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Sanquin
Sanquin Blood Supply Foundation
respects the fundamental principles
of the International Red Cross.

Sanquin Scientific Report 2005

Scientific Report 05

Sanquin

Blood and Beyond

Scientific Report 2005

Contents

Introduction

In 2005 Sanquin continued strengthening its research portfolio in the broad area of transfusion medicine and blood related research. Facing a diminishing amount of research funding from its own resources, extra effort was put in securing research funding from outside, from research councils and charities. In competition with other research groups, Sanquin researchers were successful in obtaining research grants and contract research commissions.

Personnel and Organization

In 2005 Professor Ernest Briët joined the Executive Board and took the responsibility for medical affairs and research & development. He is professor of medicine at the Academic Medical Center, University of Amsterdam and was appointed part time professor of epidemiology of blood transfusion at the Leiden University Medical Center. Prof. Briët also acts as Director of Sanquin Research.

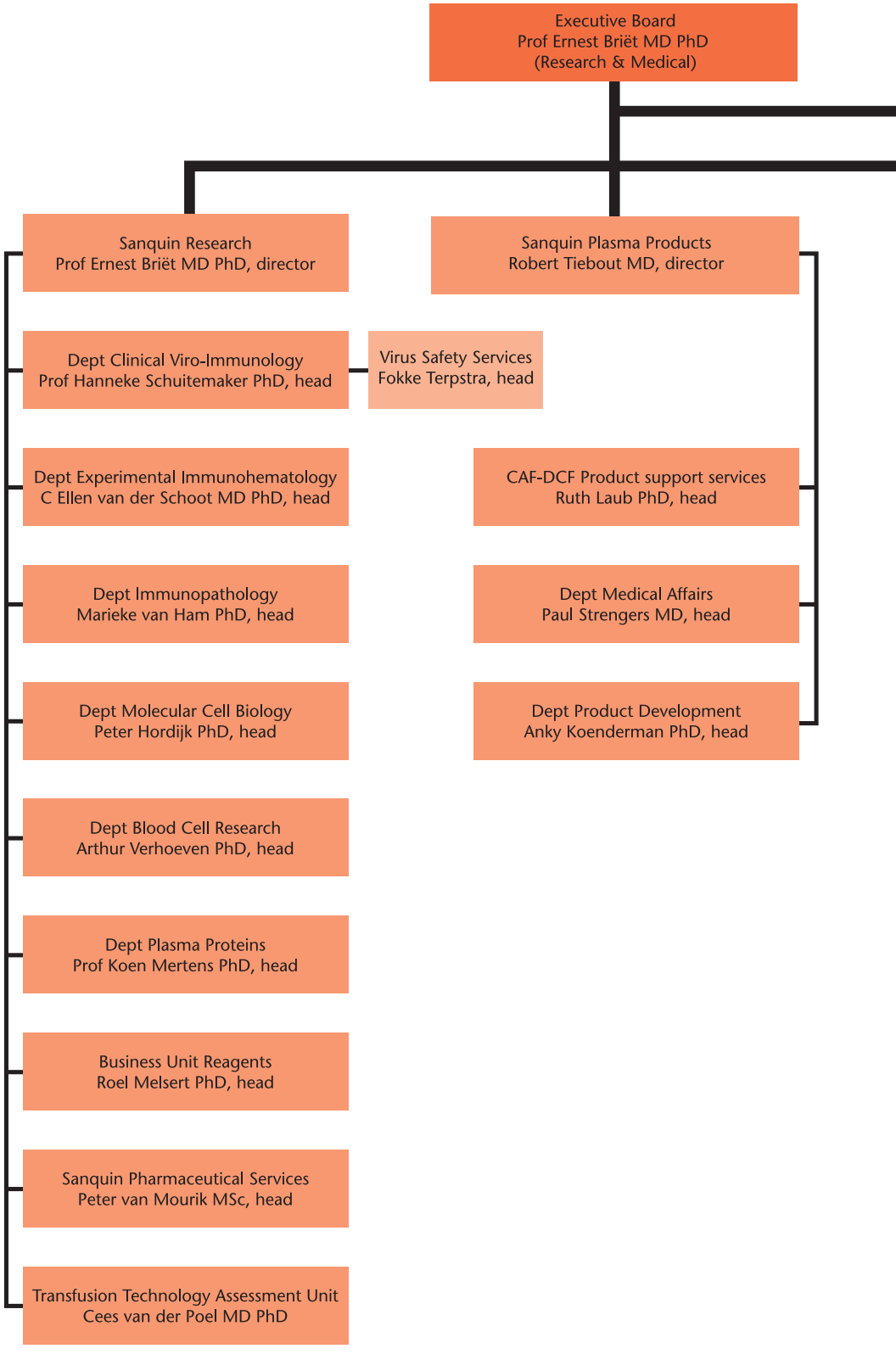
The research group of Ronald van Ree (Allergy) left Sanquin to continue its work at the Academic Medical Centre of the University of Amsterdam.

