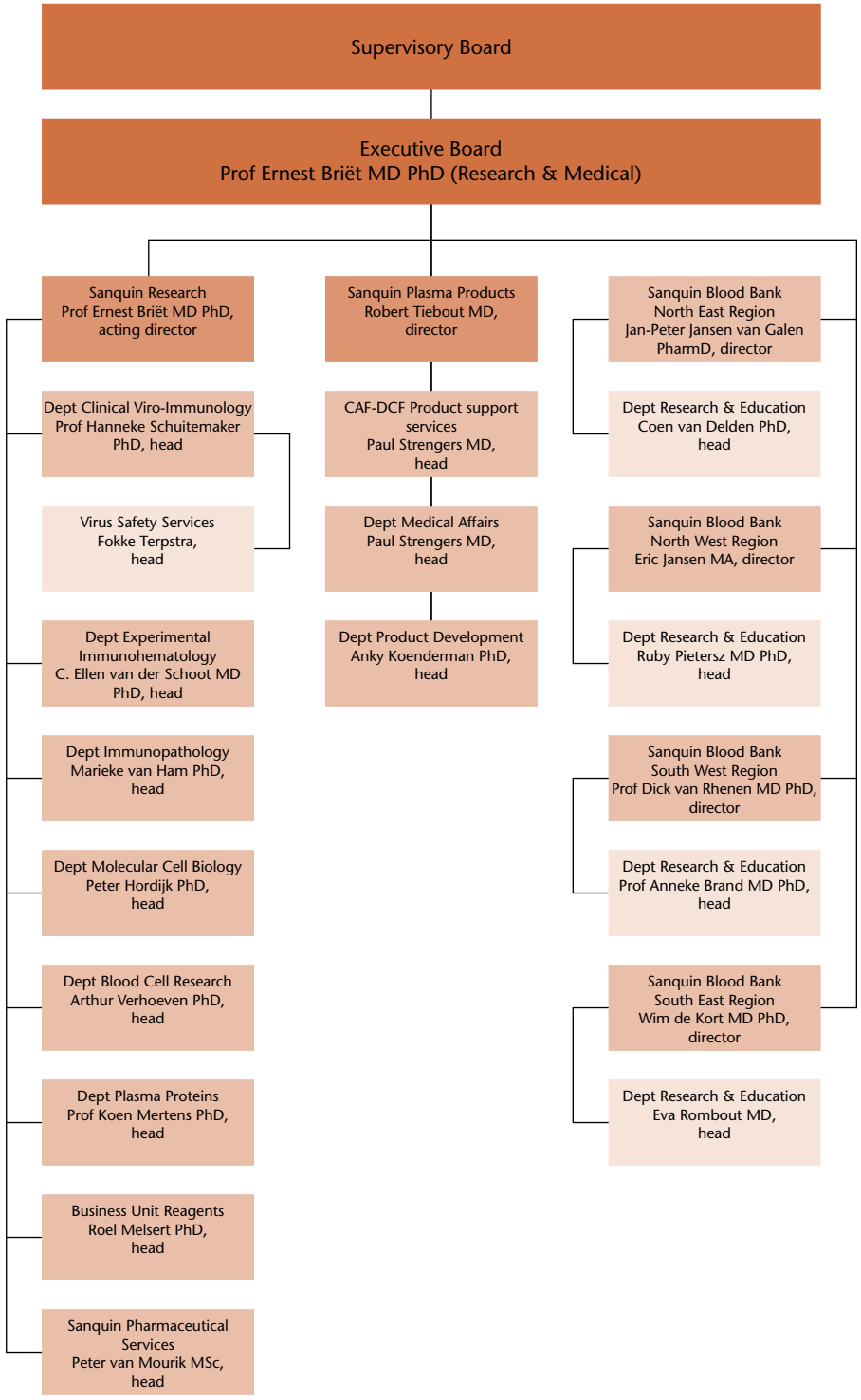
Personnel changes

In May 2004 Professor Frank Miedema, Director Sanquin Research, and his group left for the University of Utrecht. Professor Eric Hack, Head of the Department of Immunopathology, also left Sanquin at the end of 2004.
Dr Hanneke Schuitemaker was appointed part time professor of Virology, especially viro-pathogenesis of AIDS, at the University of Amsterdam Medical Centre.

At the end of 2004 it was announced that the Sanquin Executive Board would be strengthened with a third member, professor Ernest Briët, who will also act as temporary director of Sanquin Research. The appointment of professor Briët will be effected early 2005.

Also at the end of 2004 the organization of Sanquin Research was changed.
The Department of Experimental Immunohematology was split into three research groups. Dr C Ellen van der Schoot became head of the new, smaller Department of Experimental Immunohematology, that also comprises the stem cell laboratory; Dr Peter Hordijk will head the Department of Molecular Cell Biology. Professor Roos and his group rejoined the Department of Dr Arthur Verhoeven that was renamed from the Department of Transfusion Technology to the Department of Blood Cell Research.
Dr Marieke van Ham was appointed head of the Department of Immunopathology.

Sanquin now consists of the following Research and Development Departments.


Supervisory Board
Executive Board
Prof Ernest Briët MD PhD (Research & Medical)
Sanquin Research
Prof Ernest Briët MD PhD, acting director
Dept Clinical Viro-Immunology
Prof Hanneke Schuitemaker PhD, head
Virus Safety Services
Fokke Terpstra, head
Dept Experimental Immunohematology
C. Ellen van der Schoot MD PhD, head
Dept Immunopathology
Marieke van Ham PhD, head
Dept Molecular Cell Biology
Peter Hordijk PhD, head
Dept Blood Cell Research
Arthur Verhoeven PhD, head
Dept Plasma Proteins
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Sanquin Pharmaceutical Services
Peter van Mourik MSc, head
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Robert Tiebout MD, director
CAF-DCF Product support services
Paul Strengers MD, head
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Paul Strengers MD, head
Dept Product Development
Anky Koenderman PhD, head
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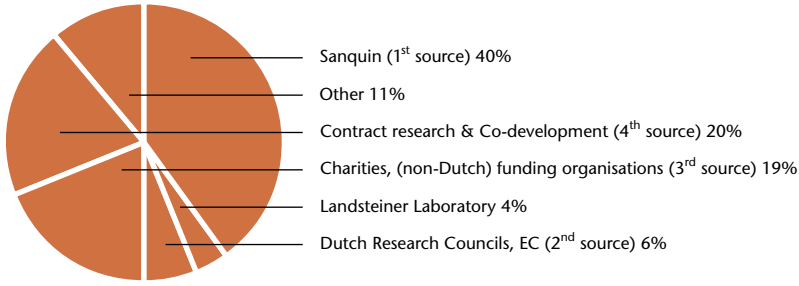
Jan-Peter Jansen van Galen PharmD, director
Dept Research & Education
Coen van Delden PhD, head
Sanquin Blood Bank North West Region
Eric Jansen MA, director
Dept Research & Education
Ruby Pietersz MD PhD, head
Sanquin Blood Bank South West Region
Prof Dick van Rhenen MD PhD, director
Dept Research & Education
Prof Anneke Brand MD PhD, head
Sanquin Blood Bank South East Region
Wim de Kort MD PhD, director
Dept Research & Education
Eva Rombout MD, head

Research funding

In 2004 Sanquin researchers were again successful in obtaining external funding (See page 133 for an overview of our sponsors). Various research groups were successful in obtaining funding from the sixth Framework Program. Contract Research income was less than in the years before. A number of co-development projects with third parties were initiated.

About 20 research projects were funded from Sanquin resources for product and process development for cellular products, after a review on quality and relevance to Sanquin's mission by the Research Programming Committee. Unfortunately over 30 proposals could not be funded.

Sources of funding of research projects (direct costs only)



Central co-ordination of research was again strengthened. Besides already existing working groups on – among others – platelet research, megakaryocytes, and transfusion transmittable infections, working groups on de dendritic cells and donor studies were started.

Research Programming Committee

Prof F Miedema PhD, Chairman (Sanquin Research, until May 2004)
C Aaij PhD (Sanquin Diagnostic Services & Sanquin Research, from May 2004)
Prof DJ van Rhenen MD PhD (Sanquin Blood Bank)
RF Tiebout MD (Sanquin Plasma Products)
JW Smeenk MSc, Executive secretary (Sanquin Corporate Staff)

Quality assessment

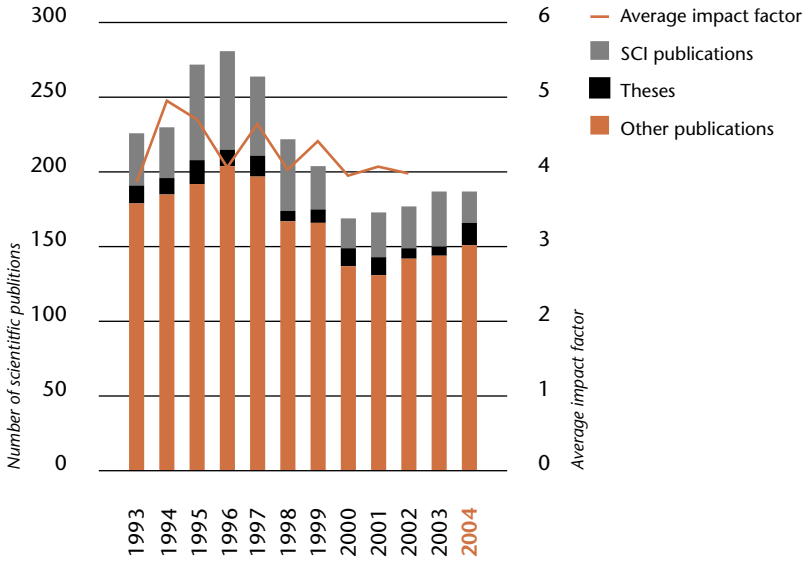
A recurrent site visit system by peer review committees was already introduced a number of years ago. The core of the peer review committees are formed by members of the Research Assessment Board, consisting of Dutch as well as international members. All research groups will be peer reviewed once in every five years. In 2004 no site visits took place.

In order to be able to maintain high quality research, human resource management aiming at a balanced academic staff profile and the accompanying management development program was continued.
The Scientific Advisory Board supervises the quality system, advises the Sanquin Executive Board on all matters concerning (co-ordination of) research and research infrastructure, and checks annually whether Sanquin's research program meets the framework of the five year planning document, that is annually drawn up by the advisory board.

Scientific publications

Year	*Total number*	*SCI publications*	*Theses*	*Average impact factor*
2004	186	150	15	3.86
2003	187	144	6	4.59
2002	177	142	7	4.70
2001	173	131	12	4.06
2000	169	137	12	4.64
1999	204	166	9	4.03
1998	222	167	7	4.41
1997	264	197	14	3.95
1996	281	204	11	4.07
1995	272	192	16	3.89
1994	230	185	11	na

Number of scientific publications



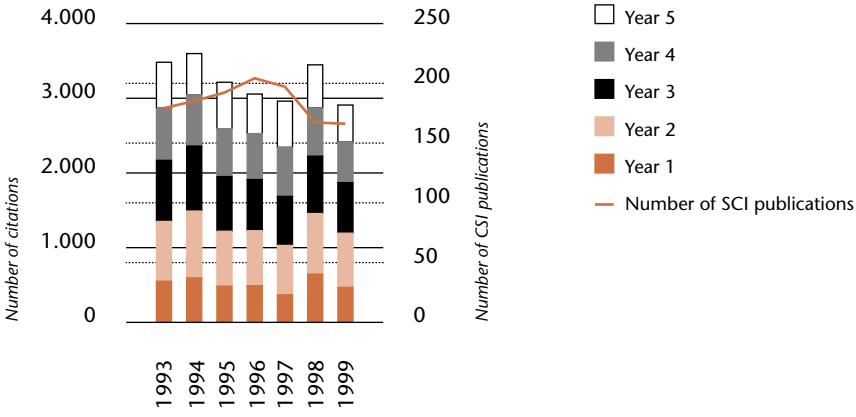
Articles* published in 1994 through 1999 annual reports cited** in five full years after publication

Publications from	*Total citations*	*Number of SCI publiccations*	*Citations in year*					
			1999	2000	2001	2002	2003	2004
1994	3599	185	552					
1995	3215	192	641	622				
1996	3057	204	685	615	530			
1997	2962	197	661	657	656	619		
1998	3448	167	646	811	768	646	577	
1999	2910	166		468	726	677	543	496

* *Only SCI publications are included*

** *Excluding self citations*

Number of citations 5 year after publication



Scientific Advisory Board

Prof EJ Ruitenberg PhD, Chairman (Utrecht University & Vrije Universiteit Amsterdam)

C Aaij PhD (Sanquin Research, a.i. from May 2004)

Prof A Brand MD PhD (Sanquin Blood Bank South West Region & Leiden University)

Prof HC Hemker MD PhD (Maastricht University)

Prof MM Levi MD PhD (University of Amsterdam)

Prof JWM van der Meer MD PhD (University of Nijmegen)

Prof DKF Meijer PhD (University of Groningen)

Prof F Miedema PhD (Sanquin Research, until May 2004)

Prof DJ van Rhenen MD PhD (Sanquin Blood Bank South West Region & Erasmus University Rotterdam)

JW Smeenk MSc, Executive secretary (Sanquin Corporate Staff)

Research Assessment Board

National members

Prof RM Bertina MD PhD (Leiden University)

Prof FC Breedveld MD PhD (Leiden University)

Prof WE Fibbe PhD (Leiden University)

Prof F Grosveld PhD (Erasmus University Rotterdam)

Prof ADME Osterhaus PhD (Erasmus University Rotterdam)

Prof JJ Sixma MD PhD (Utrecht University)

International members

Prof D Anstee MD, PhD (University of Bristol, United Kingdom)

Prof R Carell PhD (University of Cambridge, Cambridge, United Kingdom)

Prof RA Flavell PhD (Yale University, New 0Haven, USA)

Prof LW Hoyer MD (American Red Cross, Rockville, MD, USA)

Prof MD Kazatchkine MD PhD (INSERM, Hospital Broussais, Paris, France)

Prof RA Koup MD PhD (University of Texas, Southwestern Medical Center, Dallas, USA)

Prof D Lane MD PhD (Imperial College School of Medicine, London, United Kingdom)

Academic affiliations

research@sanquin.nl
www.practical training.sanquin.nl
www.scs.sanquin.nl

Sanquin research departments attract many students who participate in scientific projects. Historically there is a strong collaboration with the Academic Medical Center (AMC) of the University of Amsterdam. Sanquin staff members participate in research programmes and curricula of the AMC Research Institute for Immunology (JJ van Loghem Institute) and the Research Institute for Infectious Diseases. This joint AMC – Sanquin Landsteiner Laboratory is housed within Sanquin premises.
At many Dutch universities, staff from various Sanquin divisions are involved in theoretical and practical training programs for undergraduate and graduate students in (medical) biology, pharmacy, medicine, and health sciences as well as laboratory technicians. Of course, Sanquin is also involved in training of specialist in blood transfusion medicine, other medical specialties, and training of nurses. Sanquin Consulting Services emphasizes on training on the job for colleagues from sister organizations in developing countries in Africa, South America, and Asia as well as the former East European Countries. Recently a postgraduate masters program was established in collaboration with the University of Groningen Medical Center, under the heading of Academic Institute for International Development of Transfusion Medicine (IDTM).
Sanquin is WHO Collaborating Organization for Transfusion Medicine.

Professorships Sanquin staff

Prof Rob Aalberse PhD (Biological immunology, Faculty of Biology, University of Amsterdam)
Prof Lucien Aarden PhD (Molecular immunology, Academic Medical Center, University of Amsterdam)
Prof Anneke Brand MD PhD (Blood transfusion medicine, Faculty of Medicine, Leiden University)
Prof Erik Hack MD PhD (Immunopathophysiology of non-specific immunity, Free University, Amsterdam)
Prof Taco Kuijpers MD PhD (Pediatric immunology, Emma Children's Hospital, University of Amsterdam)
Prof Koen Mertens PhD (Pharmaceutical plasma proteins, Faculty of Pharmacy, Utrecht University)
Prof Frank Miedema PhD (Immunology of AIDS, Academic Medical Center, University of Amsterdam)
Prof Dick van Rhenen MD PhD (Blood transfusion medicine, Faculty of Medicine and Health Sciences, ErasmusUniversity Rotterdam)
Prof Dirk Roos PhD (Non-specific immunology, Academic Medical Center, University of Amsterdam)
Prof Hanneke Schuitemaker PhD (Virology, especially viro-pathogenesis of AIDS, University of Amsterdam)
Prof Cees Smit Sibinga MD PhD (International development of transfusion medicine, University of Groningen)

CAF-DCF professorships

Prof Michel Delforge MD PhD (CAF-DCF professor in Hematology and Stem Cell Plasticity, Catholic University of Leuven)
Prof Jacques Pirenne MD PhD (CAF-DCF professor in Abdominal Transplant Surgery, Catholic University of Leuven)

Research lines

Hematology

Alloimmunization against blood group antigens

Principal investigators
C Ellen van der Schoot
MD PhD
Dept Experimental
Immunohematology
(e.vanderschoot@sanquin.nl)
Masja de Haas MD PhD
Dept Experimental
Immunohematology
(m.dehaas@sanquin.nl)

In the last year the projects dealing with the molecular characterization of the Rhesus system have been finished. Especially, variation in the Rh-locus in Negroid was studied. A non-invasive diagnostic method based on fetal DNA circulating in maternal plasma for RhD typing, based on detection of RHD sequences has been tested on more than 2500 D-neg pregnant women. A diagnostic accuracy of more than 99% has been reached. This study shows the feasibility of screening D-neg women to restrict antenatal immunoprophylaxis to women carrying D-pos fetuses. Fetal DNA was found to be fragmented in fragments below 300 bp. Furthermore, a new technical approach to decrease the aspecific background amplification from maternal DNA applying PNA-probes specific for the maternal allele has been developed. Based on these results it will become possible to develop genotyping assays for all SNP-defined blood groups. In a collaborative European study we are developing a so-called 'blood group-chip'. For that purpose, a multiplex PCR methodology has been developed in which gene fragments carrying the polymorphic nucleotides specific for 19 blood group antigen systems are simultaneously amplified. In a pilot study the feasibility of a micro-array set up (applying ASO-probes spotted on glass) for blood group typing has been shown. Research on placental FcRn has been started again. GFP-FcRn transfected cell-lines have been produced and mutant IgG molecules are being made to study the FcRn mediated transport of IgG in the placenta. The role of IgG-Fc receptor (the activating *FCGR2A*, *3A* and *3B* as well as the inhibiting *FCGR2B*) polymorphisms in blood cell destruction has been further investigated.

Key publications

Beiboer SH, Wieringa-Jelsma T, Maaskant-Van Wijk PA, van der Schoot CE, van Zwieten R, Roos D, den Dunnen JT, de Haas M. Rapid genotyping of blood group antigens by multiplex polymerase chain reaction and DNA microarray hybridization. Transfusion. 2005; 45(5):667-79.

Hemker MB, Cheroutre G, van Zwieten R, Maaskant-van Wijk PA, Roos D, Loos JA, van der Schoot CE, von dem Borne AE. The Rh complex exports ammonium from human red blood cells. Br J Haematol. 2003; 122(2):333-40.

Molecular blood group polymorphisms

Principal investigator
Petra Maaskant-van Wijk PhD
Dept Research and Education
South West Region
(petra.maaskant@bloodrtd.nl)

The aim is to evaluate the application of molecular blood group typing of red cell and platelet antigens for blood bank purposes (donor typing) and for diagnostic and therapeutic purposes, in particular for patients with a non-Caucasian origin or for (multi)transfused patients with (multiple) antibodies.

Rh-D zygosity

The possibility of determining RHD zygosity with a PCR-RFLP method was evaluated for different ethnic groups, Blacks from South Africa, Blacks from Ethiopia, Blacks from Curacao, Asians from South Africa and Caucasians. Knowledge about paternal RHD zygosity is of clinical interest for RhD alloimmunized RhD negative pregnant women. The risk of an affected child is 100% when the father is homozygous for RHD, but when the father is hemizygous for RHD, there is a 50% chance that the child will not be affected. The RFLP method was compared to a newly developed real-time quantitative PCR. Sequence analysis of the discrepancies revealed mutated Rhesus boxes that hamper zygosity determination by detection of the RHD-locus in non-Caucasians.
In collaboration with the Blood Bank Shangdong from China the genetic background of RhD negativity in a Chinese Han population was investigated. The RHD gene deletion, RHD-CE-D hybrid genes and a novel 933C>A mutation were found to be the three mechanisms that caused RhD negativity in our samples. The 1227 G>A Del mutation appeared to be the major cause of discrepancies between genotyping and phenotyping strategies, favoring genotyping of D-samples.

Other blood group antigens

With respect to donor typing automated Pyrosequencing assays were developed and validated for HPA-1, -2, -3, -5 and -15. Patients with thrombocytopenic syndromes benefit from the constant availability of an HPA typed donor pool in order to be transfused with HPA-matched blood products. Therefore blood banks should maintain an HPA-typed donor pool of apheresis-platelet donors. For optimal management of matched platelet transfusions, these donors should also be typed for HLA. Donors that

A European Consortium was formed to demonstrate the use of molecular genetic techniques to genotype a large cohort of individuals in order to demonstrate the accuracy and improvement of this technology.

contribute to the Dutch HLA-typed donor file as platelet and/or bone marrow donor were genotyped for HPA-1, -2, -3, -5 and -15 providing a continuous availability of HPA typed platelets. Automated DNA isolation and assays to genotype red cell antigens are currently being developed.
For the development and implementation of a real 'high-throughput' system, the transfusion medicine DNA micro-array, there is collaboration with Sanquin Research/Diagnostic Services, the Blood Bank North West Region and JT den Dunnen (Human and Clinical Genetics, Leiden). A robust multiplex PCR was developed to amplify and fluorescently label gene fragments to type for red cell antigens and HPAs. A pilot study with HPA genotyping was performed with 94 donors.
A European Consortium, of which the Blood Bank South West Region is a sub-contractor, was formed to demonstrate the use of molecular genetic techniques to genotype a large cohort of individuals drawn from across the EU in order to demonstrate the accuracy and improvement of this technology over standard serological testing. First prototype of this micro array has been developed in 2004.

Key publications

Beiboer SH, Wieringa-Jelsma T, Maaskant-Van Wijk PA, van der Schoot CE, van Zwieten R, Roos D, den Dunnen JT, de Haas M. Rapid genotyping of blood group antigens by multiplex polymerase chain reaction and DNA micro array hybridization. Transfusion 2005; 45(5):667-79.

Grootkerk-Tax MG, Maaskant-van Wijk PA, van Drunen J, van der Schoot CE. The highly variable RH locus in nonwhite persons hampers RHD zygosity determination but yields more insight into RH-related evolutionary events. Transfusion 2005; 45(3):327-37.

Qun X, Grootkerk-Tax MG, Maaskant-van Wijk PA, van der Schoot CE. Systemic analysis and zygosity determination of the RHD gene in a D-negative Chinese Han population reveals a novel D-negative RHD gene. Vox Sang 2005; 88(1):35-40.

Granulocyte activation

NADPH oxidase

Principal investigators
Prof Dirk Roos PhD
Dept Blood Cell Research
(d.roos@sanquin.nl)
Prof Taco W Kuijpers MD PhD
Dept Blood Cell Research and Emma Children's Hospital AMC
(t.w.kuijpers@amc.uva.nl)

Phagocytic leukocytes generate reactive oxygen species as a defense against pathogenic micro-organisms. The enzyme responsible for this reaction is an NADPH oxidase. In resting, non-phagocytosing cells, the various subunits of this enzyme are located in different compartments, ensuring inactivity of this enzyme. When the cells are activated by binding of opsonized micro-organisms to various surface receptors, the oxidase subunits assemble into a complex in the plasma membrane of the phagocyte, allowing access of NADPH to the active site in the enzyme, donation of electrons from NADPH and reduction of molecular oxygen to superoxide. We have found that priming of neutrophils for oxidase activation through Toll-like receptors (TLR) can take place on the cell surface before ingestion of the micro-organism, but also on the membrane of the phagosomal vacuole that has engulfed the micro-organism during the process of phagocytosis. This latter process can be seen as 'sensing the contents of the phagosome' for adequate cellular reaction. We have also obtained evidence that the lag time in oxidase activation seen when neutrophils are incubated with unopsonized zymosan can be taken away by prior priming with TLR ligands. Perhaps the binding of opsonized zymosan to the complement receptor CR3 and to the IgG receptor FcγRIIa primes in itself for oxidase activation or enhances the interaction with TLR's.

The activation of the NADPH oxidase is controlled by several small Rho- or Ras-like GTPases, one of which may be Rap1A. We have set up methods to measure the activation of this protein and are now evaluating a role for Rap1A in deactivating the oxidase. Moreover, we are also investigating the role of Rap1A in neutrophil adhesion, and the possibility that the activation of this protein is defective in patients with the syndrome of Leukocyte Adhesion Deficiency type I-variant. A second branch of this project aims at crystallizing the cytoplasmic part of gp91phox, the catalytic subunit of the NADPH oxidase. For this purpose we are trying to create gp91phox mutants that can be produced by micro-organisms in a correctly folded state.

In collaboration with the Biophysical Engineering Group of Dr C Otto from the Technical University in Twente, we have studied by single-cell Raman and fluorescence

microscopy the involvement of lipid bodies in neutrophils in the oxidase activation. We found transient, gp91-phox-dependent association of lipid bodies with phagosomes containing latex particles, suggesting that the arachidonic acid that is known to be involved in NADPH oxidase activation is derived from these lipid bodies. This idea will be tested with phopholipase-deficient phagocytic cells.