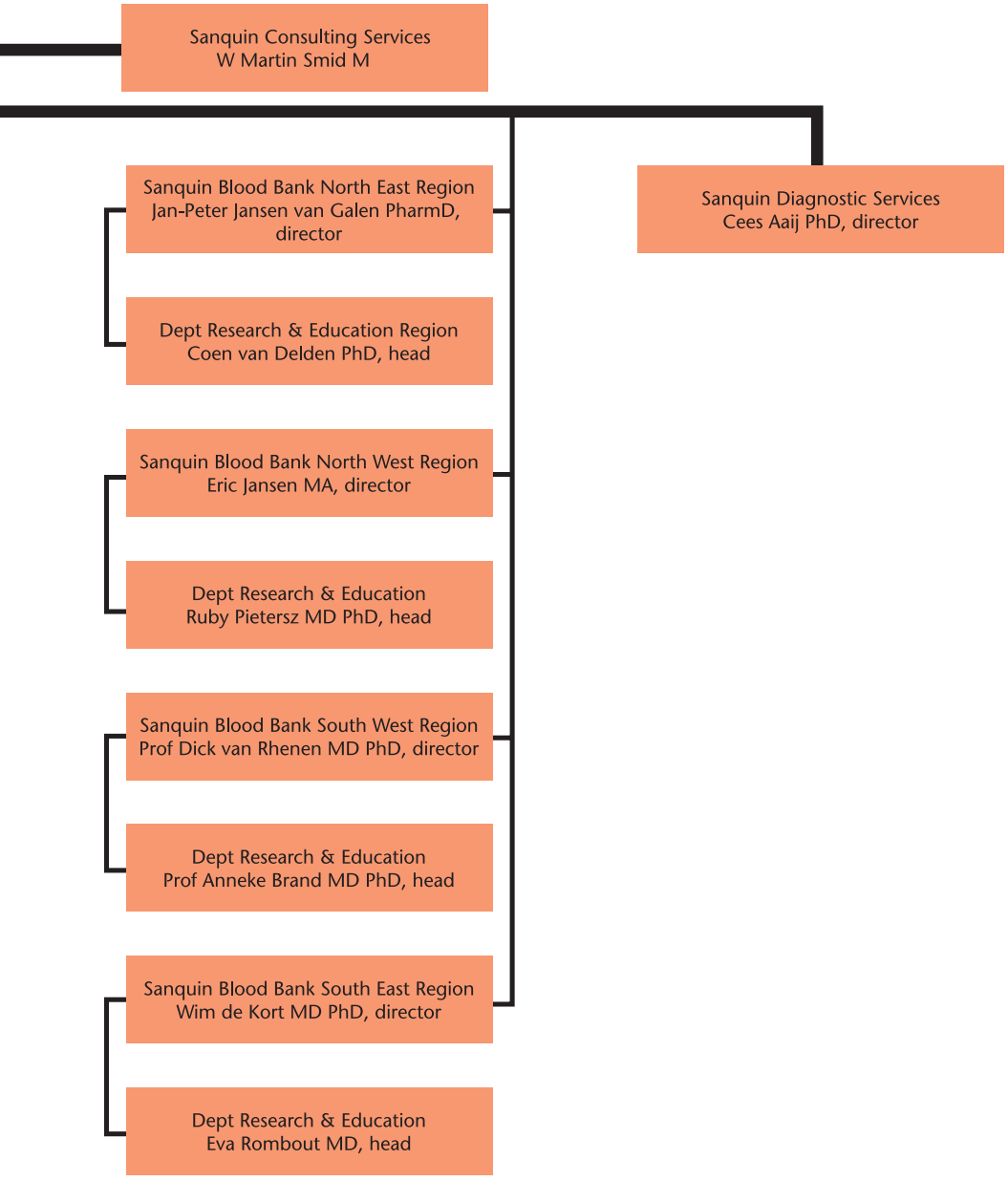
In 2005 the new departmental structure of Sanquin Research was consolidated. Sanquin Research has now six research departments.