Patients with a deficiency of the leukocyte NADPH oxidase suffer from chronic granulomatous disease (CGD). In about 70% of CGD patients, mutations are found in the X-chromosome-linked *CYBB* gene, which encodes the gp91-phox protein. Recently, we found a novel mutation in this gene (in heterozygous form) in an 80-year old lady who suffered from CGD since her 66th. This is strange, for several reasons. One is that female carriers of mutations in *CYBB* are usually without clinical symptoms, unless the X chromosome that carries the wild-type gene is inactivated in most leukocytes. The second reason is that she had been symptom-free for 66 years, although X-CGD is usually manifested at an early age. We therefore investigated whether the X-chromosome inactivation was skewed and indeed, we found that almost all neutrophils were devoid of NADPH oxidase activity and the mutation was expressed in RNA in almost all neutrophils. However, this skewed X inactivation was not a general phenomenon because it was not found in the epithelial cells from a buccal smear. Moreover, the *CYBB* mutation was not found in the DNA obtained from the buccal smear, and was probably also absent in her germ-line cells because we did not find it in the seven of her nine children that we investigated. Thus, this patient shows a somatic mosaic of mutated and non-mutated cells. We then investigated the DNA from her leukocytes, separated according to survival time in the blood. We found the mutation expressed (in heterozygous form) in all short-living myeloid cells (neutrophils and monocytes), in part of her medium-living naïve T lymphocytes but not in her long-living memory T lymphocytes. We conclude that the mutation in *CYBB* originated in her bone marrow during her life time, which then through clonal expansion became expressed in all blood cells derived from that clone.

Key publications

Van Bruggen R, Anthony E, Fernandez Borja M. Roos D. Continuous translocation of Rac2 and the NADPH oxidase component p67phox during phagocytosis. J Biol Chem 2004; 279:9097-102.

Bionda C, Li XJ, van Bruggen R, Eppink M, Roos D, Morel F, Stasia M-J. Functional analysis of two-amino acid substitution in gp91phox in a patient with X-linked flavocytochrome b558-positive chronic granulomatous disease by means of transgenic PLB-985 cells. Hum Genet 2004; 115:418-27.

Van Manen HJ, Kraan YM, Roos D, Otto C. Intracellular chemical imaging of heme-containing enzymes involved in innate immunity using Resonance Raman Microscopy. J Phys Chem B 2004; 18:18762-71.

Wolach B, Scharf Y, Gavrieli R, de Boer M, Roos D. Unusual late presentation of X-linked chronic granulomatous disease in an adult female with a somatic mosaic for a novel mutation in CYBB. Blood 2005; 105:61-6.

Opsonization

For efficient uptake into phagocytic cells, most micro-organisms need to be covered with antibodies and/or complement components, a process called opsonization. In this context we are investigating the importance of mannose-binding lectin (MBL) in the opsonization of zymosan (yeast particles) and various bacteria species. In the past year, we have optimized methods to determine the deposition of MBL, ficolin, MASP1, MASP2, C3, C3bi, IgG and IgM on zymosan and bacteria after incubation with fresh human serum. Moreover, we are now optimizing methods to separate high-molecular complexes of MBL from lower MW complexes. With these assays we will study the relation between MBL complex size and efficiency of complement activation. With a specific anti-C1q $(Fab)_2$ antibody we can block complement activation via the classical (antibody-mediated) pathway, with specific anti-MBL $(Fab)_2$ monoclonal antibody the lectin pathway and sera with Properdin or Factor D deficiency enable us to evaluate the importance of the alternative pathway and the amplification loop. We have also developed a Taqman assay to genotype MBL, and we are now correlating MBL genotype with complex size and plasma concentration. We are also involved in several clinical studies regarding MBL deficiency and susceptibility for

disease manifestations, such as fever in the neonatal period, juvenile rheumatoid arthritis, acne, cystic fibrosis, and fever in oncology patients after chemotherapy and/or irradiation. In addition, we are following MBL levels, C3 activation potential and zymosan opsonization in similarly treated pediatric oncology patients supplemented with purified MBL, in a collaborative phase-II study with the Academic Medical Centre and the Serum Staten Institute in Copenhagen, Denmark.

Kawasaki disease

Kawasaki Disease (KD) is a pediatric vasculitis of unknown etiology, but generally believed to be initiated by an infectious agent. Coronary arteries are often affected, resulting in coronary artery aneurysms in 25–30% of untreated children. Intravenous immunoglobulin (IVIG) reduces the incidence of these aneurysms with 40–70%. The striking differences in annual incidence between Japan (90 per 100,000) and America and Europe (10 per 100,000) suggest that genetic influences play an important role in KD. Moreover an increased risk is observed in siblings and twins. From our previous study in 90 KD patients we know that mutations in the MBL gene influence the risk of development of coronary artery lesions in children under the age of one year.
To study genetic variation further in KD we conducted an association study in 170 KD patients and 300 controls and analyzed Single Nucleotide Polymorphisms (SNPs) in candidate genes. Since October 2003 a collaboration exists with the laboratory of Stephen Chanock, 'Section on Genomic Variation, Pediatric Oncology Branch, National Institutes of Health, Bethesda, MD, USA'. Genotyping of the SNPs was performed by M Breunis in this laboratory. The first results from this study show an association between the occurrence of KD and SNPs in the *VEGF*, *CCR2* and *CCR5* genes.
We have also investigated SNPs in the FCGR genes but no association with the occurrence of KD was observed. In a cohort of 40 Kawasaki patients we did obtain evidence by Lightcycler technology that the number of intact *FCGR2B* genes, encoding the inhibitory Fcγ receptor type IIb, maybe less than two in 20 of these patients compared to only 5 of 40 controls. At this moment we are investigating whether copy number polymorphisms (CNPs) in the FCGR genes play a role in KD.

Besides Lightcycler technology we are also developing a Multi Ligation-dependent Probe Amplification (MLPA) assay to confirm these results.

Key publication

Biezeveld MH, van Mierlo G, Lutter R, Kuipers IM, Dekker T, Hack CE, Newburger JW, Kuijpers TW. Sustained activation of neutrophils in the course of Kawasaki disease: an association with matrix metalloproteinases. Clin Exp Immunol 2005; 141:183-8.

Apoptosis

Apoptosis of neutrophils is an important mechanism of regulating the duration of an inflammatory response. In the past year we have further evaluated the role of mitochondria in these cells in the apoptotic process. Although these organelles in neutrophils contain very little cytochrome c, caspase 9 is normally activated in these cells, and the pro-apoptotic factors Smac/DIABLO and Omi/HtrA2 are normally released. Moreover, Bid and Bax are normally activated and translocate to the mitochondria during spontaneous or Fas/TNF-induced apoptosis. We are now trying to set up assays for in-vitro induction of apoptosis with recombinant proteins, to evaluate the importance of mitochondrial and non-mitochondrial activation pathways. We also found that human neutrophils contain massive amounts of caspase-10, although the importance of this enzyme in neutrophil apoptosis is unknown. Presently, we are studying the activation of caspase-10 under various conditions, and suppression of caspase-10 expression in differentiating myeloid cells will teach us the importance of this process.

We have also studied 23 patients with neutropenia suffering from Schwachman-Diamond Syndrome. The neutropenia was apparently not due to accelerated apoptosis or to decreased chemotaxis, but may instead be caused by the consequences of mutations in the SBDS gene. The SBDS protein is supposed to be involved in RNA processing, which may be essential for correct myeloid differentiation. We have expressed this protein in recombinant form and have raised antibodies against it that may help us to unravel the cause of the clinical symptoms in these patients.

Key publications

Maianski NA, Maianski AN, Kuijpers TW, Roos D. Apoptosis of neutrophils. Acta Haematol 2004; 111:56-66.

Maianski NA, Geissler J, Srinivasula SM, Alnemri EA, Roos D, Kuijpers TW. Functional characterization of mitochondria in neutrophils: a role restricted to apoptosis. Cell Death Different 2004; 11:143-53.

Kuijpers TW, Maianski NA, Tool ATJ, Becker K, Plecko B, Valianpour F, Wanders RJA, Pereira R, van Hove J, Verhoeven AJ, Roos D, Baas F, Barth PG. Neutrophils in Barth Syndrome (BTHS) avidly bind Annexin-V in the absence of apoptosis. Blood 2004; 103:3915-23.

Blink E, Maianski NA, Alnemri ES, Zervos AS, Roos D, Kuijpers TW. Intramitochondrial serine protease activity of Omi/HtrA2 is required for caspase-independent cell death of human neutrophils. Cell Death Different 2004; 11:937-9.

Maianski NA, Roos D, Kuijpers TW. Bid truncation, Bid/Baxtargeting to the mitochondria and caspase activation associated with neutrophil apoptosis are inhibited by granulocyte colony-stimulating factor. J Immunol 2004; 172:7024-30.

Signaling in transendothelial migration

Principal investigator

Peter Hordijk PhD

Dept Molecular Cell Biology

(p.hordijk@sanquin.nl)

The group's main research theme, leukocyte-endothelium interactions, has been further expanded in the year of 2004, by the start of two projects, funded by the Netherlands Heart Foundation and by Sanquin. In general, the groups' research now deals with two main research lines (i) the control of hematopoietic cell migration and (ii) regulation of endothelial integrity. Both lines of research are closely related to a broad range of (patho) physiology, including (chronic) inflammation, stem cell homing and tumor cell metastasis. Common denominators in both research lines are the SDF1-CXCR4 axis, intracellular signaling through Rho-like GTPases and the dynamics of the actin cytoskeleton.

Surprisingly, we found that prolonged inhibition of these MAP kinases resulted in a dramatic potentiation of SDF-1-induced chemotaxis.

Control of cell migration

Leukocyte chemotaxis is initiated by chemo attractants such as SDF-1. SDF-1 acts through its G-protein coupled receptor CXCR4 and is an important, if not crucial, determinant of hematopoietic stem cell migration and homing to the bone marrow. In addition, SDF-1 is involved in migration of inflammatory and tumor cells. We have extended our initial work on the control SDF-1 induced chemotaxis by analyzing the role of the p42 and p38 MAP kinases in stem cell migration, using HL60 cells as a model. These kinases are activated rapidly following SDF-1 stimulation and were found in other cells to be involved in cell adhesion and migration. Using pharmacological inhibitors, we indeed found that these kinases are required for efficient SDF-1-induced, integrin-mediated adhesion and chemotaxis. Surprisingly, we found that prolonged inhibition of these MAP kinases resulted in a dramatic potentiation of SDF-1-induced chemotaxis. This was an unprecedented result, the molecular basis of which we are presently analyzing in more detail. Whatever this mechanism is, this result represents a very novel way of stimulating hematopoietic cell migration, the applicability *in vivo* will be analyzed in the near future.

A complementary line of research has been initiated which focuses on the role of the Slit chemo repellents and their Robo receptors. Slit has been shown to interfere with SDF-1 mediated hematopoietic cell migration, through an as yet unknown mechanism. A recently funded project has started in October 2004 in which the expression of and signaling by the Slit-Robo axis represents the central theme.

Within the context of an ongoing project, funded by the Netherlands Asthma Foundation, we discovered that activation of the small GTPase Rap-1 potentiates chemotaxis of primary monocytes and of the monocytic cell line U937. This occurs through the Rap activator Epac, which is in turn activated by cAMP. This topic has, in collaboration with the group of K Jalink at the NKI, been taken further using Epac knock-down approaches and analyzing Epac localization and activation in living, migrating cells. This finding represents the second novel approach we have found to potentiate leukocyte chemotaxis. Within the same project we have started further analyses on the role of the microtubule cytoskeleton in epithelial integrity and found, surprisingly, that loss of microtubules in these cells promotes cell-cell contact. This finding will be further analyzed in detail in 2005.

Signaling through Rho like-GTPases is a key aspect of cell migration. We have found that the Rac1 GTPase associates, through its C-terminal targeting domain, to the Rac/CDC42 activator α-PIX. This interaction is mediated by the α-PIX SH3 domain and a series of prolines in Rac1 and is required for Rac1 localization at the membrane and for its activation by α-PIX. The relevance of this interaction for Rac1 function is currently being analyzed in more detail.

Control of endothelial integrity

Within the context of our long-standing interest in endothelial cell-cell adhesion, we have initiated a series of experiments directed at the role of the microtubule cytoskeleton in the control of endothelial integrity. We found that loss of microtubules results in a rapid yet reversible loss of endothelial electrical resistance. This was dependent on the RhoA-Rho kinase pathway whereas its reversal (i.e. the restoration of cell-cell contact) is potentiated by activation of the Epac-Rap pathway. We are presently performing additional experiments to further map the relevance of various signaling events in VE-cadherin dynamics.

Novel information on control of VE-cadherin-mediated adhesion came from studies using blocking antibodies. These antibodies reduce endothelial integrity by initiating outside-in signaling. We found that this signaling involves Rac1 and Reactive Oxygen Species and requires a specific tyrosine kinase called Pyk2. This is a redox-sensitive kinase, which is recruited to cell-cell junctions upon loss of cadherin function. Blocking its function using a dominant negative version prevented the loss of endothelial integrity, induced by the antibodies.

We have previously identified endothelial signaling, triggered upon antibody-mediated activation of VCAM-1, which results in a transient loss of cell-cell adhesion. Our current models imply that this signaling is also initiated upon leukocyte binding to endothelial cells. One of the first responses of endothelial cells to leukocyte adhesion is the formation of so-called 'docking structures', membrane protrusions that partly engulf the leukocyte and that serve to concentrate endothelial adhesion and signaling molecules. We have developed the use of coated, fluorescent beads as a model for the initiation of these structures and are presently defining the molecular requirements and consequences of the formation of these docking structures.

Finally, a recently funded project (Netherlands Heart Foundation) has started in the second half of 2004. This project is aimed at defining the way in which the adhesion molecule VCAM-1 can initiate signaling in the endothelial cells. The hypothesis is that this happens through specific protein-protein interactions, mediated by the large extra cellular domain or the short intracellular tail, or both. We have set-up a proteomic approach in which novel endothelial proteins that bind VCAM-1 specifically, are being identified by SDS-PAGE in combination with mass spectrometry. This approach will provide us with new data on the initial events that accompany leukocyte binding to endothelial cells and that are crucial for efficient crossing of the endothelial barrier.

Key publications

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Red cell research

Oxygen delivery by human erythrocytes

Principal investigators
Arthur J Verhoeven PhD
Dept Blood Cell Research
(a.verhoeven@sanquin.nl)
Dirk de Korte PhD
Dept Blood Cell Research
(d.dekorte@sanquin.nl)

The collaborative project with the Dept of Physiology of the Amsterdam Medical Center to determine red cell function *in vivo* was continued. In addition to the rat model with measurement of the micro vascular oxygen tension in the gut, a model was developed with measurements in the kidney. The main difference between the two models is that the kidney is a much more oxygen demanding organ, with oxygen dependency already at a hematocrit of 37% (in contrast to 15% for the gut).

A new *in vitro* test for measurements of oxygen binding and release by hemoglobin (Hb) in intact erythrocytes was developed.

The results as obtained in the gut model were also found in the kidney model, with a clear effect of storage of the erythrocytes. Freshly isolated human erythrocytes were able to maintain oxgygen tension, whereas standard erythrocytes stored for 5–6 weeks did not.
Within the project, a new *in vitro* test for measurements of oxygen binding and release by hemoglobin (Hb) in intact erythrocytes was developed. This was done to avoid the disadvantages of existing methods, using lysed erythrocytes, thereby excluding the plasma membrane as regulatory parameter, or saturation of RBC suspensions with gas mixtures of oxygen and nitrogen often inducing foaming and partial hemolysis. We were able to achieve deoxygenation by addition of procatechuic acid (PCA) and PCA dioxygenase. This reaction does not produce hydrogen peroxide. Oxygen concentration was measured with a Clark-type oxygen electrode in a stirred vessel at 37 °C. In the absence of RBC, complete deoxygenation was achieved within 3 minutes. With RBC present, oxygen consumption decreased below pO_2 values of 100 mm Hg due to the release of oxygen by the RBC. Complete deoxygenation was achieved after about 10 min without a significant increase in Met-Hb or hemolysis. Oxygen release correlated with changes in 2,3-DPG. In some experiments, hemoglobin saturation was measured in parallel, enabling determination of the p50 value without lysis of the cells.

Key publication

Raat NJ, Verhoeven AJ, Mik EG, Gouwerok CW, Verhaar R, Goedhart PT, de Korte D, Ince C. The effect of storage time of human red cells on intestinal microcirculatory oxygenation in a rat isovolemic exchange model. Crit Care Med 2005; 33(1):39-45.

Red cell aging and survival: Improvement of blood processing and storage conditions on ageing and *in vivo* survival of red blood cells

Principal investigator
Harry J Bos PhD
Dept Research and
Education South East Region
(h.bos@sanquin.nl)

Red blood cell concentrates (RCC) (standard product) have been stored up to 52 days under blood bank conditions. Every week samples were taken from each RCC for determination of red blood cell (RBC) concentration, Hb, Ht, MCV, MCH, MCHC, pH, pO_2, pCO_2, HCO_3^+, Na^+, K^+, glucose, free Hb, hemolysis, LDH, ATP, 2,3-DPG and IgG-binding. During storage 2,3-DPG decreases very rapidly, ATP and Na^+ decrease

steadily, MCV, hemolysis, LDH and K^+ increase. These products meet the official guidelines, even when stored up to 49 days, however, the ATP content after 35 days is less than 75% of the starting value. Conclusions at this time are that the products meet the official requirements during storage up to 35 days, but statistical analysis have to be completed.

The RBCs collected in project 03-006 (Therapeutic erythrocytapheresis as treatment for hemochromatosis patients) are normally discarded. In December 2004 we started a longitudinal study in order to investigate the *in vitro* quality of RBCs of hemochromatosis patients collected by apheresis or whole blood donation. The RBCs are stored in SAGM under blood bank conditions up to 50 days.

We also determined whether any changes occur in Diego antigen reactivity during RBC storage. Our data suggest that storage-related changes in the areas of the band 3 molecule that partake in the generation of senescent cell antigen, do not affect the conformation required for maintaining Diego blood group activity. From this we conclude that immunological removal of RBCs after transfusion is not mediated by reactions involving the Diego blood group system. In a pilot experiment we have also tested the antigen reactivity of several other Band 3 antibodies during storage. It seems that antibodies which react positive with Band 3 or parts of it, have stronger reactions with RBCs stored for 37 days than RBCs stored for 2 days.

In collaboration with the research group of Salzer and Prohaska (Vienna), vesicles of stored RBCs (1, 13 and 42 days) were analyzed for their quantity, size and protein content. There are some interesting results, especially the implication that calcium- and storage-induced vesiculation might share similar mechanisms (at least concerning the micro vesicular fraction).

In order to study the *in vivo* survival of stored RCC after transfusion into hemato-(onco-)logic patients a protocol for the Committee of Human-related Research (CHR) has been designed. An approval of the CHR of the Radboud University Nijmegen Medical Center (RUNMC) has been given in November 2004 and the study has started in January 2005. In this cross-over study patients will receive 2 RCC of different storage times. One RCC will have been stored for 0 – 10 days, the other RCC will have been stored for a 25 – 35 days. The survival of the transfused RBCs will be calculated based on flowcytometric determination of autologous and transfused RBCs by measuring antigen differences between donor and recipient.

The above described research is performed in collaboration with the Dept of Biochemistry, RUNMC, Dept of Transfusion Services, RUNMC, Dept of Hematology, RUNMC and Rijnstate Hospital, Arnhem (Dr. GJCGM Bosman, Dept of Biochemistry, RUNMC; Prof WJ de Grip, Dept of Biochemistry, RUNMC; Dr JM Werre, Dept of Transfusion Services, RUNMC; Dr NPM Schaap, Dept of Hematology, RUNMC and FL Willekens, Rijnstate Hospital, Arnhem).

Key publications

Luten M, Roerdinkholder-Stoelwinder B, Bos HJ, Bosman GJCGM. Survival of the fittest?- Survival of stored RBCs after transfusion. Cell Mol Biol 2004; 50(2):197-203.

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Hemostasis and thrombosis

Biosynthesis of the factor VIII-von Willebrand factor complex

Principal investigators
Jan A van Mourik PhD
Dept Plasma Proteins
(j.vanmourik@sanquin.nl)
and Jan J Voorberg PhD
Dept Plasma Proteins
(j.voorberg@sanquin.nl)

While Factor VIII and von Willebrand factor (VWF) circulate in plasma in a non-covalent complex, it has remained controversial whether or not cells exist that are capable of expressing the combination of both proteins. It has been generally accepted that the liver is a major site of factor VIII synthesis. We have previously demonstrated that factor VIII mRNA synthesis occurs both in the liver and in non-hepatic tissues such as kidney and brain. Hepatic factor VIII synthesis is primarily confined to cells lining the sinusoids. These cells, however, are devoid of VWF mRNA. The lack of co-localization of factor VIII and VWF synthesis in these and other liver cells supports the view that complex assembly occurs after the constituent proteins have entered the circulation. This issue has been further explored in hepatic tissue of patients with severe liver disease. Because liver failure often is associated with elevated factor VIII levels in plasma, the possibility was addressed that this would be associated with increased expression of hepatic factor VIII mRNA. This, however, could not be confirmed by quantitative gene expression analysis. However, we did find enhanced hepatic synthesis of VWF mRNA, as well as increased plasma levels of VWF. By virtue of its stabilizing effect on factor VIII, VWF may cause the elevated factor VIII levels in liver disease. We further found reduced levels of mRNA encoding low density lipoprotein receptor-related protein (LRP). This suggests that impaired LRP-dependent clearance of factor VIII (see below) may also contribute to elevated factor VIII in severe liver failure.

As for VWF, is has been established that it is synthesized in vascular endothelial cells, where it is stored in typical organelles, the Weibel-Palade bodies. Besides VWF, these Weibel-Palade bodies contain other proteins, including P-selectin and the chemotactic cytokine interleukin-8 (IL-8). Upon stimulation by agonists such as thrombin, Weibel-Palade bodies undergo exocytosis, resulting in release or surface expression of their contents. An interesting question is which intracellular pathways drive the trafficking of Weibel-Palade body subsets in endothelial cells. One potential mechanism involves the small GTP-binding protein Ral. We have previously shown

that activation of endothelial cells by thrombin results in transient cycling of Ral from its inactive GDP-bound to its active GTP-bound state. In addition to Ca^{2+}-elevating secretagogues such as histamine and thrombin, release of WPB is also observed after administration of cAMP raising substances such as epinephrine and vasopressin. We observed that activation of Ral was also observed following stimulation of endothelial cells with epinephrine or forskolin. Maximal activation of Ral was achieved at 15 to 20 minutes. The gradual increase in activated Ral in response to epinephrine is compatible with the slow onset of VWF secretion observed on stimulation with this agonist. Inhibition of protein kinase A resulted in a reduction of VWF release and also interfered with activation of Ral on stimulation with epinephrine. These results suggest that protein kinase A-dependent activation of Ral regulates cAMP-mediated exocytosis of Weibel-Palade bodies in endothelial cells.

Key publications

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Structure and function of enzyme-cofactor complexes

Principal investigator
Prof Koen Mertens PhD
Dept Plasma Proteins
(k.mertens@sanquin.nl)

One characteristic of the coagulation cascade is that it comprises several serine proteases that act in combination with a non-enzymatic cofactor. We focus on the complex of activated factor IX (factor IXa) and its cofactor factor VIII, and in particular on the relation between cofactor binding and enhancement of factor X activation by factor IXa. By protein engineering studies of the factor IXa protease domain (the heavy chain), we previously demonstrated that multiple surface-exposed loops in the protease domain limit the accessibility of the substrate binding groove. This makes enzyme activity strictly dependent on the assembly with its cofactor factor VIII.

Our studies have further demonstrated that factor VIII binding involves two helical structures in the factor IXa protease domain (residues 301–303 and 330–339). The latter helix comprises two residues, R333 and R338, that are part of an extended basic exosite that further comprises K293, K341, K400 and K403. This exosite has previously been implicated as being the heparin binding site of factor IXa. We found that the same site also contributes to the interaction with low-density lipoprotein receptor-related protein (LRP). This is particularly interesting since LRP inhibits the activity of the factor IXa/factor VIIIa complex, presumably by interfering in enzyme-cofactor assembly. This raises the possibility that factor IXa binds factor VIIIa, and in particular the A2 domain, by the same anionic exosite that also binds heparin and LRP. This hypothesis has been addressed by site-directed mutagenesis, employing purified Ala substitution variants that were expressed in mammalian cells. All mutants displayed reduced heparin binding in surface plasmon resonance studies. Residual binding varied between 40% for single substitutions at positions 341 and 338 and ≤5% for replacements at 333, 400 and 403. Positions 293, 333, 400 and 403 also contributed to association with the Factor VIII A2 domain, although no single substitution abolished complex assembly completely. The same substitutions had little or no effect on the reactivity towards a synthetic peptide substrate or on the interaction with antithrombin. Factor X activation, however, was impaired for all mutants. In the absence of factor VIIIa, factor IXa activity was reduced by between 10% for the 338 substitution and 75–85% for the 333 and 333/403 double replacements. In the presence of factor VIIIa, mutations at positions 293, 341, 400 and 403 were associated with a 20–45% reduced activity, while substitutions of R333 and R338 in the helix proved totally different. While the R338A substitution displayed supernormal activity, the activity of the R333A substitution and the 333/403 double mutant was <1%. Kinetic analysis revealed that the low activity was largely due to substrate inhibition, suggesting defective formation of the enzyme/cofactor/substrate complex. We conclude that factor IXa residues K293, K400 and K403 of the anionic exosite contribute to the interaction with both factor X and the factor VIII A2 domain, while the anionic helix residues R333 and R338 predominantly determine the extent of catalytic rate enhancement. As such, this helix may be a molecular switch that controls the enzymatic activity of factor IXa within the coagulation cascade.

Key publications

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Rohlena J, Kolkman J.A, Boertjes RC, Mertens K, Lenting PJ. Residues Phe-342 to Asn-346 of activated coagulation factor IX contribute to the interaction with low-density lipoprotein receptor-related protein. J Biol Chem 2003; 278:9394-401.

Inhibitory antibodies in hemophilia

Principal investigator
Jan J Voorberg PhD
Dept Plasma Proteins
(j.voorberg@sanquin.nl)

Coagulation factor replacement therapy of hemophilia may be complicated by the formation of inhibitory or neutralizing antibodies (inhibitors). This side-effect occurs in approximately 25% of the patients with severe hemophilia A, and in about 5% in patients with severe hemophilia B. Inhibitor development in patients with mild hemophilia is relatively rare. Whereas inhibitor development occurs in 25% of patients with severe hemophilia A, factor VIII inhibitors are less frequently observed in mild and moderate hemophilia A. Inhibitor formation in these patients commonly occurs after intensive factor VIII replacement therapy. Although inhibitor titers are usually low and inhibitors often disappear spontaneously, patients can suffer from severe life-threatening bleedings. Most of these patients have low amounts of circulating factor VIII, which has been suggested to provide tolerance to infused factor VIII. This can explain the lower risk of inhibitor development in mild/moderate hemophilia A patients. Interestingly, the risk of inhibitor development is higher for patients with missense mutations in the A2 domain or around the C1/C2 junction of factor VIII. An example of a mild hemophilia A mutation with a high incidence of inhibitor formation is the missense mutation Arg593 to Cys in the A2 domain of factor VIII. Several patients with this genotype that developed anti-factor VIII antibodies have been described. At present, it is unclear why a subset of patients with this genetic defect develops inhibitory antibodies and others do not. Both treatment-related and genetic factors may contribute to inhibitor development in these patients. We addressed the role of human leukocyte antigen (HLA) class II alleles in inhibitor development in patients with an Arg593 to Cys mutation by HLA genotyping.

Our data suggest that inhibitor development in mild hemophilia A patients with an Arg593 to Cys mutation is not linked to HLA class II profile.

In the group of inhibitors patients raised frequencies of HLA-DRB1*01 and HLA-DQB1*05 were observed that did not reached statistical significance. Our data suggest that inhibitor development in mild hemophilia A patients with an Arg593 to Cys mutation is not linked to HLA class II profile.

Key publication

Van den Brink EN, Bril WS, Turenhout EAM, Zuurveld M, Bovenschen N, Peters M, Yee TT, Mertens K, Lewis DA, Ortel TL, Lollar P, Scandella D, Voorberg J. Two classes of germline genes both derived from the VH1 family direct the formation of human antibodies that recognize distinct antigenic sites in the C2 domain of factor VIII. Blood 2002; 99:2828-34.

Cellular receptors involved in clearance of factor VIII and factor IX

Principal investigators
Sander B Meijer PhD
Dept Plasma Proteins
(s.meijer@sanquin.nl)
Prof Koen Mertens PhD
Dept Plasma Proteins
(k.mertens@sanquin.nl)

The identification of the mechanism involved in the clearance of coagulation factors VIII and IX is a continuous challenge. Dysfunction of these mechanisms may cause elevated levels of these coagulation factors, and as such be a novel risk factor for developing venous thrombosis. A few years ago, we and others observed that factor VIII binds to the low-density lipoprotein receptor-related protein (LRP). This receptor is a member of the LDL-receptor family, which is involved in the binding and cellular uptake of a variety of ligands. We further observed that LRP binds to factor IXa, but not to its non-activated precursor factor IX. This suggests that LRP interacts with structure elements that become surface-exposed upon factor IX activation, possibly the same sites that also are involved in factor IXa function.

We have employed a large panel of factor IX variants with substitutions in various surface loops in the protease domain in order to map the LRP binding site. This approach revealed that LRP binding involves an extended region of basic amino acids that surround residue Asn-346. A number of potentially important residues have been identified, and the role thereof is currently analyzed in more detail. One particularly striking observation is that LRP binding inhibits factor IXa activity towards its natural

This suggests that apart from LRP, also other RAP-sensitive receptors contribute to the regulation of factor VIII levels in the circulation.

substrate factor X. This suggests that LRP, apart from its role in the cellular uptake of factor IXa, contributes to the regulation of factor IXa within the coagulation cascade. As for factor VIII, we identified residues within the light chain that contribute to the high affinity binding of factor VIII to LRP. By using a combination of synthetic peptides, recombinant antibody fragments, and factor VIII/factor V hybrid molecules, we have demonstrated that LRP binding involves the residues 1811–1818 in the A3 domain. This suggests that the LRP binding site is partially overlapping with a factor IXa interactive region, which is known to involve the same surface-exposed part of the factor VIII A3 domain.

We have further provided evidence that the endocytic receptor LRP indeed regulates factor VIII clearance *in vivo*. For this purpose we have used a mouse model of conditional hepatic LRP deficiency. This model takes advantage of the so-called 'cre-lox-P' technique for targeted disruption of the LRP gene. Upon inactivation of the LRP gene, mice developed significantly higher factor VIII plasma levels than their non-deficient controls, and these persisted for at least 6 weeks. LRP deficient mice further displayed longer factor VIII half-life in infusion studies using purified human factor VIII. Adenovirus-mediated overexpression of the endocytic receptor antagonist Receptor Associated Protein (RAP) also resulted in an increase of factor VIII levels. Surprisingly, this occurred both in normal mice and in mice that were lacking hepatic LRP. This suggests that apart from LRP, also other RAP-sensitive receptors contribute to the regulation of factor VIII levels in the circulation. In line with this observation, others and we have demonstrated that the LDL-receptor family member's very-low density lipoprotein receptor (VLDLR) and megalin (LRP2) can bind factor VIII *in vitro*. A physiological role of megalin in the clearance of factor VIII from the circulation seems unlikely since this receptor is mainly identified on the surface of cells that are not in direct contact with plasma. VLDLR is, however, present on the endothelial cells of the vascular wall. To address the physiological relevance of VLDLR in the catabolism of FVIII, we employed knockout mouse models for VLDLR and LRP alone and in combination. The data showed that VLDLR deficiency did not affect the plasma level of endogenous factor VIII. This model system further revealed that factor VIII clearance from the circulation was in fact slightly accelerated in the absence of VLDLR.

As a consequence, it is questionable that VLDLR plays an important role in the catabolism of factor VIII. The identification of the RAP-sensitive receptors involved in the clearance of factor VIII remains, therefore, a major challenge.

Key publications

Bovenschen N, Boertjes RC, van Stempvoort G, Voorberg J, Lenting PJ, Meijer AB, Mertens K. Low density lipoprotein receptor-related protein and factor IXa share structural requirements for binding to the A3 domain of coagulation factor IXa. J Biol Chem 2003; 278:9370-7.

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Inflammation and sepsis

Immunoglobulins

Principal investigator
Prof Rob C Aalberse PhD
Dept of Immunopathology
(r.aalberse@sanquin.nl)

Intravenous immunoglobulin (IVIg) is being used not only for replacement therapy in patients with antibody deficiency, but also in other conditions such as idiopathic thrombocytopenia, Kawasaki syndrome and Guilain-Barre. In applications other than replacement therapy, the mechanisms of action are largely uncertain. Possibilities are: effects due to an increased level of total IgG, effects of IgG dimers and effects of specific antibodies (for example: cytokine neutralization).

Biological properties of intravenous immunoglobulin

Research on the biological properties of IvIG was centered on the interaction with Fc-receptors. In cooperation with the Dept of Experimental Immunohematology, specific PCRs were set up to measure expression of the different Fc-gamma receptors II (FcγRII) on cells, which include the activating FcγRIIa and the inhibitory FcγRIIb. The ratio between these activating and inhibitory receptors was determined on various cell types. On neutrophils this ratio was found to correlate with elastase release upon triggering with IgG-dimers. This technology is being used to screen patients with and without side effects upon IvIG infusion.

Biochemical and structural aspects of IVIg

Fragmentation of IgG during prolonged storage at elevated temperatures was investigated using domain-specific immunoblotting and N-terminal sequencing. The results indicated that neutrophil elastase is likely to be the main culprit. The stability of the IgG dimers present in IVIg was investigated by repeated fractionation by size-exclusion chromatography. The results indicated heterogeneity among the dimers: some dimers dissociated rapidly, whereas others were substanti ally more stable. Biacore analysis of dimer formation using Fab and Fc fragments showed an interaction between Fab fragments and Fc fragments, but not between soluble Fab and coated Fab, which does not support the hypothesis that idiotype-anti-idiotype interactions are an important cause of dimer formation.

Structural and functional properties of human IgG4

Human IgG4 has been found to exchange half-molecules with other IgG4 in the blood, which usually results in asymmetric antibodies (i.e. with two different antigen-combining sites). Such an exchange reaction is not observed upon mixing IgG4 antibodies in buffer, which suggests that the process, which involves breaking disulphide bonds as well as strong hydrophobic interactions, is catalyzed in-vivo. Factors involved in this exchange reaction are being investigated.

Role and specificity of IgM in ischemia-reperfusion

A novel project on the role and specificity of IgM in ischemia-reperfusion was started together with the Dept of Experimental Surgery of the AMC, and the immunoglobulin group of the Dept of Immunopathology.

Key publications

Van Mirre E, Teeling JL, van der Meer JW, Bleeker WK, Hack CE. Monomeric IgG in intravenous Ig preparations is a functional antagonist of FcgammaRII and FcgammaRIIIb. J Immunol 2004; 173(1):332-9.

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Auto-immune Diseases

Principal investigator
Prof Lucien Aarden PhD
Dept of Immunopathology
(l.aarden@sanquin.nl)

The Auto-immune Diseases research aims to identify mechanisms that underlie the formation of auto-antibodies. The goal is to verify the hypothesis that impaired clearance of apoptotic cells may result from defects in the proteins contributing to the clearance and lead to an increased risk for the formation of auto-antibodies against nuclear antigens which in their turn may lead to systemic lupus erythematosus (SLE). When apoptotic T cells are incubated with plasma or serum a number of plasma proteins binds to late apoptotic cells. As a side effect we noticed that incubation with plasma lead to rapid removal of nucleosomes from the dead cells. In the absence of serum this takes days but with as little as 5% serum the removal of nucleosomes is completed in 30 minutes. In a large percentage of SLE sera this activity seems to be

We have set up an assay to measure antibodies to citrullinated human fibrinogen and verified its diagnostic and prognostic value in early arthritis.

absent. We are working on the identification of this plasma protein. Work on the role of Toll-like receptors in the stimulation of inflammatory cytokines was continued. We have now identified a serum factor that is instrumental in stimulating IL-8 production in TLR4/CD14 transfected HEK 293 cells. Patients with RA have antibodies to citrullinated proteins. We have set up an assay to measure antibodies to citrullinated human fibrinogen and verified its diagnostic and prognostic value in early arthritis. As model for antibody formation in auto-immune conditions, antibody formation to Infliximab in patients with rheumatoid arthritis (RA) was investigated. Infliximab (anti-TNF monoclonal antibody) treatment is nowadays a standard treatment of RA, but clinical responses become limited over time in most patients. In cooperation with the Dept of Rheumatology of the VU Medical Center, the AMC, Slotervaart Ziekenhuis and the Jan van Breemen Institute levels of Infliximab and anti-Infliximab antibodies were measured in RA patients. Formation of Infliximab-neutralizing antibodies indeed seems to be major cause for diminished clinical efficacy. This research is now extended to other anti-TNF agents such as Enbrel and Adalumimab.

Key publications:

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Inflammation

Principal investigator
Prof Lucien Aarden PhD
Dept of Immunopathology
(l.aarden@sanquin.nl)

The Inflammation Research group until recently headed by Prof CE Hack, focuses on identification of novel activation products of the classical pathway of complement and on the development and clinical evaluation of complement inhibitors. Previously, it was found that covalent fixation of activated C4 and C3 to C1q occurred during classical pathway activation and not during other activation processes. A differential antibody sandwich ELISA was optimized, and levels of these novel activation products were measured in various diseases. Patients with rheumatoid arthritis (RA) were demonstrated to exhibit higher expression levels of these activation parameters.

Immune regulation

Principal investigator
S Marieke van Ham PhD
Dept of Immunopathology
(m.vanham@sanquin.nl)

A major inhibitor of classical complement pathway is C1-inhibitor, a serpin. The work on the structure and function of the C1-inhibitor was continued. Based on previous results of a study of a genetic deficiency of C1-Inhibitor a novel recombinant mutant of C1-inhibitor was designed, as were mutants that lack any carbohydrate group. These mutants have been successfully expressed in *Pichia pastoris* and were demonstrated to be functionally active. Currently, the mutants are being purified in large quantities for *in vivo* rabbit studies to compare pharmacokinetics and dynamics of the mutants to the full length recombinant protein. Identification of the function of some other serpins in is an additional topic of this research-line. In this project the role of granzymes and granzyme-inhibiting serpins in the innate and adaptive immune system are being studied. The activity of granzyme B is regulated by the human intracellular serpin SERPINB9. It was demonstrated that mast cells express both granzyme B, perforin and SERPINB9. In addition, GrB and perforin produced by the mast cell line HMC-1 were demonstrated to be active in hemolytic assays. These findings point to a novel cytolytic mechanism for human mast cells in host defense and/or tumor rejection. A novel protease inhibitor of human neutrophil elastase, cathepsin G and proteinase 3, termed Fahsin, was expressed in *P. pastoris*. Purified

recombinant Fahsin was demonstrated to be a tight-binding reversible inhibitor of human neutrophil elastase and insensitive to chemical and biological oxidation.

Key publications

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Immunology

Immunopathology of HIV infection

Principal investigator
Prof F Miedema PhD
Dept Clinical Viro-Immunology
(h.schuitemaker@sanquin.nl)

T cell dynamics in children

Current understanding of how the T cell pool is established in children and how this is affected by HIV infection is limited. It is widely believed that the thymus is the main source for T cells during childhood. Here we show, however, that healthy children had an age-related increase in total body numbers of naive and memory T cells, whereas absolute numbers of T cell receptor excision circles (TRECs) did not increase. This suggests that expansion of the naive T cell pool after birth is more dependent on T cell proliferation than was previously recognized. Indeed, the proportion of dividing naive T cells was high, especially in younger children, which is consistent with expansion through proliferation, in addition to antigen-mediated naive T cell activation leading to formation of the memory T cell pool. In untreated children infected with HIV-1, total body numbers of T cells and TRECs were low and stable, whereas T cell division levels were significantly higher than in healthy children. We postulate that in children infected with HIV, similar to adults infected with HIV, continuous activation of naive T cells leads to erosion of the naive T cell pool and may be a major factor in lowering $CD4^+$ T cell numbers.

Rates of CD4 T cell decline in HIV infection

To study whether immune status prior to HIV seroconversion predicts CD4 T cell decline during HIV infection. Prospective cohort study including 51 injecting drug users (IDU) who were HIV negative at study entry and seroconverted for HIV during follow-up. Cryopreserved peripheral blood mononuclear cells obtained before HIV seroconversion were used to measure naive ($CD45RO^-CD27^+$), memory ($CD45RO^+CD27^+$), and total CD4 T cell numbers, the fraction of dividing $Ki67^+CD4^+$ T cells, and CD4 T cell receptor excision circles (TREC). The effect of pre-seroconversion immune status, as defined by these markers, on the rate of CD4 T cell decline during HIV infection was assessed using linear regression for repeated measurements. IDU with low pre-seroconversion CD4 T cell TREC contents lost CD4 T cells at a significantly faster rate during HIV infection than those with a high CD4 T cell TREC content. IDU with higher pre-seroconversion CD4 T cell

A dichotomy between TREC contents in CD4 and CD8 T cell populations in HIV-1 infection and indicate that thymus function in younger subjects is preserved at early and intermediate stages of HIV infection.

numbers had a significantly steeper CD4 T cell decline in the first 3 months of HIV infection, but their CD4 T cell counts remained higher throughout HIV infection. Intermediate levels of pre-seroconversion dividing $Ki67^{+}CD4^{+}$ T cells were associated with a significantly steeper CD4 cell decline than high levels. IDU with the highest pre-seroconversion drug-injecting frequencies showed slower CD4 T cell decline than those who injected less. No correlation was present between pre-seroconversion immune markers and the pre-seroconversion duration or intensity of drug use. We conclude that among IDU, immune status prior to HIV infection as measured by TREC content affects the disease course after HIV seroconversion.

T cell production during HIV infection

We assessed de novo T cell generation by determining T cell receptor-rearrangement excision circles (TRECs) based on patient age and on stage of HIV-1 infection. TRECs were measured in purified CD4 and CD8 T cells of a large cohort of HIV-1 infected subjects (n = 297) with chronic infection but no previous antiretroviral treatment and of a control group of HIV-negative subjects (n = 120). HIV-1 infected subjects were stratified on the basis of CD4 T cell counts in 3 groups, early-stage disease (more than 500 CD4 T cells), intermediate-stage disease (200 – 500 CD4 T cells), and late-stage disease (fewer than 200 CD4 T cells). Compared with the control group, CD8 TREC contents were severely reduced ($P < .001$) in HIV-1 infected subjects regardless of the stage of HIV disease. In contrast, CD4 TREC contents were significantly increased ($P = .003$) in HIV-1 infected subjects during early-stage disease, similar at intermediate-stage disease, and severely reduced only at late-stage disease. We show that the increase in CD4 TRECs was mostly limited to younger (younger than 45 years) patients at early-stage disease. Our results demonstrate a dichotomy between TREC contents in CD4 and CD8 T cell populations in HIV-1 infection and indicate that thymus function in younger subjects is preserved at early and intermediate stages of HIV infection.

Loss of T cell repertoire diversity in HIV infection

Loss of CD4 T cells is the hallmark of HIV infection, but it remains unclear to what extent it also reduces the diversity of the CD4 T cell repertoire. Quantification of

HIV-induced changes in CD4 T cell diversity can give important insights into the mechanisms whereby HIV induces a CD4 T cell loss, i.e. whether only T cells recognizing specific antigens are lost or whether T cell loss occurs irrespective of antigen-specificity. Therefore, we have made detailed T cell repertoire analyses of HIV-infected individuals and healthy controls using T cell receptor (TCR) complementarity determining region 3 (CDR3) spectratyping (the so-called immunoscope technique) combined with extensive sequencing of peaks of a certain TCR length within the immunoscope profiles of the Vb8 and Vb3 families. All patients were therapy-naive and seroconverted between 1.5 and 3 years before the date of analysis. Using different measures of T cell diversity, we found that early in infection, the naive CD4 T cell repertoire of HIV-infected patients tended to be less diverse than the naive repertoire of healthy individuals, suggesting that HIV-induced T cell loss occurred antigen-specifically. Inter-individual differences in memory T cell diversity were much larger, and there was no clear correlation between the diversity of the memory T cell pool and HIV infection. Importantly, some HIV-infected individuals showed undisturbed immunoscope profiles while their T cell diversity appeared to be reduced when analyzed by TCR sequencing. Our data therefore also stress the need to analyze T cell repertoires in greater detail than just at the immunoscope level as is typically done.

Limitations of the use of CD31 as a marker of thymic output

CD31 has been proposed as a marker that can be used to discriminate between ($CD31^+$) true recent thymic emigrants and ($CD31^-$) naïve $CD4^+$ T cells that have undergone peripheral T cell proliferation. Consistent with this idea, the percentage of $CD31^+$ T cells in the naïve $CD4^+$ T cell pool was found to decline with age, and the TREC content of $CD31^+$ naïve $CD4^+$ T cells was shown to be consistently higher than that of their $CD31^-$ counterparts. We found, however, that the TREC content of the $CD31^+$ naïve $CD4^+$ T cell pool declines with age, indicating that even $CD31^+$ naïve $CD4^+$ T cells undergo peripheral T cell proliferation, and that CD31 can thus not be used to identify recent thymic emigrants.

We studied how the dynamics of $CD31^+$ and $CD31^-$ naïve $CD4^+$ T cells are altered during HIV-infection and in other conditions of chronic immune activation. In contrast to the anticipated decrease in the fraction of $CD31^+$ cells within the naïve $CD4^+$ T cell

Results suggest that in young mice a constant output of T cells from the thymus is necessary to maintain the peripheral naive T cell pool.

pool, chronic immune activation frequently led to significant increases in these CD31 fractions. Proportions of naïve CD31$^+$ cells in the *total* CD4$^+$ T cell pool were, however, always reduced during chronic immune activation, implying enhanced maturation or death of CD31$^+$ naïve CD4$^+$ T cells. These studies on the dynamics of CD31$^+$ and CD31$^-$ naïve CD4$^+$ T cells provide novel insights into the possibilities and limitations of the use of CD31 as a CD4$^+$ T cell thymic proximity marker.

Depletion of CD4$^+$ T cells in HIV infected patients

The question of how CD4$^+$ T cells are being depleted in HIV infected patients is still extremely topical. In this project we test the hypothesis that in HIV patients chronic immune activation is the driving force for depletion of the naive T cell pool and thereby of CD4$^+$ T cell depletion. In the last year we have focused on the contribution of the peripheral naïve T cell division and thymic output in the maintenance of the naïve T cell pool in a mouse model with progressive peripheral T cell depletion, namely CD70 TG mice. In this system we compare T cell death and proliferation rates, are as measured with static markers (KI-67 and annexin V) and determined dynamically (BrdU /D2O labeling), in the presence and absence of thymic output (sham or thymectomized mice).
Our preliminary results showed that in contrast to the situation in humans, thymectomy of young (6 weeks-old) mice leads to a 50 percent reduction in naïve peripheral CD4 and CD8 T cell numbers. In agreement with this observation labeling studies in sham thymectomized mice showed that despite a low number of KI67$^+$ naive T cells, approximately 30% proportion of the naive T cells was labeled during a 2-week labeling period. These results suggest that in young mice a constant output of T cells from the thymus is necessary to maintain the peripheral naive T cell pool. Currently we are investigating the age dependency of this mechanism.

Analysis of the role of HIV specific CD4$^+$ T cells in progression towards AIDS

In the last couple of years a host of studies on the functionality of Human Immunodeficiency virus (HIV)-specific CD8$^+$ T cells during HIV infection has been published. Loss of HIV-specific CD8$^+$ T cell functionality during progressive HIV-1 infection is currently widely accepted. However, the explanation for this loss of

function is still not clear. The contribution of HIV-specific $CD4^+$ T helper cells in controlling HIV-1 infection and maybe also for sustain of $CD8^+$ responses was investigated. This prompted us to investigate the function of HIV-specific $CD4^+$ T cells in different patient groups, at several stages of infection to see whether indeed, loss of HIV-specific $CD4^+$ T cell function may be the causal mechanism behind the lack of control of HIV by $CD8^+$ T cells and progression to AIDS.

To further investigate the importance of HIV-specific $CD4^+$ T cell function in controlling HIV-1 infection, cytokine production, proliferation and phenotype of HIV-specific $CD4^+$ T cells was analyzed in a prospective cohort study and related to HIV-specific $CD8^+$ T cell functionality during the natural course of HIV-infection. Slow and rapid progressors to AIDS with a median follow-up of respectively 118 and 57 months were included. Early in infection, equal numbers of gag-specific IFNγ-, IL-2- and IL-2&IFNγ-producing $CD4^+$ T cells were found in slow and rapid progressors. During HIV-1 infection, cytokine producing $CD4^+$ T cells decreased in rapid progressors, in 3 out of 7 individuals to undetectable levels, and this was paralleled by a decrease in proliferative capacity. This suggested that the presence of HIV-specific $CD4^+$ T cell responses early in HIV-1 infection did not protect against rapid progression to AIDS in treatment naive patients. Furthermore, the observed loss of $CD4^+$ T cell function in rapid progressors was specific for HIV, as CMV-specific $CD4^+$ T cell functionality did not change during progressive HIV infection. We extended these analyses to a group of 96 HIV-infected seroconverters and could demonstrate using survival analyses that, indeed, the presence of HIV-specific $CD4^+$ T cells early in HIV-1 infection was not related to progression to AIDS. Only minor evidence for a role of $CD4^+$ T cell help in preservation of CTL function was found, suggesting that loss of HIV-specific $CD8^+$ T cell function in individuals progressing to AIDS can not be exclusively explained by lack of $CD4^+$ T cell help.

The effect of HAART on the function of HIV specific $CD4^+$ cells

Previously, it was reported that highly active antiretroviral therapy (HAART) during primary HIV-1 infection may rescue HIV-1 specific $CD4^+$ T cell responses. We set out to determine the duration of this preserved response, and investigated the long

term effect of HAART during acute infection on HIV-specific $CD4^+$ T cell function related to possible immune control during subsequent therapy interruption. Absolute numbers, but not percentages, of gag-specific $IFN\gamma^+$, $IL\text{-}2^+$, or $IL\text{-}2^+\&IFN\gamma^+$ $CD4^+$ T cells were increased in treated compared to untreated individuals up to 2 years after seroconversion. HAART during acute HIV-1 infection was associated with lower viral load, but did not result in increased proliferation of HIV-specific $CD4^+$ T cells. One out of five individuals who discontinued therapy showed evidence for immune control. However, also patients that failed to control viremia had measurable proliferative HIV-specific $CD4^+$ T cell responses and preserved numbers of cytokine producing $CD4^+$ T cells. Thus, early HAART during primary HIV-1 infection results in higher numbers of HIV-specific $IFN\gamma^+$ and $IL\text{-}2^+$ producing $CD4^+$ T cells, but this preservation in 4 out of 5 patients was not associated with control of viremia upon treatment interruption.

The effect of long-term HAART during chronic HIV-1 infection on HIV-specific $CD4^+$ T cell function was studied and compared to $CD4^+$ T cell responses against the persistent herpes viruses CMV and Epstein-Barr virus. To this end, HIV- and herpes virus-specific cellular immune responses were measured longitudinally in 10 seroconverters with long-term follow-up including 50 months of successful HAART. Initiation of HAART resulted in a transient increase of HIV-specific $IL\text{-}2^+\&IFN\gamma^+$ $CD4^+$ T cells, and to a lesser extent $IL\text{-}2^+$ $CD4^+$ T cells. Long-term HAART resulted in an increase in HIV-, CMV-, and EBV-specific $CD4^+$ T cell proliferative capacity. This suggests that the improved proliferative response is not specific for HIV, but reflects a more general improvement of anti-viral immune responses which is induced by HAART.