Research Assessment and Quality Control

Bibliometric analysis

In 2004 Sanquin commissioned a Bibliometric analysis of its research output over de years 1992 – 2003. The analysis - performed by the Leiden University Center for Science and Technology Studies - showed that Sanquin overall scored well above world average on all the main indicators. The results (f.i. subfield indicator 1.37) were comparable with that of the mean score of the Dutch University Hospitals (1.35; 1.0 being the world average). A benchmark with another European Blood Supply establishment also showed Sanquin to perform very well. Further analyses showed that scientists citing Sanquin themselves also where members of relatively high scoring research groups.


Executive Board
Prof Ernest Briët MD PhD
(Research & Medical)
Sanquin Research
Prof Ernest Briët MD PhD, director
Sanquin Plasma Products
Robert Tiebout MD, 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
CAF-DCF Product support services
Ruth Laub PhD, head
Dept Immunopathology
Marieke van Ham PhD, head
Dept Medical Affairs
Paul Strengers MD, head
Dept Molecular Cell Biology
Peter Hordijk PhD, head
Dept Product Development
Anky Koenderman PhD, head
Dept Blood Cell Research
Arthur Verhoeven PhD, head
Dept Plasma Proteins
Prof Koen Mertens PhD, head
Business Unit Reagents
Roel Melsert PhD, head
Sanquin Pharmaceutical Services
Peter van Mourik MSc, head
Transfusion Technology Assessment Unit
Cees van der Poel MD PhD


Sanquin Consulting Services
W Martin Smid M
Sanquin Blood Bank North East Region
Jan-Peter Jansen van Galen PharmD, director
Dept Research & Education Region
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
Sanquin Diagnostic Services
Cees Aaij PhD, director

Scientific Advisory Board

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 drawn up by the advisory board.

On December 31, 2005 the SAB consisted of:

Prof E Briët MD, PhD (Chairman, Sanquin Executive Board & Universities of Amsterdam and Leiden)

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

C de Visser PhD (Nefarma)

Prof RRP de Vries (Leiden University)

Prof DE Grobbée MD PhD (Utrecht University)

Prof MM Levi MD PhD (University of Amsterdam)

Prof DKF Meijer PhD (University of Groningen)

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

JW Smeenk MSc, Executive secretary (Sanquin Corporate Staff)

Research programming committee

In 2005 the Research Programming Committee (RPC) consisted of four members, the directors of Sanquin Plasma Products (RF Tiebout MD), Diagnostic Services (C Aaij PhD) and Sanquin Research (Prof E Briët MD PhD), and Prof DJ van Rhenen MD PhD on behalf of Sanquin Blood Bank divisions, supported by an executive secretary (JW Smeenk MSc).

The RPC advises Sanquin Executive Board on the main themes of the research program as well as the selection of research projects funded by Sanquin itself. In 2005 actions were taken to further improve the quality of research proposals to be submitted to external funding agencies and charities by introducing internal review meetings.

Site visits

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. In 2005 no site visits took place. Following the advice of the Scientific Advisory Board the focus of the site visits was changed from a departmental set up to a more subject related focus, involving research departments from all Sanquin divisions. These subjects correspond with the research lines described in this report. Site visits according to this new scheme will start in 2006.