The role of EBV-specific $CD4^+$ T cells in maintaining control over EBV infection

HIV-infected individuals have a highly increased incidence of (EBV-positive) B cell non-Hodgkin's lymphomas (AIDS-NHL) due to uncontrolled EBV-driven B cell proliferation because of loss of functional EBV-specific $CD8^+$ T cells. Since $CD4^+$ T cells seem to play an important role in the functional maintenance of $CD8^+$ T cells, we aimed to document the $CD4^+$ T helper cell response against EBV in AIDS-NHL patients.

Therefore, we set up a method combining ex vivo expansion of specific T cells with flow-cytometric analysis of IFNγ production to study EBV-specific $CD4^+$ T cells after stimulation with overlapping peptide pools from a latent (EBNA1) and a lytic (BZLF1) EBV protein. In a cross-sectional study, untreated HIV-infected individuals had a lower $CD4^+$ T cell response to both EBNA1 and BZLF1 as compared to healthy EBV carriers and HAART-treated HIV^+ subjects. This suggests loss of EBV-specific $CD4^+$ T cells due to HIV infection, while HAART might restore this response. In addition, we found an increase in EBNA1-specific $CD8^+$ T cell responses in HAART-treated subjects. Interestingly, numbers of EBV-specific $CD4^+$ and $CD8^+$ T cells were inversely correlated with EBV viral load, suggesting an important role also for EBV-specific $CD4^+$ T cells in the control of EBV infection. Next, we studied the role of EBV-specific $CD4^+$ T cells in the maintenance of control over EBV-infected cells *in vivo*. To this end, $CD4^+$ and $CD8^+$ memory T cells directed against EBNA1 and BZLF1 were studied longitudinally in 9 progressors to NHL, 4 progressors to non-EBV-related AIDS and 4 slow progressors to AIDS. In all 3 groups, we observed a decline of EBV-specific memory $CD4^+$ and $CD8^+$ T cell responses during HIV infection. However, whereas latent antigen EBNA1-specific $CD4^+$ T cells were lost well before diagnosis in all subjects who developed an AIDS-related NHL (and EBNA1-specific $CD8^+$ T cells were significantly lower compared to the other groups), these cells were better preserved in progressors to non-EBV-related disease and slow progressors. Loss of EBNA1-specific T cell immunity thus might be important for progression to NHL. Interestingly, BZLF1-specific T cells were not lost in all progressors to NHL, suggesting a difference in the function of these cells in the surveillance of EBV-infected B cells.

The incidence of EBV-related malignancies in HIV-infected subjects has declined since the introduction HAART. Earlier studies indicated an improvement of EBV-specific $CD8^+$ T cell function after initiation of HAART. We investigated the effects of long term HAART on immune activation and EBV load on one side, and EBV-specific T cell responses on the other side. These parameters were studied in 10 subjects from early in HIV infection up to 5 years after HAART. All individuals responded to HAART by a decline in HIV viral load, a restoration of total $CD4^+$ T cell numbers, and a decline in T cell immune activation. In contrast, EBV load remained unaltered, even after 5 years

Results support the idea that chronic activation of the immune system can lead to an increase in the number of EBV-infected B cells, without altering inter-individual differences in EBV set point.

of therapy, although a decline in both $CD4^+$ and $CD8^+$ T cells specific for lytic EBV protein BZLF1 suggested a decreased EBV reactivation rate. In contrast, after 5 years of treatment, latent EBV antigen EBNA1-specific $CD4^+$ and $CD8^+$ T cell responses were restored to levels comparable with healthy individuals. In the same study subjects, we studied the long-term effect of HAART on HIV-specific $CD4^+$ T cell responses in comparison to specific $CD4^+$ T cell responses against the persistent herpes viruses Cytomegalovirus (CMV) and EBV. Long-term HAART resulted in an increase in HIV- and CMV-specific $CD4^+$ T cell proliferative capacity. Thus, restoration of EBNA1-specific $CD4^+$ T cell responses appears to be related to a general improvement of immune function.

Alteration of the EBV set point after HIV infection; role of immune activation

We previously observed that the high EBV load levels in HIV-infected individuals reflect an altered EBV set point after HIV-seroconversion, probably due to EBV reactivation. As EBV is strictly dependent on the normal B-cell biology, and HIV infection is characterized by chronic immune activation of both B and T cells, we aimed to study whether activation of the immune system could be a determinant of an elevated EBV load. EBV load and immune activation markers were studied in individuals with a chronically activated immune system, amongst whom HIV seronegative and positive homosexual men and healthy and HIV positive Ethiopian subjects. The groups with a more activated immune system, as measured by expression of CD38 and HLA-DR on T cells, had a higher EBV load. Furthermore, in HIV-infected homosexual men, increases in EBV load over time correlated with increases in immune activation, but not with changes in absolute $CD4^+$ T cell numbers. Most interestingly, there was a strong correlation between EBV load before and after HIV seroconversion, and also before and after treatment of HIV by antiretroviral therapy. These results support the idea that chronic activation of the immune system can lead to an increase in the number of EBV-infected B cells, without altering inter-individual differences in EBV set point.

Loss of CMV-specific $CD4^+$ T cell cytokine production and proliferative capacity precedes progression to HIV-related CMV end-organ disease

To define function and phenotype of CMV-specific $CD4^+$ T cells in HIV-infected individuals with different clinical end-points to identify which factors might be related to progression to CMV end-organ disease, we performed a longitudinal analysis of CMV-specific $CD4^+$ T cells in progressors to AIDS with CMV end-organ disease (AIDS-CMV) compared to long-term asymptomatics (LTA) and progressors to AIDS with opportunistic infections (AIDS-OI). We investigated production of IFNγ and IL-2, phenotype, and proliferative capacity of CMV-specific $CD4^+$ T cells after stimulation of PBMC with CMV lysate. Numbers of CMV-specific IFNγ-producing $CD4^+$ T cells were higher than IL-2-producing $CD4^+$ T cells. In LTA and progressors to AIDS-OI, numbers of IFN-producing $CD4^+$ T cells remained detectable during follow-up, but decreased sharply in individuals progressing to AIDS-CMV a year before onset of CMV end-organ disease. In parallel, CMV-specific IL-2-production and proliferative capacity were lost in progressors to AIDS-CMV. Most CMV-specific cytokine-producing $CD4^+$ T cells were of the $CD27^-$ phenotype. Initially, the majority of the $IFN\gamma^+CD4^+$ T cells were of the $CD45RO^+CD27^-$ effector subset, but during progression to AIDS-CMV a shift in phenotype to the highly differentiated $CD45RO^-CD27^-$ subset was observed. Our data indicate that loss of CMV-specific cytokine production and proliferative capacity is associated with progression to AIDS-CMV. Accumulation of $CD4^+$ T cells with a $CD45RO^-CD27^-$ phenotype suggests that persistent antigen exposure drives differentiation of CMV-specific $CD4^+$ T cells towards a non-IL-2-producing, poorly proliferating, and highly differentiated 'effector' subset, which eventually also fails to produce IFNγ in patients developing AIDS-CMV.

Development of HLA class II tetrameric constructs for direct visualization of CMV-specific $CD4^+$ T cells

We also developed tools to study CMV-specific $CD4^+$ T cells in more detail. To this end, we set up an expression system to produce HLA-class II-tetrameric complexes. A Schneider cell expression system is used to produce correctly folded HLA class II tetramers with a specific (CMV) peptide covalently linked to the class II-α-chain. A DRB1-0301 construct was made. For this DR3 molecule, 12 CMV helper epitopes

were predicted for presentation in HLA-DR3. As a control a known DR3-restricted peptide from mycobacterium Tuberculosis (MTB) was also coupled to the DR3 construct. So far, only one of the tested predicted CMV epitopes showed a response in healthy CMV carriers. This peptide was included in the DR3 construct. The DR3-peptide transfected Schneider cells showed stable expression of DR3 monomers. These were purified and tetramerised. Correctly folded DR3 complexes were produced which could be used to stain a DR3-retricted MTB-specific T cell clone and CMV-specific T cell lines, respectively. However, in CMV-positive individuals only few specific T cells could be detected using the tetramer.

In HIV-1-infected children CMV rather than HIV triggers the outgrowth of effector $CD8^+CD45RA^+CD27^-$ T cells

The way $CD8^+$ T cells differentiate during chronic infection in children is unclear. In a cohort of HIV-1-infected children treated with HAART, we analyzed the absolute lymphocyte numbers of various subsets of $CD4^+$ and $CD8^+$ T cells. Prior CMV, but not EBV or VZV infection correlated with an increased number of $CD8^+CD45RA^+CD27^-$ effector T cells at baseline as well as an increased state of T cell activation as defined by HLA-DR and CD38 expression. The expansions of $CD8^+CD45RA^+CD27^-$ T cells persisted over time, independent of the HIV response to HAART. Numbers of $CD8^+CD45RA^+CD27^-$ T cells were significantly higher in patients with CMV replication, as reflected by persistent urinary CMV shedding and periodic CMV DNAemia. These patients also showed an increase in CMV-specific antibodies, compared to those without CMV shedding. The number of CMV-specific IFNγ-producing $CD8^+$ T cells were lower in children who persistently shed CMV compared to those who did not. CMV-specific $CD4^+$ T cell responses could be detected at similar levels regardless of shedding.
In conclusion, in HIV-1-infected children expansions of total $CD8^+CD45RA^+CD27^-$ T cells seems to be related to persistent replication of CMV, which potentially contributes to chronic immune activation in pediatric HIV-1 infection.

Key publications

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Antigen presentation

Principal investigator
S Marieke van Ham PhD
Dept of Immunopathology
(m.vanham@sanquin.nl)

Antigen presentation Research addresses the question how the humoral immune response is regulated by MHC class II-mediated antigen presentation in B cells and dendritic cells.
On one hand we continued our research on the regulation and cellular mechanisms that lead to efficient class II MHC molecules in normal and malignant B cells in cooperation with the Depts of Hematology and Pathology of Vrije Universiteit Medical Center. In human B cells, effective class II-Ag presentation depends on MHC class II, but also on HLA-DM (DM) and HLA-DO (DO), the chaperones regulating the composition of the peptide repertoire. We have now demonstrated that multiple and independent aberrations in the expression of these proteins in malignant B cells correlate with the disease status in B cell chronic lymphocytic leukemia abnormal. In addition, we have identified aberrancies in the T cell compartment of these patients. Currently, we are investigating the relationship between the aberrant antigen presentation pathway in the malignant B cells and the observed T cell deviations. In addition, we are currently generating B cell line systems expressing the various components of the class II antigen presentation pathway tagged to fluorescent

reporters to study the dynamics of B cell-mediated class II antigen presentation in life cells.

In the past year, we have started an extensive project in collaboration with the Dept of Experimental Immunohematology to develop clinically approved, validated and cost-efficient monocyte-derived dendritic cell products, which will serve as product in clinical trials for tumor vaccination and possibly tolerizing therapy in autoimmune disease and transplantation. We have set up the methods to generate both immature and mature DCs from monocytes has been set up as well as assays to measure the phenotype of immature and mature dendritic cells by flowcytometry. In addition we are now able to test the functionality of the immature and mature dendritic cells using cytokines assays, dextran-FITC uptake assays to determine the capacity of the cells to endocytose antigen and T cell proliferation assays to determine the antigen presentation and T cell activation potential of the DC generated. We are currently investigating which closed system isolation of monocytes is optimal for DC generation in collaboration with the Blood Bank North West Region.

Finally, research on the use of tetrameric MHC class II molecules as tools to monitor antigen specific T cells in relation to antibody formation against therapeutic proteins was continued and transfer of the technique to Sanquin Reagents for commercialization of the product was ensured.

Key publication

Chamuleau MED, Souwer Y, van Ham SM, Zevenbergen A, Westers TM, Berkhof J, Meijer CJ, van de Loosdrecht AA, Ossenkoppele GJ. Class II-associated invariant chain peptide expression on myeloid leukemic blasts predicts poor clinical outcome. Cancer Res 2004; 64(16):5546-50.

Immunomodulation of blood transfusions in transplantation tolerance

Principal investigator
Prof Anneke Brand MD PhD
Dept Research and Education South West Region (anneke.brand@bloodrtd.nl)

Pretransplantation blood transfusions impair the allograft rejection against donor grafts. Neither the mechanism, nor the factors in the blood product relevant to this effect are known. In 2004 a prospective study started in recipients of combined kidney-pancreas transplantation. Patients deliberately receive a 1 HLA-DR shared red blood cell concentrate with buffy-coat. At regular intervals after transplantation the establishment of (donor specific) regulator T cells is evaluated. This research is done in collaboration with Prof FHJ Claas, Leiden University Medical Center.

Key publication

Roelen D, Brand A and Claas FHJ. Pretransplant blood transfusions revisited: a role for $CD4^+$ regulatory T cells? Transplantation 2004; 77:26-8.

Allergy

Principal investigators
Ronald van Ree PhD
Dept Immunopathology (r.vanree@sanquin.nl)

The Allergy Research group focuses on the humoral immune response against inhalant and food allergens. This work is carried out with essentially four different aims: improvement of diagnostic tests, development of novel strategies for allergen-specific immunotherapy, identification of factors that determine the biological activity of specific IgE antibodies and establishing the role of the hygiene hypothesis in the development of allergy.

The study of the IgE response against food allergens is carried out in the frame of EU fifth and sixth framework projects and focuses on the improvement of food allergy diagnosis by replacing extracts by purified (recombinant) food allergens. Clinical impact of individual allergens has been shown to differ. It has been demonstrated that this is largely dependent on the degree of resistance of these allergens against digestive enzymes in the gastro-intestinal tract. This knowledge is the basis for new diagnostic tests with improved clinical relevance. For the development of novel strategies for immunotherapy recombinant allergens are also evaluated to replace

extract-based products. This work is carried out as contract research for allergen manufacturers. Recombinant Fel D 1 has been produced in yeast and subsequently been chemically modified to reduce allergenicity. Preparations are being made to start clinical trials to treat cat-allergic patients. For the study of the biological activity of specific IgE antibodies basophil histamine release tests have been performed with many different purified natural and recombinant food and inhalant allergens. Affinity and epitope valency of interactions between IgE and allergens are important factors for biological activity. This work is mainly carried out by comparing food-sensitized patients with and without clinical allergy and by comparing children from developed and developing countries sensitized to house dust mite (hygiene hypothesis). The work on the hygiene hypothesis is done in collaboration with the Leiden University Medical Center (Dr Maria Yazdanbakhsh).

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Van Ree R. Clinical importance of cross-reactivity in food allergy. Curr Opin Allergy Clin Immunol 2004; 4:235-40.

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Blood transmitted infections

Virological aspects of AIDS pathogenesis

In vivo evolution of X4 human immunodeficiency virus type 1 variants

Principal investigator
Prof Hanneke Schuitemaker
PhD
Dept Clinical
Viro-Immunology
(h.schuitemaker@sanquin.nl)

Human immunodeficiency virus type 1 (HIV-1) variants that use coreceptor CXCR4 (X4 HIV variants) in addition to CD4 evolve from HIV-1 variants with a restricted CCR5 coreceptor usage (R5 HIV variants). Early after their first appearance *in vivo*, X4 HIV-1 variants additionally use CCR5. The ability to use CCR5 in addition to CXCR4 is generally lost late in infection. We studied whether this evolution of the coreceptor repertoire is also reflected in a changing sensitivity of X4 variants to CXCR4 antagonists such as peptide T22 and the synthetic compound AMD3100. We observed differences in the concentrations of CXCR4 antagonists needed to suppress replication of X4 HIV variants from different patients. In general, late X4 HIV variants were less sensitive to AMD3100 than early R5X4 HIV variants. The differences between early R5X4 HIV variants and late X4 variants were less pronounced for T22-mediated inhibition. These results suggest an ongoing evolution of X4 virus variants toward more efficient usage of the cellular entry complex.

Key publication

Koning FA, Jansen CA, Dekker J, Kaslow RA, Dukers N, van Baarle D, Prins M, Schuitemaker H. Correlates of resistance to HIV-1 infection in homosexual men with high-risk sexual behaviour. AIDS 2004; 18(8):1117-26.

Increased sensitivity to CD4 binding site-directed neutralization following *in vitro* propagation of HIV-1 on primary lymphocytes.

Principal investigator
Prof Hanneke Schuitemaker
PhD
Dept Clinical
Viro-Immunology
(h.schuitemaker@sanquin.nl)

We previously reported the adaptation of the neutralization-sensitive human immunodeficiency virus type 1 (HIV-1) strain IIIB to a neutralization-resistant phenotype in an accidentally infected laboratory worker. During long-term propagation of this resistant isolate, designated FF3346, on primary peripheral blood leukocytes *in vitro*, an HIV-1 variant appeared that had regained sensitivity to neutralization by soluble CD4 (sCD4) and the broadly neutralizing monoclonal antibody b12. When an early passage of FF3346 was subjected to limiting-dilution culture in peripheral blood mononuclear cells, eight virus variants with various degrees of neutralization resistance

were isolated. Two of them, the sCD4 neutralization-resistant variant LW_H8 (res) and the sCD4 neutralization-sensitive variant LW_G9 (sens), were selected for further study. Interestingly, these two viruses were equally resistant to neutralization by agents that recognize domains other than the CD4 binding site. Site-directed mutagenesis revealed that the increased neutralization sensitivity of variant LW_G9 (sens) resulted from only two changes, an Asn-to-Ser substitution at position 164 in the V2 loop and an Ala-to-Glu substitution at position 370 in the C3 domain of gp120. In agreement with this notion, the affinity of b12 for monomeric gp120 containing the N164S and A370E substitutions in the background of the molecular clone LW_H8 (res) was higher than its affinity for the parental gp120. Surprisingly, no correlation was observed between CD4 binding affinity for monomeric gp120 and the level of neutralization resistance, suggesting that differences in sCD4 neutralization sensitivity between these viruses are only manifested in the context of the tertiary or quaternary structure of gp120 on the viral surface. The results obtained here indicate that the neutralization-sensitive strain IIIB can become neutralization resistant *in vivo* under selective pressure by neutralizing antibodies but that this resistance may be easily reversed in the absence of immunological pressure.

Key publication

Beaumont T, Quakkelaar E, van Nuenen A, Pantophlet R, Schuitemaker H. Increased sensitivity to CD4 binding site-directed neutralization following *in vitro* propagation on primary lymphocytes of a neutralization-resistant human immunodeficiency virus IIIB strain isolated from an accidentally infected laboratory worker. J Virol 2004; 78(11):5651-7.

Reduced Trim5α mRNA levels in MDM coincided with susceptibility to HIV-1 infection, indicating that Trim5α is involved in the post-entry restriction of HIV-1 in macrophages.

Correlates of resistance to HIV-1 infection in homosexual men with high-risk sexual behavior

Principal investigator
prof Hanneke Schuitemaker
PhD
Dept Clinical
Viro-Immunology
(h.schuitemaker@sanquin.nl)

Correlates of HIV resistance were investigated in participants from the Amsterdam Cohort of Homosexual men who have remained HIV seronegative despite high-risk sexual behavior.

We studied *in vitro* HIV-1 susceptibility and adaptive and innate immunity in 29 high-risk seronegative (HRSN) and 15 HIV-negative pre-seroconversion (pre-SC) homosexual men from the same Amsterdam Cohort Study (ACS) who seroconverted to HIV-1 positive during active follow-up. Host genetics were compared between HRSN and HIV-positive ACS participants.

In vitro susceptibility for a CCR5–using (R5) HIV-1 variant was lower while RANTES production levels were higher in HRSN, but no differences in coreceptor expression were observed between HRSN and pre-SC controls. Reduced R5 *in vitro* susceptibility of two HRSN tested was restored to normal levels by addition of antibodies against beta-chemokines. A higher proportion of HRSN carried the SDF-1 3'A variant and HLA-A*11, A*31 and Cw*15 alleles. ELIspot analysis with HIV-1 peptide stimulation revealed low frequencies of HIV-1-specific CD8 interferon-gamma producing cytotoxic T cells in both HRSN and pre-SC controls. From this study we conclude that the lower *in vitro* R5 susceptibility of cells from the HRSN men was due to beta-chemokine mediated inhibition of virus replication. The presence of HIV-1 specific cytotoxic T cells in both HRSN and pre-SC participants may signify exposure to the virus rather than protection from infection. Host genetic characteristics and other factors affecting innate immunity may contribute to differential resistance to HIV-1 infection among exposed seronegative individuals.

Post entry restriction of the HIV-1 based lentiviral vector

Principal investigator
Neeltje Kootstra MD PhD
Dept Clinical
Viro-Immunology
(n.kootstra@sanquin.nl)

HIV-1 depends on host cell factors for infection and replication. Accordingly, differential expression and species variation of these cellular factors play an important role in the cellular tropism of the virus. Recently, Trim5α, a component of cytoplasmic bodies, has been identified as the simian restriction factor Lv1 and the human restriction factor Ref1. Although the mechanism of this restriction is still unclear, the viral determinant involved is located in the cyclophilin A (CyPA) binding region of the

gag-capsid protein, and mutations in this region can make the virus resistant to Lv-1. HIV-1 group O and HIV-2 are highly related to HIV-1 (group M) and similar to group M HIV-1. In this study we analyzed whether these viruses were resistant to Ref1 and Lv-1. When the CyPA binding region of HIV-1 group O and HIV-2 was placed into the wild type HIV-1 group M backbone, the virus became resistant to Ref1, however resistance to Lv1 was only observed with the HIV-2 CyPA binding region. To analyze which part of the CyPA binding region was responsible for the differential resistance to Lv1 and Ref1 additional chimeric viral vectors were constructed. Resistance to Lv1 and Ref1 could not be attributed to specific amino acid changes or regions in the CyPA binding region, suggesting that conformational changes in the binding loop are most likely responsible for the observed differential resistance to Lv-1 and Ref1. HIV-1 infection in monocyte derived macrophages (MDM) is also restricted at a post-entry step depending on the cellular differentiation stage. We measured the level of Trim5α mRNA during differentiation of monocytes into MDM. TRIM5α levels varied between donors but in all donors the Trim5α mRNA levels decreased during the first 5 days of differentiation. Reduced Trim5α mRNA levels in MDM coincided with susceptibility to HIV-1 infection, indicating that Trim5α is involved in the post-entry restriction of HIV-1 in macrophages.

Quality, safety and efficiency

Pathogen detection and inactivation

Pathogen inactivation in platelet concentrates

Principal investigators
Dirk de Korte PhD
Dept Blood Cell Research
(d.dekorte@sanquin.nl)
Frank van Engelenburg PhD
Dept Clinical
Viro-Immunology
(f.vanengelenburg@
sanquin.nl)

In collaboration with CAF/DCF (Brussels), we studied the use of UV-C illumination as delivered by a lab scale apparatus developed by CAF. In this apparatus, various experimental parameters were investigated for their effect on virus inactivation and platelet quality. The amount of residual plasma was found to be the most important factor, with opposite effects on viral kill and platelet quality. Smaller volumes (resulting in shorter light paths) had a positive effect on virus kill, with some negative effect on platelet quality.

As most important side effect of UV-C illumination, it was noted that platelet counts dropped by about 30% in the presence of 10% plasma. Recent experiments with IIb/IIIa antagonists have shown that this decrease is caused by aggregation, due to IIb/IIIa activation and subsequent fibrinogen binding. Activation of IIb/IIIa could be prevented by addition of forskolin (elevating intracellular cAMP) and suppressed by 30% plasma. Moreover, aggregation does not occur in the complete absence of plasma, showing that plasma exerts both a beneficial effect (at high concentration) by suppressing IIb/IIIa activation and a detrimental effect by enabling fibrinogen binding. It remains to be determined how this knowledge can be used for a procedure applied on whole units of platelets.

Measurements of the platelet metabolism after UV-C treatment revealed a significant drop of the mitochondrial membrane potential (MMP) after 5 days of storage, in conjunction with an increase in lactate formation. The resulting drop in pH, however, could largely be prevented by using a synthetic medium with a high buffer capacity (PASIII-M). It remains to be determined whether the drop in MMP is caused by a damaging effect on the mitochondrial compartment or to an increased rate of ATP consumption, stimulating mitochondrial respiration.

In most studies on viral inactivation, BVDV was used as model for enveloped viruses. In an extensive study, also inactivation of other viruses was studied. With 10% plasma present, the order of sensitivity for UV-C was VSV > CPV > BVDV > PSR. With the latter virus, about 3 log kill was achieved. Inactivation of extra cellular HIV was only 1.85 log, whereas cell-associated HIV (as present in infected H9 cells) was almost

A broad-range 16S rDNA PCR assay was developed.

insensitive towards UV-C treatment. The latter results strongly limit the potential of UV-C treatment for blood bank practice and therefore new avenues strategies have to be pursued to enhance HIV inactivation.

Key publication

Li J, de Korte D, Woolum MD, Ruane PH, Keil SD, Lockerbie O, McLean R, Goodrich RP. Pathogen reduction of buffy coat platelet concentrates using riboflavin and light: comparisons with pathogen-reduction technology-treated apheresis platelet products. Vox Sang 2004; 87:82-90.

Detection of bacterial contamination of blood products: 16S rDNA PCR

Principal investigator
Henk Reesink MD PhD
Dept Research
and Education
North West Region
(h.reesink@sanquin.nl)

Based on real-time PCR technology a broad-range 16S rDNA PCR assay was developed and optimized in 2003 in collaboration with the Vrije Universiteit Medical Center Dept of Microbiology (head Prof Vandenbroucke-Grauls).
Compared to automated culturing (current gold standard) the assay was shown to be 100% specific and 100% sensitive. The assay enables detection of 50 CFU/mL and the results are obtained in a timely fashion (within 4 hours). A paper is in preparation.
Aside from the 16S rDNA PCR assay, a PCR test was also developed to estimate the number of residual white blood cells in leuko-reduced PCs. This HLA-DQA1 assay was shown to be more sensitive than flow cytometry and very suitable for use in a high throughput setting. The paper has been published in 2004.
Since the 16S rDNA assay can not distinguish between viable and non-viable bacteria, another assay based on RT-PCR was developed. The preliminary results were encouraging and the method may find application in studying the effect and resistance of bacteria to antibiotics. A patent application and a manuscript describing the method are in preparation.

Propionibacterium is frequently associated with contamination of PCs. By means of AFLP fingerprinting attempts were made to trace the source of contamination. This method may prove very valuable in providing information about bacteria contaminating blood products; a paper is prepared. This project received a grant from Sanquin until November 2004, a new grant application has been submitted to further explore applications for this interesting assay.

Impedance measurement

Principal investigator
Henk Reesink MD PhD
Dept Research
and Education
North West Region
(h.reesink@sanquin.nl)

The project of impedance measurement for detection of bacteria in platelet concentrates granted by the European Commission in the Fifth Framework of demonstration projects has been delayed. Sanquin Blood Bank North West Region has been assigned as administrative coordinator for the Consortium consisting of Magen David Adom blood center in Tel Aviv and a company in Israel, a company in Germany, the Dept of Microbiology of the Slotervaart Hospital and Sanquin Blood Bank North West Region. The initially proposed sensors were not sensitive enough and will be further developed. Therewith the investigations in the blood centers and microbiology laboratories with samples of platelet concentrates spiked with various species of bacteria will start in 2005.

Key publication

Peters RP, Mohammadi T, Vandenbroucke-Grauls CM, Danner SA, van Agtmael MA, Savelkoul PH. Detection of bacterial DNA in blood samples from febrile patients: underestimated infection or emerging contamination? FEMS Immunol Med Microbiol 2004; 42(2):249-53.

Functional characteristics of photochemically treated platelets

Principal investigator
Prof Dick van Rhenen
MD PhD
Dept Research
and Education
South West Region
(dick.van.rhenen@
bloodrtd.nl)

A photochemical treatment (PCT) process using the psoralen compound amotosalen HCL (S59) and long wavelength ultraviolet (UVA) light was developed for inactivation of infectious pathogens and leukocytes. The effect of PCT on functional characteristics of the platelets was evaluated *in vitro*. Platelet concentrates (PC) treated photochemically using the experimental T-bag S59 Reduction Device (SRD) (n=4) or the commercially available Wafer SRD (n=4) were compared with control platelet concentrates in plasma/PAS III (n=4). Overall quality was estimated by: pH, pO_2, pCO_2, HCO_3^-, platelet count, mean platelet volume, plasma glucose, plasma lactate, and total ATP), the function (expression of p-selectin, hypotonic shock response and aggregation capacity using collagen, thrombin and ADP as agonists), apoptosis (caspase 3 and annexin V) and lysis (plasma LDH). No differences were found for the product quality parameters, for p-selectin expression, hypotonic shock response and the apoptosis parameters ($p>0.05$). PCT using the experimental T-bag SRD led to a significant decrease in aggregation capacity with collagen and thrombin ($p<0.05$), whereas no decrease

with the commercially available Wafer SRD was found. In addition a significant increase in plasma LDH was found using the experimental T-bag SRD in contrast to the commercially available Wafer SRD ($p<0.05$). The results show no evidence that photochemical treatment in combination with the commercially available Wafer SRD affect the quality of platelet concentrate in plasma/PAS III.

Key publication

Jansen AJG, van Vliet HHDM, Vermeij J, Beckers EAM, Leebeek FWG, Sonneveld P, van Rhenen DJ. Functional characteristics of photochemically treated platelets. Transfusion 2004; 44(3):313-9.

Photodynamic sterilization of cellular blood products with porphyrin

Principal investigator
Prof Anneke Brand MD PhD
Research and Education
South West Region
(anneke.brand@bloodrtd.nl)

Research on red cell and stem cell sterilization using a porphyrin (Tri-P-4) shows limited red cell damage *in vitro* and sufficient inactivation of non-enveloped viruses. We found however increased IgG-binding to red cells, a phenomenon also observed by others using different methods of photodynamic treatment of red cells. We further focus on the observed increase in IgG binding on Tri-P-4 treated red cells. The level of this defect seems located at the cell membrane and its nature is further studied. Red cell antibody formation was the reason that clinical studies using other methods of red cell Photo-activation by Cereus/Baxter were abrogated. This is a collaboration with Leiden University Medical Center Dept of Immunohematology (D Roelen) and Sanquin Research (A Verhoeven, F Engelenburg and J Lagerberg).

Key publication

Trannoy LL, Lagerberg JWM, Dubbelman TMAR, Schuitmaker JJ, Brand A. Positively charged porphyrins: a new series of photosensitizers for sterilization of RBCs. Transfusion 2004; 44:1186-96.

S-59 platelet inactivation (EuroSPRITE)

Principal investigator
Prof Dick van Rhenen
MD PhD
Dept Research
and Education
South West Region
(dick.van.rhenen@
bloodrtd.nl)

The Blood Bank South West Region participated in a European study on the effect of S-59 platelet inactivation in collaboration with Baxter Healthcare and Cerus Corporation (euroSPRITE study *in vitro* and *in vivo* research was conducted to the safety and effectiveness of transfusions with S59 photochemically treated platelets). Photochemical treatment inactivates the DNA/RNA present in the blood product and this inactivates the viruses, bacteria, protozoa and leukocytes. The results are promising. A national study to investigate the performance of S-59 platelets in clinical practice in collaboration with the Dutch Hemato-oncological Research Federation (HOVON), is in preparation.

Key publication

Van Rhenen DJ, Gulliksson H, Cazenave JP, Pamphilon D, Davis K, Flament J, Corash L. Therapeutic efficacy of pooled buffy coat platelet components prepared and stored with a platelet additive solution. Transfusion Medicine 2004; 14:289-95.

Prion research

Principle investigator
Frank van Engelenburg
PhD
Dept Clinical
Viro-Immunology
(f.vanengelenburg@
sanquin.nl)

The prion research line continued to focus on the development of assays for infectious prions in blood and assays to monitor prion protein in the production process in collaboration with the Dept of Virus Safety Services. The assay for the infectious prion protein and the assay for the protein EDRF, which is considered to be a surrogate marker for prion disease, were further optimized. A panel of healthy donors was tested to establish the normal range for these parameters. In addition, patients suffering from various neurological diseases were tested. This demonstrated that in some neurological diseases EDRF levels are elevated compared, indicating that the EDRF assay may be of diagnostic value for neurological disorders.

Validation of the model virus approach

Principle investigator

Frank van Engelenburg PhD
Dept Clinical
Viro-Immunology
(f.vanengelenburg@
sanquin.nl)

The preclinical evaluation phase of development of plasma derivatives includes validation of the virus reducing capacity of putatively virus inactivating of removing steps. In addition novel technologies have been developed for pathogen reduction or blood components like platelets. Virus validation studies are typically performed using a series of relevant and model viruses. This approach has been appreciated for many years, but recently the model virus approach has been debated again since differences between the resistance of human parvovirus B19 (B19) and frequently used model viruses for B19, like canine parvovirus (CPV) were described by a few groups. In those studies B19 appears to be significantly less resistant than the model viruses. Therefore the use of model viruses may lead to underestimation of the virus reducing capacity of virus inactivating steps for the relevant virus. To facilitate comparison between B19 and model viruses, we compared the virus reducing capacity of a series of different virus reducing steps. Since we have no *in vitro* culture system available for B19, we used a highly viraemic plasma from a blood donor for spiking and real-time PCR as a read-out. Such experimental set-up is readily suitable for evaluation of virus removal steps. E.g. we compared the virus removing capacity of precipitation of Cohn's fraction III and of nanofiltration steps. In particular conflicting results were found for the latter step: nanofiltration of intravenous immunoglobulin resulted in >6.2 $\log_{10}$ reduction for B19, and >4.1 & ≤ 5.0 $\log_{10}$ for CPV. This difference can be explained by the presence of anti-B19 antibodies improving the removal of B19, but not CPV, which is not cross-neutralized by B19 antibodies. For prothrombin complex concentrate 4.9 $\log_{10}$ reduction was found for B19, and >6.3 $\log_{10}$ for CPV. However, when the B19 sample was treated with DNase I complete removal was demonstrated suggesting that the residual reactivity was due to the presence of free B19 DNA. The B19-real time-PCR system is currently modified to also facilitate evaluation of virus inactivating steps. Due to physico-chemical treatment, the viral capsid can destabilize and the viral nucleic acid becomes susceptible to nuclease treatment. This destabilization could serve as a surrogate marker for loss of infectivity. We have designed a method to treat B19 with a combination of double-stranded and single-stranded DNA nucleases, and are using this method to evaluate virus inactivating steps, like heat treatment, low pH treatment, and sanitization steps.

To further validate the model virus approach we are also setting up systems for hepatitis B virus and hepatitis C virus and compare removal of these viruses by various virus reducing steps with that of their respective model viruses.

Improving materials and methods for blood bank processing

Counting residual leukocytes in blood components

Principal investigator
Ruby Pietersz MD PhD
Dept Research
and Education
North West Region
(r.pietersz@sanquin.nl)

In search for an accurate method for counting low levels of white blood cells (WBCs) a real-time (rt) PCR was developed, using the Magnapure for DNA extraction and an albumin probe for amplification. The PCR was performed on a Taqman ABI prism 7000. With this method DNA from WBCs could be discriminated from cell-free DNA. Various amounts of cell-free DNA were observed in plasma of individual donors and in blood products from these donors. These results were published in 2004.
Spiking experiments with various amounts of 'male' WBCs into double filtered 'female' platelet concentrates (PCs) indicated that fragments of 'male' WBCs occur in PCs. For these experiments a specific rt PCR detecting exclusively DNA of 'male' WBC, an albumin rt PCR detecting both 'female' and 'male' DNA and flowcytometry for measuring intact WBCs were used to calculate the amounts of WBC fragments. Asfor the cell-free DNA, WBC fragments were not removed by WBC reducing filters. Furthermore, when WBCs were spiked in PBS an increasing amount of DNA, derived from WBC fragments, were observed during storage, while the amount of intact WBCs decreased. When plasma spiked with WBCs was stored for >48 hours at room temperature before filtration the amount of WBC fragments in the product increased and reached levels $>5 \times 10^6$ WBC equivalents. It is known that that dose of intact WBCs may induce HLA immunization. The results about WBC fragments also resulted in a paper which was published in 2004.
Further studies showed that in buffy coat derived PCs a significantly higher amount of WBC fragments was found than in PCs prepared by the whole blood soft-spin platelet-rich plasma (PRP) method, commonly used in the USA. About these results a short

article has been submitted in 2004. The investigations were performed in collaboration with Sanquin Research Dept of Immunohematology (Dr Ellen van der Schoot).

Key publications

Dijkstra-Tiekstra MJ, Pietersz RNI, Reesink HW, van der Schoot CE. Influence of cell-free DNA in plasma on real-time polymerase chain reaction for determination of residual leucocytes in platelet concentrates. Vox Sang 2004; 86:130-5.

Dijkstra-Tiekstra MJ, van der Meer PF, Pietersz RNI, de Wildt-Eggen J. Multicenter evaluation of two flow cytometric methods for counting low levels of white blood cells. Transfusion 2004; 44:1319-24.

Mohammadi T, Reesink HW, Vandenbroucke-Grauls MJE, Savelkoul PHM. Real-time amplification of HLA-DQA1 for counting residual white blood cells in filtered platelet concentrates. Transfusion 2004; 44:1314-8.

Beckman N, Sher G, Masse M, Richter E, Ringwald J, Rebulla P, van der Meer PF, Justica B, Walker B, Rowe G. Review of the quality monitoring methods used by countries using or implementing universal leukoreduction. Transf Med Rev 2004; 18:25-35.

Bacterial contamination of platelet concentrates

Principal investigator
Dirk de Korte PhD
Dept Blood Cell Research
(d.dekorte@sanquin.nl)

During 2002 and 2003, a pilot study with diversion of the first blood volume during collection of whole blood units was performed. No large problems by the new collection system were met in practice and a reduction of 50% was shown for the bacterial contamination of platelet concentrates (PC) prepared from 5 pooled buffy coats. Based on these results, diversion became obligatory nationwide in the Netherlands starting July 1, 2004.

Prior to the implementation of diversion, a total of 25,487 PC units were tested, with 273 initial positive signals (1.07%). After implementation of diversion, a total of 35,165 PC units were tested, with 150 initial positive signals (0.43%). The percentage initial positive units was very comparable to the value of 0.37% obtained in the pilot

study with 6749 units. Both the frequency of Coagulase Negative Staphylococci (from 0.30% to 0.11%) and Diphteroid rods (from 0.54% to 0.19%) were significantly decreased by the intervention studied.

Key publications

de Korte D, Marcelis JH, Verhoeven AJ, Soeterboek AM. Diversion of first blood volume results in a reduction of bacterial contamination for whole-blood collections. Vox Sang 2002;83(1):13-6.

de Korte D, Curvers J, de Kort WLAM, Hoekstra T, van der Poel CL, Beckers EAM, Marcelis JH. Effects of skin disinfection method, deviation bag and bacterial screening on clinical safety of platelet concentrates in The Netherlands. Transfusion (accepted for publication).

Improving materials and methods for storage of blood components

Crypreservation of red blood cells

Principal investigator
Johan Lagerberg PhD
Dept Blood Cell Research
(j.lagerberg@sanquin.nl)

Research on the crypreservation of red blood cells has focused on improvement of the high glycerol/-80 °C method. For this method, a closed system to glycerolize and deglycerolize is now available, that may allow the storage time after thawing of previously frozen red blood cells to be longer than 24 hours.
Glycerol was added to a final concentration of 40% and red blood cells were frozen and stored at -80 °C for at least 4 weeks. After thawing and deglycerolization, the cells were resuspended in additive solution. Two additive solutions, SAG-M and AS-3 were tested for their ability to maintain the quality of thawed red cells during storage at 2–6 °C. Based on the limit of 0.8% hemolysis, the maximal storage time of thawed red cells in SAG-M was 2 days, while in AS-3 hemolysis remained below 0.8% for at least 21 days. However, the ATP content of the red cells, which correlates with *in vivo* recovery, was better maintained in SAG-M as compared to AS-3. The faster decline of ATP could be the lower pH of AS-3, which results in a lower intracellular pH. Further studies with various washing and/or additive solutions, will be undertaken to prevent

lowering of the intracellular pH in order to maintain the metabolic parameters of thawed red cells. Preferably, these solutions should be already in use for medicinal products to allow rapid application in clinical practice.

Erythrocyte storage solutions

Principal investigator
Dirk de Korte PhD
Dept Blood Cell Research
(d.dekorte@sanquin.nl)

During preparation and storage of red cell concentrates (RCC), levels of 2,3-DPG levels fall rapidly. Last year, we reported on an additive solution allowing maintenance of high 2,3-DPG levels without concurrent ATP decline, but requiring washing steps. We now present results for RCC in the new solution, prepared according to standard blood bank methods, hence without washing. Our new solution (PAGGG-M containing gluconate instead of saline, pH 8.2) is based on the 'chloride-shift' principle demonstrated 10 years ago. By lowering extra cellular chloride, intracellular chloride leaves the cell in exchange for OH^- ions making the cytosol more alkaline, which favors 2,3-DPG formation.

During storage in PAGGG-M at 4 °C, the RCC showed an increase in 2,3-DPG from 14 to 24 µmol/g Hb in the first 21 days, followed by a gradual decrease to 12 µmol/g Hb after 35 days. After 42 days, the level of 2,3-DPG was still 6 µmol/g Hb. During storage, the ATP level remained unchanged after an initial increase from 5 to 6 µmol/g Hb during the first week. Over the whole storage period the degree of hemolysis was very low, with a maximum of 0.2% after 42 days. Measurements of intracellular pH indicated that the chloride shift may not be the only factor contributing to these favorable results.

In a rat model with exchange transfusions with human RBC (see elsewhere in this report), RBC stored for 5–6 weeks in the new medium showed an oxygen delivery in the kidney comparable to fresh cells, whereas RBC stored in SAGM for 5–6 weeks showed a decreased oxygen delivery (collaboration with the group of Prof Ince at Academic Medical Center, University of Amsterdam).

Results indicate that the JC-1 signal in human platelets also reflects changes in the cytosolic ATP/ADP ratio, offering a rapid and sensitive way of monitoring platelet metabolism

Key publications

De Korte D, Verhoeven AJ: Quality determinants of erythrocyte destined for transfusion. Cell Mol Biol 2004; 50:187-95.

Hilarius PM, Ebbing IG, Dekkers DW, Lagerberg JW, de Korte D, Verhoeven AJ: Generation of singlet oxygen induces phospholipid scrambling in human erythrocytes. Biochemistry 2004; 43:4012-9.

Platelet metabolism during storage

Principal investigator
Dirk de Korte PhD
Dept Blood Cell Research
(d.dekorte@sanquin.nl)

Platelets may be stored for 7 days in plasma prior to transfusion. During storage, however, several biochemical parameters change ('platelet storage lesion') although the relevance of these changes for *in vivo* functionality is unknown. In our project, the metabolism of platelets during *in vitro* storage is studied and in particular the role of mitochondrial metabolism. As reported on in 2003, a flow cytometric method has been validated to measure mitochondrial membrane potential with the fluorochrome JC-1 as indicator. Using this method, we now investigated the effect of different substrates on the JC-1 signal, in conjunction with the cytosolic ATP/ADP ratio. In the presence of glucose and pyruvate, the JC-1 ratio was almost 5, in the absence of any substrate this value was 1.5 ± 0.3 ($n = 3$). With mitochondrial substrates only, intermediate values were found. The JC-1 signals correlated very well with the cytosolic ATP/ADP ratio, again indicating that JC-1 fluorescence reliably reflects the mitochondrial membrane potential. Only in platelets with an ATP/ADP ratio below 1, phosphatidyl-serine exposure was readily detectable. These results indicate that the JC-1 signal in human platelets not only detects damage to the mitochondria, but also reflects changes in the cytosolic ATP/ADP ratio as induced by substrate variation. This result, in line with the existing theory on mitochondrial oxidative phosphorylation, offers a rapid and sensitive way of monitoring platelet metabolism.

Key publication

Verhoeven AJ, Verhaar R, Gouwerok EG, de Korte D. The mitochondrial membrane potential in human platelets: a sensitive parameter for platelet quality. Transfusion 2005; 45(1):82-9.

Platelet activity en viability: process automation

Principal investigators
Eva Rombout MD
Dept Research
and Education
South East Region
(e.rombout@sanquin.nl)
Joyce Curvers PhD
Dept Research
and Education
South East Region
(j.curvers@sanquin.nl)

In collaboration with Gambro/BCT, we have conducted two studies regarding automation of blood processing and platelet preparation. The OrbiSac BC apparatus (designed for semi-automatic pooling, centrifugation and filtration of buffy coat pools) was tested for platelet yield and efficacy. Although we could not confirm the observation that automation leads to less variation between platelet counts in pooled products, we found platelet count and concentration to be higher (and the machine to be more efficient) in producing pooled products compared to the currently used manual method. The Orbisac BC system was compatible with three storage solutions tested (i.e. plasma, Composol or T-Sol).

A second study in cooperation with Gambro/BCT concerned the automatic separation of whole blood units on a prototype of the so-called Atreus. (Single donor) platelet products were obtained from the whole blood separated by the Atreus machine and subsequently pooled and stored for up to 7 days under standard blood bank conditions. The products thus produced were compared to the conventionally prepared pooled trombocytes in plasma (TC). No significant changes were observed between the conventional TC and the automated pooled TC for pH, metabolites (glucose, lactate, LDH) or aggregation responses. Only a slight increase of CD62-positive platelets was observed in the Atreus prepared pools. The Atreus prepared pools met the requirements according to the current guidelines. The separated red cells and plasma units also met requirements.

Key publication

Curvers J, van Pampus EC, Feijge MA, Rombout-Sestrienkova E, Giesen PL, Heemskerk JW. Decreased responsiveness and development of activation markers of PLTs stored in plasma. Transfusion 2004; 44(1):49-58.

A non-radioactive method for survival studies of transfused platelets

Principle investigator
John Scharenberg PhD
Dept Research
and Education
South West Region
(john.scharenberg@
bloodrtd.nl)

In 2004 a project to develop a non-radioactive method of *in vivo* platelet survival started. A panel of informative monospecific monoclonal HLA-class I antibodies was selected and these antibodies were evaluated for suitability to detect reliably small platelet populations by flowcytometry assay, to be able to distinguish between platelets of different origin. Hybridomas (provided by dr A Mulder, Dept of Immune Haematology and Blood transfusion, Leiden University Medical Center) are grown and supernatant is purified and labeled with Alexa Fluor-488 dye, which is spectrally similar to fluorescein. Four different human monoclonal antibodies against different HLA types are validated to detect 2–3% of transfused platelets expressing the corresponding HLA antigen in blood samples of thrombocytopenic patients using flowcytometry (EPICS XL; BeckmanCoulter). We were able to monitor the concentration of three different transfused platelet populations simultaneously. The assay is reproducible and accurate in the mixing experiments. The next step is to extend the panel of antibodies to determine *in vivo* survival of transfused platelets in >90% of patients according to HLA-type.

Key publication

Tomson B, Scharenberg J, Mulder A, Brand A. Measurement of *in vivo* survival of allogenic blood platelets with a non-radioactive labeling method using antigen-specific monoclonal antibodies against HLA-antigens. Platelets 2004; 15:271-2.

Platelet storage

The transfusion efficacy of platelets stored in Platelet Additive Solution II (PAS II) versus plasma

Principle investigator
Prof Anneke Brand MD PhD
Dept Research
and Education
South West Region
(anneke.brand@bloodrtd.nl)

Utilization of platelet additive solutions (PASs) for storage of platelets has several advantages, however only one randomized study testing the clinical efficacy has been published. This study compared the efficacy of transfusions with platelets stored in Platelet Additive Solution II (PAS II) versus plasma and showed that CCIs after transfusion with platelets stored in PAS II were significantly lower. Major drawbacks of this study were the exclusion of patients with clinical factors known to increase platelet consumption and a limited number of patients. A multi-centre, randomized study to investigate clinical efficacy of platelets stored in PAS II versus plasma, also including patients with factors of increased platelet consumption was performed in 2004 to evaluate non-inferiority of PAS-II stored platelets compared to plasma stored platelets. Statistical analysis will be based on a one-sided non-inferiority model. Inferiority is defined as a >15% decrease of mean 1- and 24-hour CCI. The study started October 2003 and will end April 2005. 183 patients have been included. 8 patients were excluded because of HLA-allo-antibodies. 716 PCs were administered and 1- and 24-hour CCI were evaluable in 82% of the transfusions. Interim-analysis in August 2004 showed a correlation between refractoriness to platelet transfusions and bleeding complications leading to the hypothesis that endothelial damage may play an important role in the increased clearance of transfused platelets. A protocol for a second an additional study is in preparation, regarding determination of some markers of endothelial damage, i.e. von Willebrand factor and its pro-peptide, VEGF, t-PA, PAI-1, soluble E-selectin and thrombomodulin, in relation to clinical parameters and survival of transfused platelets. Collaboration: Leyenburg Hospital, The Hague.

Key publication

Kerkhoffs JL, van Wordragen R, Eikenboom H, de Vries R, Brand A. A randomized study of the efficacy of transfusions with platelets stored in platelet additive solution versus plasma: A first impression of bleeding episodes and refractoriness. Najaarsconferentie NVvH 2004: 55-6.

Despite the complexity of the phagocytosis assay, it is cost- and time-effective; because it had a significant correlation with GPIb expression.

Biochemical and biophysical changes in platelets during storage

Principle investigator
Joyce Curvers PhD
Dept Research
and Education
South East Region
(j.curvers@sanquin.nl)

We continued work on storage related changes in platelets stored under blood bank conditions. In previous studies we have observed a slight decrease in ADP responsiveness of platelets during storage in plasma. This decreased responsiveness is likely caused by ADP-receptor desensitization. During storage under blood bank conditions, platelets continuously secrete granule contents including ADP, also observed from increasing CD62 expression on platelet membranes during storage. Upon activation by the secreted ADP, platelets become refractory to restimulation.
ADP receptor function *in vivo* is protected from desensitization by so-called nucleotidases, present on endothelial cells and leukocytes, which for example degrade ADP to AMP. These enzymes appear to be present also *in vitro* in plasma stored platelets. However, we found that platelets stored in synthetic medium lack this enzymatic activity to preserve ADP responsiveness during storage. Moreover reconstitution in plasma of platelets stored in synthetic media did not yield full recovery of the ADP responsiveness. We have found that mainly the purigenic G-protein coupled ADP receptor P2Y12 was desensitized. This receptor is necessary for full activation and amplification of the platelet response.

Key publication

Curvers J, van Pampus CM, Feijge MAH, Rombout-Sestrienkova, Giesen PLA, Heemskerk JWM. Decreased responsiveness and development of activation markers of PLTs stored in plasma. Transfusion 2004; 44:73-82.

'Hibernation' project

Principal investigator
Ruby Pietersz MD PhD
Dept Research
and Education
North West Region
(r.pietersz@sanquin.nl)

The first study experiments were performed to investigate whether metabolic suppression can be used to preserve platelet function during prolonged storage. Washed human platelets (plts) were incubated without glucose and with antimycine A to block energy generation. Metabolic suppressed plts (MSP) were stored for 72 hours at different temperatures to find the optimal storage temperature. Controls were incubated with 5 mM glucose and stored at 22 and 4 °C. Following metabolic recovery with glucose, MSP stored at 37, 22 and 4 °C showed (i) an increase in basal P-selectin expression (PSE) reaching >40% after about 2, 20 and 48 hours,

(ii) a decrease in TRAP-induced PSE inversely related to the increase in basal PSE, (iii) a decrease in TRAP induced aggregation reaching <30% after about 4, 24 and >72 hrs. When compared with control suspensions, MSP stored at 4 °C better preserved a low basal PSE and in addition showed a better adhesion to surface coated-von Willebrand Factor and fibrinogen in a flow chamber. In conclusion metabolic suppression prior to storage at 4 °C contributes to better preservation of plt function.
The next experiments assessed whether MSP (at 4 °C) could prevent binding to and phagocytosis by macrophages. Plt binding and phagocytosis is thought to be mediated via cold-induced clustering of plt GPIb that is recognized by hepatic macrophage complement type 3(Mac-1) receptors. Plt binding to macrophages was determined by measuring double positive particles ($CD42b^+/CD14^+$); phagocytosis by measuring mepacrine-labelled THP-1 cells and expressed as % of total number of THP-1 cells, by flow cytometer analysis. Controls were plts in glucose-containing buffer stored at 22 °C (C 22 °C) or cooled plts stored at 0 °C (C 0 °C). Fresh plts showed <5% binding and phagocytosis. After 40 min, binding of MSP increased to 25±5%, and after 48 hours storage to 30±4%. Compared with MSP (100%), the binding of control plts after incubation at 22 °C was about 30% higher and after incubation at 0 °C about 70% higher. Phagocytosis of MSP increased to 26±7 % and after 48 hours storage to 32±9%. Compared with MSP (100%), phagocytosis of control plts after incubation at 22 °C was about 45% higher and after incubation at 0 °C about 75% higher. Analysis before and after incubation with glucose showed that metabolic recovery induced a 40% drop in phagocytosis. GPIb clustering is known to be blocked by beta-N-acetyl-hexosaminidase (GlcNAc). In C 22 °C GLCNac decreased phagocytosis by 50 % but not with MSP. These data show that metabolic suppression attenuate plt-macrophage interaction.