On December 31, 2005 members of the Research Assessment Board were:

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 Haven, 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 (Vaccin Research Center, National Institutes of Health, Bethesda, USA)

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

Accreditation

In April 2005 the departments of Virus Safety Services, Clinical Monitoring, and Blood Transfusion Technology received reaccredidation of the Central Committee for Clinical Chemistry Laboratories and the Dutch Accreditation Council. The Stem cell-laboratory received its ISO 9001 certificate. In December Virus Safety Services received the Endorsement of Compliance with OECD GLP principles.

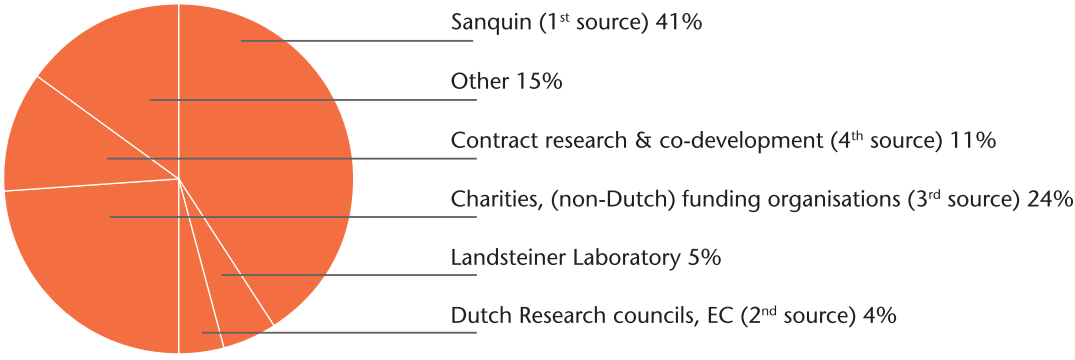
Spring Seminar

In 2005 the first in a new series of biennial conferences was organized under the heading of Sanquin Spring Seminars. In April over 250 scientists met in Amsterdam for the Sanquin Spring Seminar on Red Blood Cell Aspects, Defects and Prospects. The next Sanquin Spring Seminar will be held in 2007 on immunoglobulins.

Funding

In 2005 Sanquin researchers were again successful in obtaining external funding (See page 146 for an overview of our sponsors). Various research groups were successful in funding from the sixth Framework Program of the European Community. Contract Research income was higher than in the years before. About ten 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 fifteen proposals could not be funded.

Sources of funding of research projects (direct costs only)



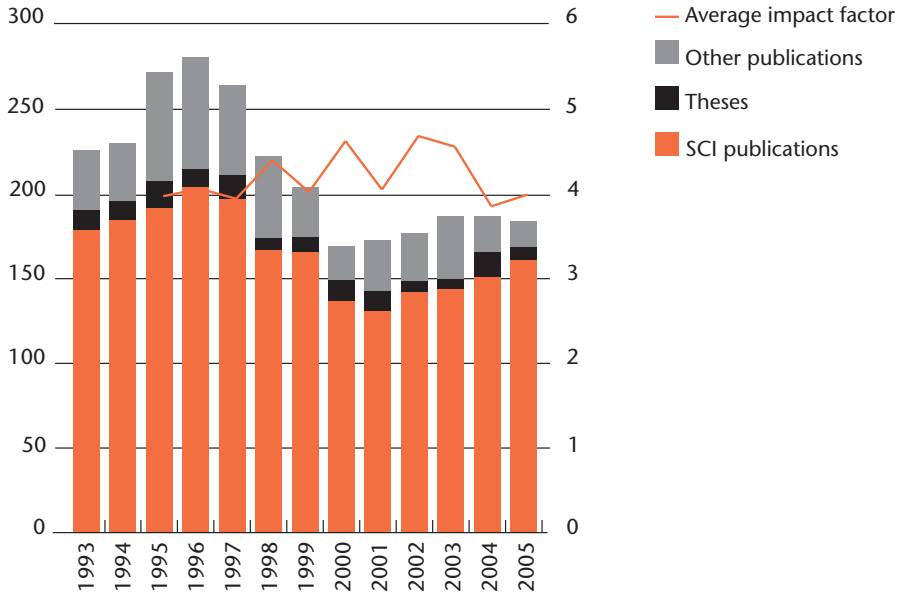
Publications

The number of publications in peer reviewed journals was slightly higher than in the last few years, with an average impact factor of 4.00. Eight PhD theses were defended.