Despite the complexity of the phagocytosis assay, it is cost- and time-effective; because it had a significant correlation with GPIb expression. Hence we replaced the GPIb assay by FACS. In pilot studies we observed that GPIb expression decreased in time due to ageing and clustering. Three populations with two major different

Results revealed no significant differences between the irradiated units and the control group and remained adequate up to 7 days.

densities were present: high density called peak1, and low density called peak2. Obviously, the higher peak 2, the more phagocytosis after prolonged storage time. The study is performed in collaboration with the Dept of Trombosis and Hemostasis of the Utrecht University Medical Centre (Prof JW Akkerman).

Storage of leuko-reduced platelets up to 7 days

Principal investigator
Ruby Pietersz MD PhD
Dept Research
and Education
North West Region
(r.pietersz@sanquin.nl)

Pursuing on the acceptation of extension of the storage time of pooled leuko-reduced platelets in plasma to 7 days at 20–24 °C with gentle agitation, the Board of Sanquin deemed it necessary to have a post authorization surveillance. The Dept of Hematology of the Utrecht University Medical Centre (Dr HC van Prooyen) participated and platelet concentrates leuko-reduced and stored in plasma for various days have been transfused to hematological patients needing platelet transfusions. Data were collected from about 200 transfusions, (unexpected) adverse reactions and increments were recorded. The number of transfusion reactions was as expected; the increments were in the same range as known from earlier studies. The results will be further analyzed and sent to the Sanquin Medical Advisory Board in 2005.

Platelet concentrates derived from pools of buffy coats, leuko-reduced and suspended in plasma were stored for up to 7 days at 20–24 °C with gentle agitation following irradiation with 25Gy. Various series of paired experiments were performed. For each experiment two 'routine' pools were pooled and divided into two equal parts. One part was irradiated either on day 1 or on day 5, the other part served as control. All platelet concentrates were stored up to 7 days and checked with parameters such as cell numbers, pH, glucose, lactate, P-selectin expression and swirling effect. Results revealed no significant differences between the irradiated units and the control group and remained adequate up to 7 days. The results have been summarized in a report that has been presented to the Medical Advisory Board of Sanquin.

Single donor leuko-reduced apheresis platelets can be harvested with equipment from various manufacturers. Recently the FDA has given clearance to Gambro (TRIMA®) to store leuko-reduced apheresis platelets in plasma for 7 days at 20–24 °C with gentle agitation. To investigate the reproducibility of the published results in our blood bank

we obtained leuko-reduced single donor apheresis platelets with the TRIMA® and stored these in plasma for up to 7 days. Parameters such as cell numbers, pH, glucose, lactate, P-selectin expression and swirling effect were used to assess quality. The results of 10 experiments were conforming to the requirements. The units maintained quality *in vitro* up to 7 days. In a second study results from platelet concentrates processed with TRIMA® will be compared with those processed with MCS+ from Haemonetics.

New functional whole blood assays to assess platelet function under flow

Principle investigator
Joyce Curvers PhD
Dept Research
and Education
South East Region
(j.curvers@sanquin.nl

From previous research we learned that thrombin generation in platelet products is highly dependent on the non-platelet fraction of these pools, likely shed micro particles. We hypothesize that patients with low numbers of circulating platelets might benefit from the presence of platelet derived microvesicles (PDMPs) in transfusion products. The formation, quantification and characterization of PDMPs are part of this newly started research project. We have adjusted an existing method for the measurement of micro particles on a flowcytometer in such a way that quantification and characterization of PDMPs is possible. We found that during storage there is an increase in PDMPs. These PDMP were highly procoagulant and able to support coagulation. In addition, PDMPs, propagated coagulation through stimulation of thrombin-dependent factor XI activation.
In vivo, PDMPs are believed to induce tissue factor (TF) expression on monocytes and/or contain TF, hereby acting as an initiator of blood coagulation. We determined that 10% of the PDMP present in platelet products expose TF. We are currently investigating whether this TF is functionally active and whether PDMP in platelet products are able to induce TF on monocytes. Also, the role of PDMP in the transfusion product will be studied in patients.

Key publication

Keuren JFW, Cauwenberghs S, Heeremans J, de Kort WLAM, Heemskerk JWM, Curvers J. Platelet ADP response deteriorates in synthetic storage media. Transfusion (accepted for publication).

New therapies and evaluation of clinical applications

New cellular therapies

Cellular therapy research

Principal investigators
C Ellen van der Schoot MD PhD
Dept Experimental Immunohematology (e.vanderschoot@sanquin.nl)
Carlijn Voermans PhD
Dept Experimental Immunohematology (c.voermans@sanquin.nl)

Projects in this research line are focused on the development of new cellular therapies. A project directed at the application of mesenchymal stem cells as support for stem cell transplantation has been started. Supernatant of MSCs were shown to alter the endothelial growth pattern and to induce the expression of CD90 in endothelial cells, which can function as an adhesion molecule. New research will be focused on the potential of blood or bone marrow derived cells for therapeutic revascularization. For that purpose the ischemic hind limb model has made operational at our department, in which sequential LDPI (Laser Doppler Perfusion Imaging) is used to monitor the normalization of blood flow after ischemia induction. In a pilot experiment it has been shown that the infusion of monocytes increased the recovery of the blood flow. Endothelial Progenitor Cells can be grown in the so-called Endocult assay. Studies to show the phenotypic make-up of EPCs growing in this assay are underway. Results so far show that for the outgrowth of these EPCs the presence of monocytes is required. Remarkably, depletion of CD34, KDR or S-Endo1 positive cells did not have any effect. Previous work has shown that stem cells can be expanded ex vivo into megakaryocytes by the combination of thrombopoietin (Tpo) and IL-1. A MEC-approved clinical trial to investigate whether these expanded cells can shorten the thrombocytopenic period after autologous stem cell transplantation has not been carried out yet, because a parallel animal study did not show any effect of these expanded cells. New studies are now focused on the effects of CD34/CD41 double positive cells. In collaboration with Marieke van Ham (Sanquin Research, Dept of Immunopathology) a new project on dendritic cells has been started.

Key publications

Tijssen MR, van der Schoot CE, Voermans C, Zwaginga JJ. The (patho)physiology of megakaryocytopoiesis: from thrombopoietin in diagnostics and therapy to ex vivo generated cellular products. Vox Sang 2004; 87 Suppl 2:52-5.

Van Buul JD, Mul FP, van der Schoot CE, Hordijk PL. ICAM-3 activation modulates cell-cell contacts of human bone marrow endothelial cells. J Vasc Res 2004; 41(1):28-37.

Beillard E, Pallisgaard N, van der Velden VH, Bi W, Dee R, van der Schoot E, Delabesse E, Macintyre E, Gottardi E, Saglio G, Watzinger F, Lion T, van Dongen JJ, Hokland P, Gabert J. Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) – a Europe against cancer program. Leukemia 2003; 17(12):2474-86.

Factors affecting proliferation and differentiation of stem and progenitor cells

Principal investigator
A Lyndsay Drayer PhD
Dept Research and Education
North East Region
(L.Drayer@sanquin.nl)

This project aims to understand in more detail the expression, regulation and function of genes controlling proliferation and differentiation of megakaryocyte progenitor cells. During the ex vivo expansion process, $CD34^+$ cells are stimulated with recombinant cytokines *in vitro* to generate partially differentiated megakaryocytic progenitor cells. This expanded population of more differentiated progenitors might be of use to reduce the period of thrombocytopenia after autologous stem cell transplantation.
In our current work we have investigated the role of mTOR and SCF during megakaryocyte progenitor expansion and differentiation.

Translational regulation plays a central role in cell proliferation, survival and cell differentiation through activation of the target of rapamycin (mTOR) signaling pathway. mTOR controls the phosphorylation status of proteins involved in initiating translational control, including ribosomal S6 kinase (p70 S6K) and eukaryotic translation initiation factor 4E-binding protein (4E-BP). Recently, the mTOR and phosphoinositide 3-kinase (PI3-K) pathways have been linked through the tumor suppressor complex TSC1/2. As the regulation of cell number and cell

Results demonstrate that the mTOR pathway is activated by TPO and plays a major role in regulating proliferation in megakaryocyte progenitors.

size are important factors during megakaryopoiesis, we investigated the role of mTOR signaling in TPO-induced proliferation and differentiation using the specific mTOR inhibitor rapamycin. The results demonstrate that the mTOR pathway is activated by TPO and plays a major role in regulating proliferation in megakaryocyte progenitors.

Stem cell factor (SCF) has a potent synergistic effect during megakaryopoiesis when administered in combination with the major megakaryocytic cytokine TPO. In the present study we investigated the signaling pathways involved in the synergistic activation of STAT5 by SCF and TPO in megakaryocytic cells. STAT5 has been shown to be critical for differentiation and proliferation of hematopoietic stem cells. Our results demonstrate that co stimulation with SCF enhances TPO-induced STAT5 signaling in megakaryocyte progenitors. This synergistic response is mediated by JAK2 and Src kinases, which leads to an enhanced and prolonged STAT5 tyrosine phosphorylation and an increase in STAT5–dependent transactivation. Identifying signaling pathways involved in the synergistic activation by multiple cytokines and determining their functions during self-renewal, proliferation, apoptosis and differentiation should help our understanding and provide means to control cell fate. These projects are performed in collaboration with Prof E Vellenga (Dept of Hematology, University Medical Centre Groningen).

Key publications

Drayer AL, Boer AK, Los EL, Esselink MT, Vellenga E. Stem cell factor synergistically enhances thrombopoietin-induced STAT5 signaling in megakaryocyte progenitors through JAK2 and Src kinase. Stem Cells 2005; 23(2):240–51.

Boer AK, Drayer AL, Vellenga E. Stem cell factor enhances erythropoietin-mediated transactivation of signal transducer and activator of transcription 5 (STAT5) via the PKA/CREB pathway. Exp Hematol 2003; 31(6):512-20.

Cord blood

Principle investigator
Yvette van Hensbergen PhD
Dept Research
and Education
South West Region
(Yvette.vanHensbergen@
bloodrtd.nl)

The purpose of this research is to increase the applications of cord blood for transplantation and transfusion. This includes improvement of the stem cell engraftment in order to apply cord blood stem cell transplantation in adult patients. Because a cord blood donor can not be requested for post-transplant immunotherapy (DLI) in case of relapse, the immunological profile of (expanded) cord blood mononuclear cells for graft versus leukemia purposes is explored as well.

With regard to enhancement of engraftment, our research focuses on the expansion and differentiation of stem cells towards megakaryocyte precursor cells. We characterized that in contrast to bone marrow and peripheral blood mobilized stem cells, the $CD34^+/41^-$ subset in cord blood contributes to 99% of *in vitro* generated megakaryocyte precursors. *Ex vivo* expansion and differentiation of cord blood $CD34^+$ cells with trombopoietin towards megakaryocyte precursors resulted in earlier platelet recovery in the NOD/SCID mice model. Transplantation with these expanded cord blood $CD34^+$ cells did not result in decreased engraftment potential, indicating that no loss of pluripotent stem cells had occurred during expansion. Besides expansion, the effect of co-transplantation with mesenchymal stem cells (MSC's) on *in vitro* differentiation and *in vivo* engraftment of megakaryocytes and platelets is investigated in the NOD/SCID mice.

In 2004 we started research on expansion of autologous cord blood erythropoietic progenitor cells towards erythroblasts for possible transfusion purposes in preterm infants. This study is associated with a clinical randomized study on the feasibility of the use of autologous cord blood red cell transfusion in preterm children (supported by a ZonMW grant). In this feasibility study two University neonatology centers participate.

Collaboration Leiden University Medical Center, Dept of Immunohematology and Blood Transfusion (Prof WE Fibbe; megakaryocyte expansion). Prof E Goulmy (immunity against minor transplantation antigens). HHH Kanhai, S Scherjon, Dept of Obstetrics; F Walther, Dept of Neonatology; and L Christiaens, Obstetrics; Prof H Brouwer, Dept of Neonatology, Utrecht Universtity Medical Center.

Key publications

Verdijk RM, Wilke M, Beslier V, Kloosteman A, Brand A, Goulmy A, Mutis T. Escherichia Coli-nitroreductase suicide gene control of human telomerase reverse transcriptase-transduced minor histocomatibility antigen-specific cytotoxic T cells. Bone Marrow Transplantation 2004; 33:963-7.

Verdijk RM, Kloosterman A, Pool J, van der Keur M, Naipal AMIH, van Halteren AGS, Brand A, Mutis T, Goulmy E. Pregnancy induces minor Histocompatibility antigen specific cytotoxic T cells: implications for stem cell transplantation and immunotherapy. Blood 1994; 103:1961-4.

Research on cellular blood products

Collection of autologous blood products by double erythrocytapheresis-cost effectiveness study

Principal investigator
E Rombout MD
Dept Research
and Education
South East Region
(e.rombout@sanquin.nl)

The project will determine the advantages of double erythrocytapheresis in the collection of pre-operative autologous erythrocytes as compared to the standard collections of whole blood. The study will asses the total reduction in the number of procedures required to obtain the pre-operative units ordered by the surgeon. Also the successfulness of erythrocytaphereses versus classical whole blood collection for these patients will be compared, since it is known that classical donations up to the number of units required is not always possible. Erytrocytapheresis will be performed on the Haemonetics MCS+ equipment as compared to whole blood collection. Since the start of the project 39 patients with double erytrocytapheresis have been included and 53 patients have donated autologous blood by classical whole blood collection. At this moment we can conclude that double erythrocytapheresis seems a feasible, well tolerated, safe and efficient procedure to collect autologous erythrocytes.
The cost effectiveness will be compared in due course. This project is performed in collaboration with Atrium Medical Centre Heerlen, Maasland Hospital Sittard, Laurentius Hospital Roermond and Viecuri Medical Centre North Limburg.
As related comparative study was performed as to the *in vitro* 'survival' quality of erythrocytes obtained by double erythrocytapheresis versus erythrocytes obtained by classical whole blood collection. This study showed that ATP levels diminish 5–10%

As a direct spin-off of the project a special out-patient clinic for hemochromatosis patients will be started.

faster in erythrocytes derived by double erythrocytaferesis. This aspect has led to a separate project in collaboration with dr D de Korte of Sanquin Research, Dept of Blood Cell Research. By the end of 2005, first results of the cost-effectiveness analysis are expected to be available for a publication.

Therapeutic erythrocytapheresis as treatment for hemochromatosis patients

Principal investigator
E Rombout MD
Dept Research
and Education
South East Region
(e.rombout@sanquin.nl)

In this project we plan to evaluate the effectiveness of erythrocytapheresis against phlebotomy, both regarding the impact on the reduction in iron overload as well as the reduction in patient 'burden'. Aspects of cost effectiveness will be included in the final analysis. The results of the study would allow decision-making based on Evidence Based Medicine on the 'best' therapeutic options available for newly-diagnosed as well as existing primary hemochromatosis patients. Formal approval of the Ethical Committee at Maastricht University Hospital was obtained. Since the start of the project the catchment-area for inclusion of patients was extended by greater collaboration with the gastroenterolo- and hepato-logists from the regional hospitals as established at a presentation of the project (October 2004) at their yearly scientific meeting. To allow inclusion of more patient's additional facilities at other blood collecting centers in the Sanquin South East Region were established. Collaboration with Prof B van Hout of the Julius Center UMCU was established for the cost-effectiveness analysis. The Julius Center also performs the randomization to assign eligible patients blindly to one of the two treatment arms, either phlebotomy or erythrocytapheresis. 8 patients eligible and willing to be randomized were included and had their first treatment. Inclusion at this rate will allow to obtain the minimum number of n=2x16 patients as required by the power calculation as planned. Our initiative has already led to a closer collaboration with the hospitals in the region. As a direct spin-off of the project, the Dept of Internal Medicine, Maastricht University Medical Center will start a special out-patient clinic for hemochromatosis patients in collaboration with the blood bank's Dept of Research and Education.

Plans are also underway to explore more extensive medical evaluation/follow-up as to the clinical effectiveness of the two treatment modalities, by more extensive cardiologic and/or hepathologic work-up by the participating medical specialists treating these patients.

Leukoreduction of red cell transfusions in major surgery

Principle investigators
Prof Anneke Brand MD PhD
Dept Research
and Education
South West Region
(anneke.brand@
bloodrtd.nl)
Joost A van Hilten PhD
Dept Research
and Education
South West Region
(joost.vanhilten@
bloodrtd.nl)

In 2004 the analysis of two large prospective randomized studies comparing leukocyte reduction of red cells and buffy-coat depleted red cells in major surgery was accomplished. In cardiac valve surgery the advantage of leukoreduction is a reduced incidence of postoperative infections associated with lower in-hospital mortality. In major general surgery the main advantage of leukoreduction of red cells is a shorter hospital stay. The factors leading to reduced hospital stay still has to be unraveled but point toward a role of leukocytes in transfused blood to organ damage. Collaboration: 19 hospitals

Key publications

Van Hilten J, van de Watering L, van Bockel J et al. Effects of transfusion with red cells filtered to remove leucocytes: randomised controlled trial in patients undergoing major surgery. BMJ 2004; 328:1281-4.

Bilgin Y, van de Watering L, Eijsman L et al. Double-blind, randomized controlled trial on the effect of leukocyte-depleted erythrocyte transfusions in cardiac valve surgery. Circulation 2004; 109:2755-60.

Reduction of blood transfusion in orthopedic surgery

Principle investigator
Prof Anneke Brand MD PhD
Dept Research
and Education
South West Region
(anneke.brand@bloodrtd.nl)

In orthopedic surgery several new approaches to reduce blood transfusions and to improve wound healing are available. In order to make evidence based choices on the usage of epoietin, several forms of autologous shed blood reinfusions, large multi-arm and multicenter studies are needed. The basic requirement for such studies is a strict transfusion protocol. We performed a randomized controlled study in three orthopedic centers comparing a uniform transfusion trigger with the current hospital triggers. The results show that a uniform transfusion trigger is feasible and can be applied across hospitals. We further accomplished a pilot study, to investigate the efficacy and feasibility of two different post-drainage reinfusion systems in elective

orthopedic surgery patients in the Leiden University Medical Center (LUMC). Both drainage systems were easily incorporated in the blood management policy of the Orthopaedic Dept. After both pilot studies a multicenter study was designed on integrated blood sparing approaches (Optimal Blood Management (Transfusie Op Maat study – TOMaat –). The feasibility of the complex design of such a study started in 2004. From May to December 2004 two participating hospitals (Leiden University Medical Center and Albert Schweitzer Hospital, Dordrecht) included 141 patients (inclusion percentage of 85%) showing that the design is feasible.

Key publications

So-Osman C. Bloed moet, maar met mate en goed! NVB-Bulletin 2004; 4:9-12.

So-Osman C, Nelissen RGHH, te Slaa RL, Coene LN, Brand R and Brand A. Is a restrictive transfusion trigger a method for blood saving in elective orthopaedic surgery? Vox Sanquinis 2004; 87,Suppl.3:52.

Metaregression analyses of red cell transfusions on leukoreduction of red cells in surgery patients

Principal investigator

Leo van de Watering MD PhD Dept Research and Education South West Region (leo.vandewatering@bloodrtd.nl)

From 1988, several RCT's were performed investigating the effects of leukoreduction of blood products in different groups of surgical patients. Some of these RCTs came up with answers that seemed to conflict with the results of other RCTs. Standard meta-analyses of these data is partly hampered by the seemingly conflicting results. We therefore performed combined patient-based analyses of the data. The electronic data files of the RCTs were uniformly recoded and entered in a single database, to facilitate multivariate analyses of all data. Individual datasets from 3875 surgical patients were collected (1630 Onco; 1272 Cardiac; 569 Vascular; 352 Orthopedic, 52 other). As different primary endpoints were investigated in the different RCTs, we focused on common endpoints, recorded in 4 or more of the RCTs. Multivariate analyses were performed on the endpoints: in-hospital mortality, 30-day mortality, hospital stay, stay on ICU, postoperative infections, and MODS (Multiple Organ Dysfunction Syndrome). The following variables were entered in the multivariate analyses: Trial arm, Hospital, Sex, Age, Type of surgery, Duration of surgery, Blood transfusions (RBC, plasma & platelets). The results are currently being analyzed.

Key publication

Van de Watering L, Loomans D, Brand A. Combined analyses of patient data from three cardiac surgery studies. P8.25; Vox Sanguinis 2004 (suppl 3); S17-S92:87.

Transfusion triggers and effects

Quality of life measurement and restriction of red cell transfusions in patients with transfusion dependent MDS

Principal investigator
Prof Dick J van Rhenen
MD PhD
Dept Research
and Education
South West Region
(dick.van.rhenen@
bloodrtd.nl)

Myelodysplastic syndromes (MDS) are clonal disorders characterized by dysplasia in at least 2 myeloid cell lines. Fatigue, assumed to be anemia-related, is one of the most important symptoms. MDS patients are treated with blood transfusions to improve health-related quality of life (HRQoL). Although improving HRQoL is the major goal of blood transfusion in MDS, the HRQoL have not been investigated empirically. The aims of the present study include: (i) Psychometric evaluation of two internationally established HRQoL measures in MDS patients, and (ii) Investigation of the association between the severity of chronic anemia and the HRQoL.
In a cross-sectional design HRQoL and chronic anemia is measured. 50 Randomly chosen MDS patients (RA, RARS, RAEB en CMMoL) completed the SF-36 (generic HRQoL), the Multi-dimensional Fatigue Inventory (MFI), and a Visual Analogue Scale for self-rated health within 24 hours after a regular hospital visit. Hemoglobin level was measured during the visit. Psychometric analysis focused on feasibility (completion time, experienced difficulties), reliability (Cronbach's alpha), scale structure (factor analysis) and construct validity (discriminative ability between groups). This study provides detailed insights in the suitability of established HRQoL measures for the evaluation of interventions in MDS patients. The questionnaires have a high feasibility and MDS patients have a worse HRQoL than age-related persons. There is a correlation found between hemoglobin level and HRQoL. Both hemoglobin value as HRQoL seem to be relevant for evaluation of the severity of chronic anemia.
MDS patients are predominantly treated with blood transfusions to improve health-related quality of life (HRQoL). Concerns about the transfusion-related complications,

Preliminary results show that a restrictive transfusion policy leads to a diminished use of red cell transfusion without an increase in cardiac complications.

such as infections, tumor behavior and immuno-modulatory effects, and the costs, necessitated a re-evaluation of the transfusion practice.
The goal of this study is to evaluate if a restrictive transfusion policy (Hb transfusion trigger <4.5 mmol/l) reduces the amount of red cell transfusion compared to a liberal transfusion trigger (Hb<6.0 mmol/l) without a decrease in HRQoL. Because of concerns about the feasibility of this study early results were analyzed and are presented in this abstract. After a run in period of 3 months (Hb transfusion trigger of Hb<6.0 mmol/l) patients are randomized for the restrictive or the liberal transfusion policy. Patients are followed then 12 months. HRQoL is measured after inclusion, after randomization, 6 weeks, 3, 6, 9, and 12 months after randomization. Also anemia related complications and red cell antibodies are scored. Hb values were blinded for the patients during the study period. From July 2002 till June 2004 15 patients were included (4 RA, 5 RARS, 4 RCMD, 1 RAEB, 1 CMMoL) in 2 general hospitals and 1 university hospital. Two patients died in the run in period. Eight patients were randomized for the restrictive transfusion policy and 5 patients for the liberal transfusion policy. The mean follow up period in the liberal group was 6,2 months (inclusive run in period) and 7,4 months for the restrictive group. Two patients in the liberal group died after randomization. One patient received growth factors.
In the restrictive group 2 patients finished the study, 1 received growth factors and 1 patient withdrew informed consent. The mean Hb level was lower in the restrictive group and after randomization about 55% reduction in amount of transfused red cells was found (1,8 units per pt per month in the liberal group vs. 0,8 in the restrictive group). No anemia related complications were found, e.g. cardiac failure and cerebro vascular ischemia nor a decrease in activity performance. There were some concerns after introduction of the restrictive transfusion policy. This preliminary results show, however, that a restrictive transfusion policy leads to a diminished use of red cell transfusion without an increase in cardiac complications or a decrease in activity performance. This study will be continued to compare HRQoL scores in both groups.

Key publication

Jansen AJ, Essink-Bot ML, Beckers EA, Hop WC, Schipperus MR, Van Rhenen DJ. Quality of life measurement in patients with transfusion-dependent myelodysplastic syndromes. Br J Haematol 2003; 121(2):270-4.

New insights into fatigue and health-related quality of life after delivery and Well-being of Obstetric patients on Minimal Blood transfusions (WOMB study)

Principal investigator

Prof Dick van Rhenen MD PhD

Dept Research and Education South West Region (dick.van.rhenen@bloodrtd.nl)

Blood loss is a common and important problem after delivery and has a strong influence on maternal morbidity postpartum. The most important symptom is fatigue, which is assumed to be anemia-related. Red blood cell (RBC) transfusions should be aimed at improving health-related quality of life (HRQoL). However, HRQoL has not been investigated empirically. In our study, three internationally established HRQoL measures were investigated in relation with postpartum hemoglobin levels in women after vaginal delivery (VD), elective cesarean section (CS) and emergency CS. In 2 university hospitals and 1 general hospital 141 randomly chosen patients (71 with VD, 36 with elective CS and 34 with emergency CS) completed the Multi-dimensional Fatigue Inventory (MFI), and the EuroQoL 5Q-D between 12–24 hours after VD and 24–48 hours after CS (t=0). These questionnaires, together with the Short Form 36, were completed 1, 3 and 6 weeks after delivery. Hb level and estimated blood loss was measured at t=0. Analysis of the questionnaires was focused on feasibility (completion time, experienced difficulties) and reliability (Cronbach's alpha). The EuroQoL, SF-36 and MFI showed a high feasibility and reliability. Patients after VD had higher physical HRQoL scores than patients after CS during the first 6 weeks postpartum. Patients after emergency CS had significant lower physical HRQoL scores than patients with VD or elective CS. Patients after VD needed three weeks and patients after elective weeks needed six weeks to achieve full recovery. No differences were found for mental HRQoL between the three groups. A significant correlation between Hb level and physical HRQoL scores was found on t=0. This correlation disappeared 1 week postpartum. No relation between blood loss and HRQoL scores was seen. HRQoL measures are complementary and are useful tools to measure HRQoL after delivery. Patients after VD needed three weeks to achieve normal HRQoL scores where patients after elective CS needed six weeks. However, patients with emergency CS

needed a longer period for full recovery. The relation between hemoglobin level and HRQoL in the first week postpartum disappeared after one week postpartum. Future clinical trials are needed to assess the effect of RBC transfusion and to confirm the role of HRQoL in the decision whether RBC transfusion is necessary.

In a multicenter randomized clinical trial 400 patients will be included (200 patients after vaginal delivery and 200 patients after caesarean section). Inclusion criteria are 12–24 hours postpartum, a blood loss of at least 1000 ml and a Hb value between 3.0 and 4.9 mmol/l. In both groups 100 patients will receive a red cell blood transfusion and 100 patients won't receive red blood cell transfusion. Primary outcome is Health Related Quality of Life. The goal of the study is to develop a more HRQoL based transfusion policy for patients after delivery, based on the HRQoL questionnaires, validated in the pilot study.

Key publication

Jansen AJG, Duvekot JJ, van Rhenen DJ. WOMB studie: Well being of Obstetric patients on Minimal Blood transfusions. NVB Bulletin 2004; 3:10-2.

Feasibility of a restrictive transfusion policy for hemato-oncological patients

Principal investigator
Prof Dick van Rhenen MD PhD
Dept Research and Education
South West Region
(dick.van.rhenen@bloodrtd.nl)

Red-cell transfusions are the cornerstone of the supportive care for hematological patients treated with intensive chemotherapy. Concerns about the transfusion-related complications, such as infections, tumor behavior and immuno-modulatory effects, and the costs, necessitate a re-evaluation of the transfusion practice. In a retrospective study, 47 patients with Acute Myeloid Leukemia (AML), treated with a combination of chemotherapy (ARA-C and Idarubicin) in the period June1997 till July 1999, were included. We determined whether a restrictive policy of red-cell transfusion (4.5–5.5 mmol/l, dependent on patient age and symptoms, n=25) leads to a diminished use of red-cell transfusions compared to a liberal transfusion policy (i.e. transfusion if Hb ≤6 mmol/l, n=22). We also investigated if both transfusion policies are comparable in terms of preventing signs and symptoms of anemia and other chemotherapy related complications.

The total number of red-cell transfusions and the number of units of red cells given per transfusion were different in both groups ($p<0.05$) in favor of the restrictive transfusion policy. No significant differences were found in the incidence of infections, duration of granulocytopenia, number of platelet transfusions or bleeding, thrombosis or response to chemotherapy.
It may be concluded that a restrictive transfusion policy, which leads to a decreased use of red-cell transfusions, is safe in supporting patients treated with intensive chemotherapy for AML.

Key publication

Jansen AJG, Caljouw MAA, Hop WCJ, van Rhenen DJ, Schipperus MR. Feasibility of a restrictive transfusion policy for patients treated with intensive chemotherapy for acute myeloid leukemia. Transfusion Medicine 2004; 14(1):33-8.

Donor studies, epidemiology and cost effectiveness

Principal investigator
Cees L van der Poel MD PhD, Sanquin Research & Julius Center, Utrecht University Medical Center (c.vanderpoel@sanquin.nl)

Transfusion Technology Assessment

While blood products for transfusion are presently very safe, technology becomes available to create even safer products. Human blood donations are processed into blood products are derived for humans, and therefore remain to be exposed to (emerging) infectious diseases. Blood transfusions can never be 100% safe. This creates opportunities for 'marketing of fear' and implementation of any new technology. On the other hand there is an increased need setting standards of efficacy parameters for blood safety. In collaboration with the department for Medical Technology Assessment (MTA) of the Julius Center of Utrecht University, Sanquin Research created the research group on Transfusion Technology Assessment (TTA). The group aims to perform independent risk assessments and health economic valuations on blood safety issues. Taking into account the processes that underlie the blood transfusion chain, the most important elements are risk analyses, costs and effects of safety interventions, statistics of blood use, and disease and survival profiles of blood recipients.

Among the remaining risk of blood transfusions, bacterial contamination of platelet transfusions is the most important. To become acquainted with the literature, the group started with a study on the costs and effects of pathogen reduction and bacterial screening of platelets, which was successfully submitted in 2004 to an international scientific journal of transfusion medicine.

Concerns for transmission of Variant Creutzfeldt-Jakob Disease raised the international question whether platelet transfusions with product derived from single donor apheresis instead of 5 whole blood donations should be preferred. An analysis of the risk reduction to recipients of platelets by implementation of 100% supply of platelets by apheresis donations was performed and reported to Sanquin. It will be submitted for publication.

Commissioned by the Council of Europe, indicators of 'The Collection, Testing and Use of Blood Products in Europe' of 2002 and 2003 were evaluated and reported in November 2004. In addition, the Council of Europe was advised on improvements

It is anticipated that international implementation of risk analyses and assessment of transfusion technologies will become state-of-art in the decision processes.

of the survey for 2004. The Survey is now a leading annual publication on blood transfusion indicators.

For charting the blood use and blood recipient profiles in the Netherlands the group developed a set of parameters, which were evaluated in the Utrecht University Hospital. In 2004 the collaboration of other academic hospitals was obtained to further optimize the required databases. It appears feasible to obtain sufficient reliable information on blood recipient profiles in this manner. In addition to in-hospital blood recipient profiles, the survival of these patients is of importance. In 2004 the collaboration of the Netherlands Statistics Office was obtained for this purpose. Participation of a relevant and representational sample of hospitals in the country is essential, and will be further elaborated in the field.
It is anticipated that international implementation of risk analyses and assessment of transfusion technologies will become state-of-art in the decision processes on transfusion safety.

Quality Assessment and Improvement Program

Principal investigator
Ton PM Los
Dept Research
and Education
North East Region
(a.los@sanquin.nl)

In 2004 the Quality Assessment and Improvement Program was continued. The assessment objective is to ensure that: (a) the interaction between Blood Bank (personnel) and customers (donors, hospital laboratories is positive, and (b) it promotes good customer service. Additional audits between Blood Bank departments and personnel) communication are subject of research.

In 2004 an additional (repeat) communication survey was carried out under the Blood Bank personnel of Sanquin Blood Bank North East Region in cooperation with Mercury Urval.

On behalf of the Board of Directors and Corporate Staff a study was carried out under the complete personnel of Sanquin regarding their opinion of the Sanquin communication magazine cirQlatie.

Furthermore an advisory study on donation frequency of Dutch blood and plasma donors was carried out on behalf of J Over, PhD, Strategic Planning Sanquin Plasma Products, and MP Janssen, MSc, Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht.

The systematic recruitment of new blood donors

Principal investigator
Tiny Hoekstra PhD
Dept Research
and Education
South East Region
(t.hoekstra@sanquin.nl)

In the past decade the number of blood donors declined steadily in The Netherlands; especially young adults are underrepresented in the donor file. Recruitment strategies are most likely to be successful when they are based on a theory-based understanding of both the cognitions, attitudes and beliefs about blood donation, and the reasons for non-donation. Three studies have been performed on determinants of blood donation. In the first study among students, it was found that the attitude towards blood donation, especially the negative affect components (e.g. pain and needle anxiety) is the most important predictor of the intention to become a blood donor. A second study was done among somewhat older subjects. In this study the questionnaire was extended with questions on altruism and motivational values, which appeared to have no effect on the intention to donate. A third study was performed in a population of working adults. Additional questions were asked on fear for blood and needles. The data of this study are currently being analyzed. The next step is to perform a content analysis of the brochures of the blood bank. In this analysis the information presented in the brochures will be compared with the results of the determinant studies in order to reveal which information might be missing. Based on this analysis, recommendations will be given to improve the effectiveness of the brochures. Subsequently, the brochure will be improved and experimentally tested. This project is performed in close cooperation with Maastricht University.

Key publication

Lemmens KPH, Abraham C, Hoekstra T, Ruiter RAC, de Kort WLAM, Brug J, Schaalma HP. Why don't young people volunteer to give blood? An investigation of the correlates of donation intentions among young non-donors. Transfusion (accepted for publication).

Donor cohort studies

Principal investigator
Tiny Hoekstra PhD
Dept Research
and Education
South East Region
(t.hoekstra@sanquin.nl)

The main goal of this project is to gain insight into the characteristics of the donor population and into the efficiency of the different processes within the blood bank that involve donors, e.g. call up of donors, donor examination and donating blood itself. The ultimate goal is to improve the quality and efficiency of blood bank donor processes. Questions we want to answer can be divided into three main categories:
(i) donor characteristics: What is the socio-economic background of the donors? What are their motives to give blood? Is there a difference in characteristics between regular donors and donors who only incidentally donate? Is there a difference in characteristics between new and long-time donors?
(ii) Dynamics of the donor population: How many new donors are signed in, what are their motives, and what did provoke them to register? How many of the new donors convert into regular donors? What are reasons why donors have their name removed from the donor register? How many inactive donors are registered?
(iii) Efficiency of blood bank processes (e.g. donor calls, donor examination): What are the overall main deferral rates? Are there differences in deferral rates in various subgroups? Does the number of deferrals affect the show up rate?
To reach our goals, a dynamic cohort of donors consisting of a random sample of all donors of Sanquin Blood Bank South East Region will be set up. Data will be collected by using both questionnaires and blood sampling. At the moment we are preparing the data collection, which is planned to start in the second half of 2005. A concept of the questionnaire is constructed and involves, for example, questions on lifestyle (e.g. smoking, sports, and nutrition), health, motivations to donate blood, and donor experiences. Data from this dynamic cohort study will be linked with routine data gathered by the blood bank (e.g. examination and donation data). This project will be executed in co-operation with research groups from several universities (Maastricht, Nijmegen and Utrecht).

It was found that Hb levels in plasma donors varied with the season, resulting in somewhat higher deferral rates for Hb during the summer.

Seasonality of Hb and donor deferral

Principal investigator
Tiny Hoekstra PhD
Dept Research
and Education
South East Region
(t.hoekstra@sanquin.nl)

Indications exist that during the summer, compared to other seasons, a higher percentage of donors is deferred due to a low hemoglobin (Hb) level. Biological explanations can be found in heat acclimatization (hemodilution) or differences in nutrition and activity patterns of donors. It can also be possible that a difference in population characteristics is responsible for the found Hb differences (selection bias). We investigated this phenomenon in a population of plasma donors. Data on HB values were extracted from the Blood Bank Information System for the years 2002 and 2003 (N=2357 plasma donors). It was found that Hb levels in plasma donors varied with the season, resulting in somewhat higher deferral rates for Hb during the summer.

The seasonal effect was also observed within subjects and thus cannot be explained by differences in donor characteristics. In the following step we will also examine data of whole-blood donors on seasonality of Hb.

Research Departments

Department of Blood Cell Research

Sanquin Research

Address

Sanquin Research

Department of Transfusion Technology

Plesmanlaan 125

P.O. Box 9190

NL-1006 AD Amsterdam

The Netherlands

T +31 20 512 33 17

F +31 20 512 33 10

w.winkel@sanquin.nl

www.bcr.sanquin.nl

Academic staff

AJ Verhoeven PhD (head)

D de Korte PhD

Prof TW Kuijpers MD PhD (AMC)

J Lagerberg PhD

Prof D Roos PhD

R van Zwieten Bsd

Post-doc fellows

R van Bruggen PhD (AMC)

DWC Dekkers PhD

KM Dolman MD PhD (AMC)

N Kamerbeek PhD

C van Moorsel PhD

PhD students

WB Breunis MD (AMC)

N Brouwer (AMC)

NA Maianski MD

BJ van Raam

Technical staff

V Aygun

J Berghuis

EF Clifford

M de Boer

S Duin

J Geissler (AMC)

JE Groen-Langeveld

CWN Gouwerok

V Groenewold

M Kleine-Go

VWK Paeper

PM Stokman-Hilarius

ATJ Tool, PhD

R Truijens-de Lange

AJ van Caldenhove

ChrEM van Rossum

JA van Rutten

R van Zwieten

M Velthuis

R Verhaar

JJH Verkuylen

R C Vlaar

HA Vuil

D Zweers

Students

M Buyne

R El Bouchaibi

I Franke

MH Jansen

GS Morren

HPH Naber

K Schulte

M Tesarova

Guests

M Biezeveld (AMC)

R Calmuschi

A Dreuniak

Y K'oker

Secretary

W Winkel

Research lines

Department of Clinical Viro-Immunology

Sanquin Research

Address

Sanquin Research
Department of Clinical
Viro-Immunology
Plesmanlaan 125
P.O. Box 9190
NL-1006 AD Amsterdam
The Netherlands
T +31 20 512 33 17
F +31 20 512 33 10
g.damhuis@sanquin.nl
www.cvi.sanquin.nl

Academic staff

J Schuitemaker PhD (head)
Prof F Miedema PhD
NA Tesselaar PhD
D van Baarle PhD
FAC van Engelenburg PhD

Postdoc fellows

J Borghans PhD
NA Kootstra PhD
(NWO fellow)
A van Leeuwen-Gorter

PhD-students

C Bronke (AMC)
I den Braber
EM Bunnik
CA Jansen
N Kloosterboer
FA Koning
M Navis
E Piriou
E Quakkelaar
E Stalmeijer-Schmidt
N Vrisekoop

Technical staff

S Alders
H Attalki
C Beugeling
BDM Boeser-Nunnink
A Boots
L Bos
N Brinkhuis
B de Boer
IM de Cuyper
L Dekker
E Gijsen
A Harskamp-Holwerda
C Koevoets
HGH Korsten
M Mangas-Ruiz
A Meinesz
CW Mollenaar
E Mul
NM Nanlohy
J Ottenhoff-Mollenaar
SA Otto
GGJ Sotthewes
FG Terpstra
F van Alphen
AE van den Blink
TJK van der Vorst
K van Dort
AC van Nuenen
Y van Remmerden
GMW van Schijndel
P van Swieten
M Westerlaken
N Willemse
CA Witte

Guests

M Roos
A Tsegaye (Ethiopia)
I Schellens
E Schrijver

Undergraduate students

JA Abarca Castellano
MJM de Graaf
D Edo
J Melief
A Sanz Sanz
N van de Lubbe
BN van Uden
MRA Welkers

Secretary

GSM Damhuis

Research lines

Department of Experimental Immunohematology

Sanquin Research

Address

Sanquin Research
Department of Experimental
Immunohemaotology
Plesmanlaan 125
P.O. Box 9190
NL-1006 AD Amsterdam
The Netherlands
T +31 20 512 33 77
F +31 20 512 34 74
a.engels@sanquin.nl
www.ihe.sanquin.nl

Academic staff

CE van der Schoot MD PhD (head)
M de Haas MD PhD
S Dohmen MD
C Voermans PhD
JJ Zwaginga MD PhD

Postdoc fellows

S Beiboer PhD
WA Noort PhD
D Thijssen-Timmer PhD

PhD students

S Dohmen MD
MB Hemker MD
J Koelewijn
N Stapleton
GHM Tax (Blood Bank South West Region)
M Tijssen
R van Beem

Technical staff

A Ait Soussan
GMA Cheroutre
IW de Jong
A de Vries-van Rossen
R Dee
F di Summa
C Homburg (50%)
J Janssen
M Kleijer
C Simons
R Smit
M Valk
OJMH Verhagen
A Vos
R Wijngaarden-du Bois
A Zadurian
L Zappeij

Undergraduate students

A Mol
T Sijswerda

Guests

M Bruin MD (Wilhelmina Childrens Hospital, Utrecht)
V de Haas MD PhD (AMC)
Xu Qun MD (Jinan, China, NUFFIC fellow)
P Maaskant-van Wijk (Blood Bank South West Region)

Technical trainees

M de Bruijn
J Hollander
GJ Jong
M Kok
S Meisner
A Tol
F Ummels
J van Nimwegen
M Vonk
F Winia

Secretaries

A Engels
M Lutkie

Research lines

- Hematology
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Department of Immunopathology

Sanquin Research

Address

Sanquin Research
Department of
Immunopathology
Plesmanlaan 125
P.O. Box 9190
NL-1006 AD Amsterdam
The Netherlands
T +31 20 512 31 71/58
F +31 20 512 31 70
f.muntar@sanquin.nl
p.denenting@sanquin.nl
www.ip.sanquin.nl

Academic staff

SM van Ham PhD (head)
Prof CE Hack
Prof RC Aalberse PhD
Prof LA Aarden PhD
D Hamann PhD
A Kummer MD PhD
PW Modderman PhD
R van Ree PhD
GJ Wolbink

Postdoc fellows

EC de Bruin PhD
DWC Dekkers PhD
R Diemel PhD
MCM Strik PhD
JA ten Brinke PhD
J van Beek
IM van den Nieuwenhof PhD
M van der Neut Kolfschoten PhD
JWAM van Oers PhD
GAC Wagenaar-Bos PhD
L Zuidmeer PhD

PhD-students

J Akkerdaas
W Bos
C Ciurana
S de Lathouder
N Diaz Padilla
G Diepenhorst
A Familian
R Kikkert
F McGrath
Y Souwer
E van Mirre
D Wouters

Technical staff

M Aalbers
R Bosch
B Bottelier
R Bron
MC Brouwer
I Bulder
ER de Groot
S de Haan
P de Heer
N Derksen
MC Dieker-Meijer
AJM Eerenberg-Belmer
J Huck
MHL Hart
T Jorritsma
ML Karsten
H Klaasse-Bos
MJ Kramer
R Manoe
M Mulder
S Notten
E Pastoors
HJAM Rensink
DI Roem-Haagsma
S Solati
AR van der Horst
J van Leeuwen
WA van Leeuwen
G van Mierlo
E Vermeulen
S Versteeg
AM Wolbink-Kamp

Undergraduate students

C Alhan
T Cramer
J Gomez-Gortes
A de Goffau
A Göktepe
P Gupta
S Jahangir
F Ligtermoet
R Paulis
JE Siljee
M Valls Seron
S van Leeuwen

Secretaries

PD den Enting
F Muntar
S Sebelon

Research lines

Department of Molecular Cell Biology

Sanquin Research

Address

Sanquin Research

Department of Molecular

Cell Biology

Plesmanlaan 125

P.O. Box 9190

NL-1066 CX Amsterdam

The Netherlands

T +31 20 512 33 77

F +31 20 512 34 74

a.engels@sanquin.nl

www.mcb.sanquin.nl

Academic staff

PL Hordijk PhD (head)

Post doc fellows

M Fernandez-Borja PhD

S Geutskens PhD

E Kanters PhD

PB van Hennik PhD

PhD students

M Lorenowicz

JP ten Klooster

JD van Buul

J van Gils

Technicians

EC Anthony

S Bruijns

S Basten

S de Haan

Secretaries

A Engels

M Lutkie

Research lines

Department of Plasma Proteins

Address

Sanquin Research
Department of Plasma
Proteins
Plesmanlaan 125
P.O. Box 9190
NL-1006 AD Amsterdam
The Netherlands
T +31 20 512 31 51
F +31 20 512 36 80
m.vergeer@sanquin.nl
www.pe.sanquin.nl

Academic staff

Prof K Mertens PhD (head)
AB Meijer PhD
JA van Mourik PhD
JJ Voorberg PhD

Post doc

AN Bovenschen PhD

PhD students

R Bierings
M van den Biggelaar
MHA Bos
C Fribourg
V Limburg
B Luken
MG Rondaij
PMW van Helden

Technical staff

RC Boertjes
MG Boon-Spijker
HJM Brinkman
PPFM Clijsters
K Gijzen
JG Hoogenboezem
PHP Kaijen
JWTM Klein Gebbinck
CA Koekman
S Kroon
E Sellink
F Stavenuiter
EM Turenhout
G van den Brink-van
Stempvoort
C van der Zwaan
MG Zuurveld

Secretaries

P Hiemstra
MJ Vergeer

Research lines

Sanquin Blood Bank North East Region

Department of Research and Education

Address

Sanquin Blood Bank
North East Region
Department of Research
and Education
Prof Rankestraat 42–44
P.O. Box 1191
NL-9701 BD Groningen
The Netherlands
T +31 50 369 55 55
F +31 50 361 90 39
c.vandelden@sanquin.nl
www.research.sanquin.nl

Academic staff

CJ van Delden PhD (head)
AL Drayer PhD
MK Elias MD PhD
APM Los
SGM Olthof
MR Tuyl

Research lines

Sanquin Blood Bank North West Region

Department of Research en Education

Address

Sanquin Blood Bank
North West Region
Department of Research
and Education
Plesmanlaan 125
P.O. Box 9137
NL-1006 AC Amsterdam
The Netherlands
T +31 20 512 38 60
F +31 20 617 80 80
d.heidsieck@sanquin.nl
www.research.sanquin.nl

Academic staff

RNI Pietersz MD PhD (head)
HW Reesink MD PhD
PF van der Meer PhD

PhD students

B Alamdary Badlou
MJ Dijkstra-Tiekstra
T Mohammadi

Technical staff

I de Cuyper
M Eijzenga-Demmendal
M Habibuw
M Kroonsberg
LAE Liefting
L Scholtalbers

Secretary

D Heidsieck

Research lines

Sanquin Blood Bank South East Region

Department of Research and Education

Address

Sanquin Blood Bank
South East Region
Department of Research and Education
Joseph Bechlaan 113
NL-6229 GR Maastricht
The Netherlands
T +31 43 387 14 56
F +31 43 387 77 80
e.rombout@sanquin.nl
www.research.sanquin.nl

Location Nijmegen

Van Leeuwenhoeklaan 4
NL-6525 HK Nijmegen
The Netherlands
T +31 24 361 65 82

Academic staff

E Rombout-Sestrienkova MD (head)
J Curvers PhD
W de Kort MD PhD
T Hoekstra PhD
J Keuren PhD
P van Noord PhD

PhD students

S Cauwenberghs
K Lemmens
M Luten

Technical staff

J Heeremans
J Klaarenbeek
E Magdeleijns
A Nillesen-Meertens
B Roerdinkholder-Stoelwinder
C Verhagen

Support staff

H Bos PhD
C van Buul PhD
J van Wersch PhD

Undergraduate students

C Bachas
R Vreugdewater

Secretary

E Francken-Titulaer

Research lines

Sanquin Blood Bank South West Region

Department of Research and Education

Address

Sanquin Blood Bank
South West Region
Department of Research
and Education
Plesmanlaan 1a
NL-2333 BZ Leiden
The Netherlands
T +31 71 568 50 53
F +31 71 568 51 91
anneke.brand@bloodrtd.nl
www.research.sanquin.nl

Location Rotterdam

Wytemaweg 10
NL-3015 CN Rotterdam
T +31 10 463 06 30
F +31 10 463 06 40

Location Dordrecht

Albert Schweizerplaats 5
NL-3318 AS Dordrecht
T +31 78 652 22 22
F +31 78 617 11 00

Academic staff

Prof A Brand MD PhD (head)
Y Hensbergen PhD
PA Maaskant-van Wijk PhD
J Scharenberg PhD
LMG van de Watering MD PhD
JA van Hilten PhD (Sanquin Corporate Staff)
Prof DJ van Rhenen MD PhD

PhD students

MY Bilgin
MB Hemker
AJG Jansen
J-L Kerkhoffs
F Schipper
C So-Osman MD
GHM Tax (Sanquin Research)
LL Trannoy
JM van Beckhoven
MM Waanders

Technical staff

F Barjiji
M Bogaerts
P de Koning Gans
L Douglas-Berger
M Feskens
A Honohan
K Koekkoek
D Loomans
J Lorinser
AJC Medenblik
M Slot
J van Wintershoven

Undergraduate students

M Jansen

Secretary

J Cabenda-Plaizier

Research lines

Product Development Departments

Product Development

Sanquin Plasma Products

Address

Sanquin Plasma Products
Department of Product Development
Plesmanlaan 125
P.O. Box 9190
NL-1006 AD Amsterdam
The Netherlands
T +31 20 512 39 35
F +31 20 512 36 10
a.koenderman@sanquin.nl
www.sanquin.nl

Academic staff

AHL Koenderman PhD (head)
M Kleijn PhD

Technical staff

J Bloem
JM Breen
GJ Derksen
IPP Prins-de Nijs
HGJ ter Hart
SNM van 't Schip
N Willem

Secretary

J de Witte-D'Souza

The product development strategy of Sanquin Plasma Products aims primarily at maintaining the state-of-the-art level of its plasma derivatives portfolio and production processes. To that end, the product and process development program is regularly evaluated and updated if needed. Besides, opportunities for development of new (plasma) products are being explored in feasibility studies which may evolve into full-blown development projects when considered to be economically feasible.

Project leader: H ter Hart (h.terhart@sanquin.nl)

In 2004, product development focused on the liquid intravenous immunoglobulin project, in which Sanquin Plasma Products is co-operating with Finnish Red Cross Blood Transfusion Service and Sanquin Oy in Helsinki. By means of a Mutual Recognition Procedure marketing authorization for Nanogam was obtained in Sweden, Norway, Denmark, Belgium, France and The Netherlands. As this product will be manufactured by Sanquin, three large scale batches of Nanogam were manufactured in Sanquin's manufacturing facility and stability studies were started. Data will be used to prepare a variation dossier for the change of the manufacturing site. Suitability studies for the use of paste II from third parties for the manufacturing of Nanogam were started.

In close collaboration with the Research Division of Sanquin, a project to characterize liquid immunoglobulin products is ongoing. Absence of immunogenicity of pepsin, used in the manufacturing of Nanogam was shown and studies on the nature of fragment formation are ongoing.

Project leader: M Kleijn (m.kleijn@sanquin.nl)

Feasibility to implement a 15 nm Planova-filtration step in the manufacturing process of Cetor® to enhance the virus safety of this high purity C1-inhibitor product was shown and three large scale batches with this improved process were manufactured. Stability studies were started. Robustness studies on the virus reducing capacity of this new step were performed and showed excellent results. Feasibility of the use of US plasma was shown.

Clinical studies in The Netherlands and USA with this virus safe Cetor® will start in 2005.

Project leader: I Prins (i.prins@sanquin.nl)

In collaboration with Laboratoire Français du Fractionnement et des Biotechnologies (LFB) in Les Ulis, France, a 15 nm Planova filtration step was implemented in the manufacturing process of Kaskadil to obtain a product comparable to Sanquin's current product Cofact®, a prothrombin complex concentrate. Shelf-life studies and virus validation studies are ongoing and clinical studies will start in 2005.
In close collaboration with Sanquin Virus Safety Services prion removal studies are started.

Project leader: A Koenderman (a.koenderman@sanquin.nl)

Another project concerns a new potential anti-HIV agent, so called negatively charged albumin. The three clinical grade batches produced so far have shown excellent stability on storage. Approval to perform a proof-of-principal studies in a limited number of AIDS patients was obtained and the studies were started end of 2004.
This development project is executed in co-operation with the University Centre for Pharmacy, University of Groningen and the International Antiviral Therapy Evaluation Centre (IATEC) of the Academic Medical Centre of Amsterdam.

Product Support Division

Central Fractionation Department of the Belgian Red Cross (CAF-DCF), Brussels

Address

CAF-DCF
De Tyraslaan 109
B 1120 Brussels
T +32 2 264 64 90
F +32 2 262 27 31
ruth.laub@
caf-dcf.redcross.be

Academic staff

P Strengers MD (Medical Director)
R Laub PhD (head)
M Di Giambattista Ig (supervisor)

Technical staff

Th Branckaert
V Hougardy
V Labrune
F Reyé
B Vandenborre

Secretary

B Kips

The main focus of the Product Support Division (former R&D) (CAF-DCF) is the quality and safety of plasma derivatives. Viral safety is the consequence of a number of interlocking actions starting with epidemiological virus surveillance of donors and pathogen-specific testing (HIV, HCV, B19) of plasma pools. Therefore much effort has been devoted to validating further NAT testing for the HAV and HBV viruses, to be implemented as release criteria. One of the main goals is also to improve pathogen safety through the development of novel complementary and robust technologies including UVC irradiation, active against the largest range of blood-transmitted viruses. The quality of plasma derivatives is assessed by state-of-the-art characterization of both immunological properties (therapeutic protein epitopes, specific immunoglobulins) and biochemical properties by steady state fluorescence spectroscopy.

Epidemiology

Epidemiological data on HIV, HCV, and HIV serological status in first-time and repeat donors show a drastic decrease in HCV incidence, and to a lesser extent HBV incidence, from 1997 to 2003.

UVC technology will further improve the viral safety of therapeutic proteins

Despite the increasing number of screening tests being introduced, ensuring the inactivation of blood-transmitted pathogens in blood-derived therapeutic proteins remains a major concern. Procedures for inactivating large amounts of different viruses, especially non-enveloped and/or heat-stable viruses, are therefore mandatory. Our department has been involved in designing a new, effective inactivation methodology.

We have shown that UVC irradiation is particularly effective against a wide range of pathogens (bacteria and viruses) that resist to common inactivation procedures at dosages preserving protein activity and integrity. UVC treatment thus appears advantageous as compared to currently recommended inactivation procedures, as shown by its high inactivation capacity for Parvo viruses, pathogens that cannot be destroyed easily by conventional methods. A fully GMP-compliant dynamic UVC irradiator was constructed and is available for further studies on proteins and cells.

In vitro cell-based assays for Erythrovirus B19 infectivity

Infections caused by human Parvo virus B19 can result in a wide spectrum of manifestations, influenced by the patient's immunological and hematological status. Although B19 has been propagated in established erythroid cell lines differentiated by erythropoietin treatment, these cells are difficult to handle and show low permissiveness and a low expression level. We report the development of two infectivity assays suitable for studying B19 biology, identifying neutralization antibodies, and evaluating virus inactivation methods, based on human erythroid cell lines cultured under hypoxia and human hepatocarcinoma cell lines.

We have exploited the fact that low oxygen pressure (hypoxia) can favor the development of the pluripotent human erythroid cell line KU812F and Parvo virus life cycles. Our results show that low oxygen pressure (6% O_2) leads to higher yields of B19 progeny and a higher level of viral transcription than observed under normal conditions (20% O_2). It takes only a few viral particles (low MOI) to cause the release of abundant infectious progeny in multiple rounds of infection. In the second *in vitro* model for B19 infectivity, we used two hepatocarcinoma cell lines (HepG2 and HuH7) that express the P blood group antigen, i.e. the receptor for B19. At low MOI (from 0.1 to 100 IU B19-DNA per 510^5 cells), the viral progeny released into the supernatant was detectable 72 h post-infection by nested PCR, at a dilution as low as 10^6. Moreover, this assay proved very useful for challenging the neutralizing capacity of human (IVIG) and rabbit antibodies (recognizing potential epitopes of VP1 and VP2, the B19 capsid proteins). Interestingly, the results show that the neutralizing capacity of two different IVIGs (Multigam and Sandoglobulin) is product-related.

Anti-inflammatory effects of UV irradiated lymphocytes

UV light has a marked capacity to modulate immune responses, and UV irradiation has found many clinical applications (photo chemotherapy for psoriasis, treatment of cutaneous T cell lymphoma, allograft rejection and graft-versus-host disease resistant to conventional approaches, and induction of immunotolerance). One of the most significant actions of UV irradiation on cells is induction of apoptosis. Phagocytosis of apoptotic cells results in secretion of IL-1Ra, an anti-inflammatory mediator controlling pathogenic mechanisms such as sepsis and inflammatory diseases. We report that

Results show that total antibodies against *Streptococcus pneumoniae* are a useful tool for evaluating immune potency in individuals.

UVC irradiation of lymphocytes in the presence of autologous peripheral blood mononuclear cells results in a remarkable increase in IL-1Ra mRNA (+340%; p=0.001) and protein (+72%; p=0.001) and induces lymphocyte apoptosis followed by phagocytosis by monocytes/macrophages, a step that preferentially activates IL-1Ra. This adds a new pathway of IL-1Ra activation to those previously described, triggered by agents such as LPS, IVIG, or GM-CSF.

Anti-Streptococcus antibodies in plasma pools, immunoglobulin, and patients

Streptococcus pneumoniae is a complex human pathogen affecting children and elderly people worldwide. It causes infection of the upper respiratory tract, bacteraemia, and meningitis and is a major cause of morbidity and mortality. The aim of this study was to assess the immune response of healthy individuals, imunodeficient patients, vaccinated patients, and patients undergoing treatment with intravenous immunoglobulins. A novel immunoassay was used to quantify the level of total and capsule-specific anti-pneumococcal antibodies. Serotype specificity towards 13 serovars was also determined with monospecific antigens. The results show a good correlation between both methodologies and demonstrate that total antibodies against *Streptococcus pneumoniae* are a useful tool for evaluating immune potency in individuals and for quantifying specific antibodies in plasma and plasma derivatives. The studies described here were performed in collaboration with Dr I Thomas (Institut Scientifique de Santé Publique – Louis Pasteur, Brussels), Dr PC Caillet-Fauquet and Prof Y de Launoit (ULB – Laboratoire de Virologie Moléculaire, Brussels). Collaborations are running also with Dr L Craciun, Prof E Dupont and Prof M Goldman (ULB – Laboratoire d'Immunologie Expérimentale, Brussels).

Key publications

Caillet-Fauquet P, Di Giambattista M, Draps ML, Sandras F, Branckaert T, de Launoit Y, Laub R. Continuous-flow UVC irradiation: a new, effective, protein activity-preserving system for inactivating bacteria and viruses, including erythrovirus B19. J Virol Methods 2004; 15;118(2):131-9.

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Medical Department

Sanquin Plasma Products

Address

Sanquin Plasma Products
Medical Department
Plesmanlaan 125
P.O. Box 9190
NL-1006 AD Amsterdam
The Netherlands
T +31 20 512 32 39
F +31 20 512 37 94
p.niekoop@sanquin.nl

Staff Medical Department

PFW Strengers MD (Director Medical Affairs and Product Development)

Medical Advisors

JJ Marcar MD
TJ Schouten MD PhD

Clinical Research Associates

A Faber
I Kleine Budde PhD
PJM Vossebeld PhD

Secretary

P Niekoop-Snijders

The Medical Department is responsible for the development and performance of clinical trials with plasma products in order to obtain marketing authorization or new indication(s) for new or authorized plasma products. The Medical Department cooperates closely with clinical investigators in the Netherlands and abroad for development and performance of the trials, i.e. with the Inter-University Working Party on the Study of Immune Deficiencies and the Hemophilia Treatment Centers.

The Medical Department is also responsible for pharmacovigilance. Pharmacovigilance is a system of activities to monitor safety of medicinal products in regular medical care in order to prevent the occurrence or recurrence of adverse drug reactions. Pharmacovigilance is performed either passive based on received reports, or active by performing post authorization safety studies (PASS) in ad random patient groups. Periodic Safety Update Reports (PSURs) are prepared to report the data on pharmacovigilance to the authorities. The Drug Safety Officer has prepared a safety report for Nonafact® for a one year review period. A safety report for Nanogam® was prepared together with Sanquin Oy in Finland for the first half-a-year review period.

The Medical Department gives medical information and advice to medical specialists, physicians, nurses and pharmacists on the usage of plasma products in order to safeguard the clinical use. To provide specific source plasma for the fractionation of Anti-Rhesus (D) Immunoglobulin, the Medical Department assists in the recruitment of new plasmapheresis donors and performs the selection of specific units of erythrocytes for immunization, to be used by Sanquin Blood Banks.

Clinical trials ongoing in 2004

Nonafact®

A multi-centre clinical trial 'Post marketing study in hemophilia B patients using Nonafact® 100 IU/ml powder and solvent for solution for injection (human coagulation factor IX) (human plasma derived factor IX product, freeze dried)', to study

the safety of treatment with Nonafact® in regular patient treatment is ongoing in five Hemophilia Treatment Centers in the Netherlands. Continuation of this clinical trial in Finland, in order to obtain the requested patient numbers, is in preparation.

Nanogam®

The final report of the clinical trial 'Kinetics, efficacy and safety of IVIG-L (human normal immunoglobulin for intravenous use) in patients with hypogammaglobulinaemia', showing the excellent efficacy and safety during treatment of 18 patients with primary immune deficiency, was used as part of the authorization application for IVIG-L. IVIG-L was authorized in Finland and via a Mutual Recognition Procedure also within the Netherlands and other European countries under the name Nanogam®.
The clinical trial 'Kinetics, efficacy and safety of IVIG-L (human normal immunoglobulin for intravenous use) in patients with hypogammaglobulinaemia' was continued to generate data on efficacy and safety over a longer period of time. A multi-centre post authorization safety study (PASS) to investigate efficacy and safety of Nanogam® in ad random patients with primary immunodeficiency is in preparation.
A multi-centre placebo-controlled cross-over clinical study, to demonstrate efficacy and safety of Nanogam® in patient with CIDP (chronic inflammatory demyelinating polyradiculoneuropathy), is in preparation.

PPSB-SD®

The 'Study on the efficacy of PPSB Solvent Detergent® and VP-VI in patients using oral anticoagulant therapy and undergoing acute cardiac surgery with a cardiopulmonary by-pass' is being carried out at the Academic Hospital of the Catholic University 'Gasthuisberg', Leuven in Belgium (in collaboration with the Medical Department of CAF-DCF cvba, the alliance partner of Sanquin).
The objective of the study is comparing the efficacy of treatment with PPSB Solvent Detergent® with the efficacy of the standard treatment with SD treated Fresh Frozen Plasma (FFP) in 40 patients. The clinical trial was finalized after including 40 patients. The final report of the study is in preparation.

MBL

A clinical trial with MBL (Mannan Binding Lectin, a product from Staten Serum Institute (SSI), Copenhagen, Denmark), entitled 'Phase II study on Mannan Binding Lectin (MBL) substitution in MBL-deficient children with chemotherapy-induced neutropenia', have been started. The objective of this trial is to investigate the pharmacokinetics and the clinical and biological effects of MBL replacement therapy in 12 MBL-deficient children during chemotherapy-induced neutropenia. The study has been started within the Academic Medical Centre in Amsterdam. In order to obtain the requested number of patients, a second centre, the Erasmus Medical Centre in Rotterdam, has been added to the study.

C1-esterase inhibitor

From 1997, Sanquin has marketing authorization for Cetor®, a highly purified C1 inhibitor concentrate, for use in cases of congenital and acquired C1 inhibitor deficiency, especially in the prophylaxis and acute treatment of angioedema. To optimize the viral safety of Cetor®, a 15 nm-nanofiltration step is introduced in the production process of Cetor®. A multi-centre study 'Pharmacokinetics, clinical efficacy and safety of C1 inhibitor concentrate (C1-esteraseremmer-N) for the treatment of hereditary (and acquired) angioedema' is in preparation in collaboration with the Academic Medical Centre in Amsterdam. This study will have to demonstrate (pharmacokinetic) equivalence between the current Cetor® product and nanofiltrated Cetor®. Furthermore, it will have to demonstrate the efficacy of the nanofiltrated Cetor® in the propylaxis and acute treatment of angioedema.

Business Unit Reagents

Sanquin Research

Address

Sanquin Research
Business Unit Reagents
Plesmanlaan 125
P.O. Box 9190
NL-1006 AD Amsterdam
The Netherlands
T +31 20 512 39 11
F +31 20 512 35 70
r.melsert@sanquin.nl
www.reagents.sanquin.nl

Staff

R Melsert MSc (head)
A Blom-Bijvoet PhD
K Keuning BSc
HJ Schoenmaker BSc
P Stam MSc
JH te Velthuis PhD
EMM van der Donk PhD
G van der Kamp BSc
RVW van Eijk PhD
WJE van Esch PhD
JWAM van Oers PhD
AJS Visser MSc

Secretary

Y Lammers

Sanquin Reagents is among the first manufacturers of blood group and immune reagents in the world. By virtue of its research facilities and diagnostic laboratories, Sanquin developed a broad range of blood group and immune reagents, including several innovative products for diagnostic use and for fundamental and clinical research. Sanquin reagents are available worldwide through a network of distributors. Sanquin Reagents is ISO 9001 and ISO 13485 certified.
Sanquin Reagents is committed to introduce new products on a continuous basis. New products are the outcome of R&D projects, some of which are executed in close collaboration with departments of Sanquin Research.

R&D projects

The project portfolio in 2004 consisted of ongoing projects in the fields of blood grouping (red cell panels, Cellbind customer automation, automation of Cellbind production), IgG subclass latex based reagents, Elispot reagents, and recombinant immunoglobulin technology and applications, a.o. lama heavy chain immunoglobulin ligands.

The following new development projects were initiated

(i) two assays to detect free, human immunoglobulin light chains (kappa, lambda) in serum and urine.
(ii) an assay to detect and quantify anti-myelin antibodies in blood of MS (Multiple Sclerosis) patients. This is a collaboration with the Free University Medical Center of Amsterdam, within NUBIN, i.e. the Neuro-Unit Biomarkers for Inflammation & Neurodegeneration. NUBIN aims to improve the diagnosis and monitoring of chronic neuroinflammatory and neurodegenerative diseases, such as Multiple Sclerosis (MS) and Alzheimer's Disease (AD).

MHC tetramers

The production of class 1 MHC tetramers was transferred from the Dept of Clinical Viro-Immunology to the BUR. The collaboration with the Netherlands Cancer Institute (NKI) in a Senter funded project was continued. In 2004, the first MHC tetramers were commercially introduced in the Netherlands by Sanquin Reagents. The NKI filed

We aim to improve the diagnosis and monitoring of chronic neuroinflammatory and neurodegenerative diseases, such as (MS) and Alzheimer's Disease.

a patent on the use of photo cleavable peptides to be used with MHC complexes. This new method is expected to simplify the production of MHC multimers to a large extent in the near future.

Other new products

The following new products were commercially introduced in 2004:
(i) an ELISA based assay for quantitation of human MBL in serum (Mannan Binding Lectin).
(ii) various Elispot reagents (human interferon gamma, IL-4, and IL-10).
(iii)16-cell panel: human red cells for serological testing, in this case for column agglutination techniques.
(iv) various MACS research products (from Miltenyi Biotec).

Services Departments

Sanquin Pharmaceutical Services

Sanquin Research

Address

Sanquin Pharmaceutical Services
Plesmanlaan 125
P.O. Box 9190
NL-1006 AD Amsterdam
The Netherlands
T +31 20 512 3549
www.pharmaceutical services.nl

Academic Staff

PC van Mourik (General Manager)
EJM Al PhD
NJJ Dekker
A de Jonge
HMG Sillekens

Technical staff

M Boer
MWG Botter-Lavrijsen
M Breman
A de Kleynen
A Griffioen-van der Zwet MSc
S Hol
T Lagendijk-Rogic
G Lynch
D Tharkar MSc
M van der Laan
B van Druten-Woudenberg
S van Houten
BJ Verdonk

Support staff

J Botterman
G Guigelaar MSc
KHM Klemann MSc

Sanquin Pharmaceutical Services (SPS) is a business unit specialized in a broad array of pharmaceutical services aiming at the development of biologicals intended for therapeutical application in humans. These services include the development of adequate production processes, contract production of mammalian cell products (monoclonal antibodies and/or r-DNA) as well as safety testing and designing validation studies for assays and processes.

Contract production

SPS has ample experience in designing production strategies and scaling up of production in compliance with EU and FDA guidelines. For this purpose SPS holds a GMP-license for the production of clinical grade pharmaceuticals including large scale fermentation, purification and sterile filling. The use of a specially developed serum-free culture medium in fermentation, guarantees a process free of concerns related to the transmission of pathogens such as prions and mammalian viruses. Several generic purification schemes for different types of proteins are available, allowing SPS to provide their clients already in an early stage of development with a validated process.
In their multipurpose plant several projects can be handled simultaneously, allowing for fast turn around times.

Biosafety testing

SPS is also experienced in conducting a broad array of biosafety tests required for the pharmaceutical release of biotech products in compliance with both EU- and FDA guidelines. For this purpose, all assays have been GCLP (Good Control Laboratory Practice) accredited. Moreover, process validation studies in order to demonstrate the reduction of (model) viruses or DNA during purification as well as the validation of client dedicated assays are part of their dedicated activities.

Virus Safety Services

Sanquin Research

Address

Sanquin Research
Virus Safety Services
Plesmanlaan 125
P.O. Box 9190
NL-1006 AD Amsterdam
The Netherlands
T +31 20 512 35 94
F +31 20 512 33 10
f.terpstra@sanquin.nl
www.vss.sanquin.nl

Academic staff

FG Terpstra (head)
FAC van Engelenburg PhD

Technical staff

AGC Boots
LM Bos
FHM Brinkhuis
E Gijsen
H Korsten
AE van den Blink
Y van Remmerden
CA Witte

Virus Safety Services (VSS) is a virology group dedicated to conducting virus validation studies of plasma-derived products and other biologicals. We offer a range of virus systems, which meet with the latest requirements of national and international regulatory bodies. VSS has more than fifteen years of experience in the field of virus validation. VSS is familiar with blood safety issues being part of a blood-product producing organization.

Virus validation studies

We have a broad experience in validation of various process steps, including the more delicate ones, like column and nanofiltration steps. We have state-of-the art BSL3 facilities, including strict separation between virus negative and virus positive areas. In 1998 full accreditation was granted by the Dutch Council for Accreditation, which participates in the European Cooperation for Accreditation of Laboratories (EAL).

VSS provides tailor-made solutions for virus validation problems. Detailed information on the virus reducing capacity of process steps is provided. Furthermore smart experimental designs are used for demonstrating robustness of process steps and overall accurate insight into viral safety of your product is achieved.

Virus systems available

Appreciating requirements from relevant guidelines, for performance of virus validation studies VSS can offer the following relevant or model virus systems.

- HIV (Human immunodeficiency virus), a relevant virus for products of human origin
- HAV (Hepatitis A virus), a relevant virus for products of human origin
- Human Parvovirus B19, a relevant virus for products of human origin
- BVDV (Bovine viral diarrhea virus), a specific model virus for hepatitis C virus
- CPV (Canine parvovirus), a specific model virus for Parvovirus B19
- EMC (Encephalomyocarditis virus), a specific model virus for hepatitis A virus
- PPV (Porcine parvovirus), a specific model virus for Parvovirus B19

- PSR (Pseudorabies virus), a general model virus for lipid enveloped DNA viruses (e.g. hepatitis B virus)
- SV40 (Simian virus 40), a general model virus for non-enveloped DNA viruses
- VSV (Vesicular stomatitis virus), a general model for lipid enveloped RNA viruses

Sponsors

Various organizations, charities and industries have contributed towards research of Sanquin by funding investigators, travel expenses, equipment or offering free materials:

Landsteiner Laboratory

Sanquin Research and the Academic Medical Center of the University of Amsterdam collaborate in the joint AMC-Sanquin Landsteiner Laboratory for Blood Transfusion Research, housed in Sanquin's premises in Amsterdam.

2nd source of funding

Dutch Medical Research Council (ZON/MW)
Netherlands Organization for Scientific Research (NWO)
European Commission

3rd source of funding (Charities, privat funding organizations, non-Dutch Research councils)

CvZ-College van Zorgverzekeraars
Chronic Gronulomatosis Disease Trust
Deutsche Forschungsgemeinschaft
Dutch AIDS Fund (SAF)
Dutch Cancer Fund/KWF
Dutch Cancer Society
Dutch Heart Foundation
Dutch Thrombosis Foundation
Foundation Jan Kornelis de Cock
Foundation for Pediatric Cancer Research
Friends of Research on MS
Gratama Stichting
Joghem van Loghem Foundation
Landsteiner Foundation for Blood Research (LSBR)
Leiden University Fund
Ministry of Public Health, Welfare and Sport
Municipal Health Services Amsterdam (GG&GD)
National AIDS Therapy Evaluation Center
National Foundation for Rheumatism
Nefkens Foundation
Netherlands Asthma Foundation
Platform Alternatieve Dierproeven
Princess Beatrix Foundation
SENTER/Novem
Stichting Fondsenwervingsacties Volksgezondheid
Tekke Huizinga Foundation

4th source of funding: Contract and co-development partners

Academic Hospital, University of Maastricht
Academic Medical Center, University of Amsterdam
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American Red Cross
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ASAC
A-Viral ASA
Baxter BioScience
Baxter Health Care
Baxter Oncology
Berna Biotech
BioMérieux Nederland
BioSafe
Biotest Pharma GmbH
Boehringer Ingelheim Pharmaceuticals Inc.
Cardiovascular Research Institute Maastricht (CARIM)
Cerus Corp

Chiron corporation
Crucell
Diaclone
DSM Biologics
Finnish Red Cross
Fresenius HemoCare
Gambro BCT
Genmab
GlaxoSmithKline
Guava Technologies
Haemonetics
HAL/Madaus
Innogenetics
Jan van Breemen Institute
Kamada
Laboratoire Français du Fractionnement et des Biotechnologies
Leiden University Medical Center
LevPharma
Macopharma
Magen David Adom
Microsafe BV
Miltenyi Biotec
Morphosis AG
Natal Bioproducts Institute
Navigant Bonville
Nefkens
NIZO laboratories
OncoMab
Ortho-Clinical Diagnostics
Pharming
PhotoBioChem
ProLacta
Région de Bruxelles-Capitale
RIVM, National Institute for Public Health and the Environment
Roche Diagnostics
Schering Corporation
Seattle Genetics
Slotervaart Hospital
Stallergéne
Staten Serum Institute
Synaps BV
Synco
Universiteit Amsterdam
University Medical Center Utrecht
Vitaleech Bioscience
Vrije Universiteit Medical Center, Amsterdam
Wageningen University and Research Center
Zentech
Zentral Laborator Bern

Other sources of funding

Ministry of Economic Affairs (WBSO)
In-corporate services

Publications

On our website www.research.sanquin.nl all our publications are listed in a searchable database. Where available, links to PubMed abstracts are included on that website.

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Alphabetically, first author

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Further information may be obtained
from
Jan Willem Smeenk
Corporate Staff Office
Sanquin Blood Supply Foundation
P.O. Box 9892
NL-1006 AN Amsterdam
The Netherlands
T +31 20 512 30 00
F +31 20 512 33 03
jw.smeenk@sanquin.nl
www.research.sanquin.nl

Editors

Prof Ernest Briët MD PhD
Prof Dick van Rhenen MD PhD
Jan Willem Smeenk MSc
Anneke de Regt MA

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Sanquin
Plesmanlaan 125
NL-1066 CX Amsterdam
P.O. Box 9892
NL-1006 AN Amsterdam
The Netherlands
Telephone +31 20 512 30 00
Fax +31 20 512 33 03
info@sanquin.nl
www.sanquin.nl

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