The number of 5 years citations of papers published in 2000 is 2699.

Scientific publications

Year	Total number	SCI publications	Theses	Average impact factor
2005	187	164	8	4.00
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
1993	226	179	12	Na



Articles* published in 1993 through 2000 annual reports cited** in five full years after publication

Publications from	*Total citations*	*Number of SCI publications*	*Average number citattions* per publication
1993	3483	179	19,5
1994	3599	185	19,5
1995	3215	192	16,7
1996	3057	204	15.0
1997	2962	197	15.0
1998	3448	167	20.7
1999	2910	166	17.5
2000	2699	137	19.7

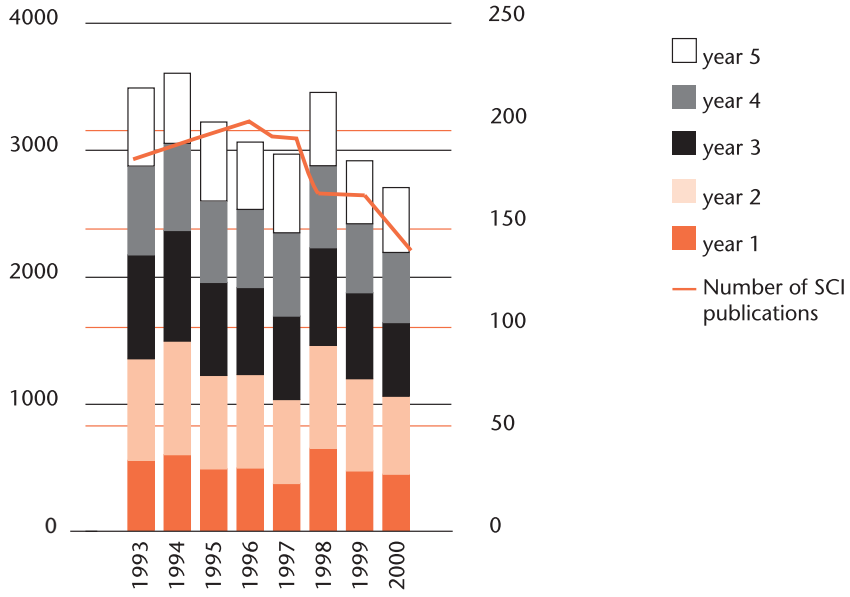
Articles* published in 1993 through 2000 annual reports cited** in five full years after publication

Citations in year

	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005
1993	551	800	818	699	615							
1994		596	894	871	686	552						
1995			484	736	732	641	622					
1996				491	736	685	615	530				
1997					369	661	657	656	619			
1998						646	811	768	646	577		
1999							468	726	677	543	496	
2000								442	614	580	552	511

* Only SCI publications are included

** Excluding self citations



Academic affiliations, education and training

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. 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 technology. Of course, Sanquin is also involved in training of specialists in blood transfusion medicine, other medical specialties, and training of nurses. Sanquin Consulting Services provides 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.

Landsteiner Laboratory

As mentioned above, there is a long standing collaboration with the University of Amsterdam in the joint AMC-Sanquin Landsteiner Laboratory. Through this collaboration Sanquin staff members participate in research programs and curricula of the AMC Research Institute for Immunology (JJ van Loghem Institute) and the Research Institute for Infectious Diseases. Sanquin staff members are heading the following research themes of the AMC research program:

- Infections, Sepsis and Inflammation Processes (prof D Roos PhD)
- Autoimmune Diseases, Allergies, Immunodeficiencies (prof RC Aalberse PhD)
- Immunogenetics & Transplantation Immunology (CE van der Schoot MD PhD)
- Virus and host factors in AIDS pathogenesis (prof J Schuitemaker PhD)

At the end of 2005 Sanquin and AMC started talks on further strengthening of this collaboration.

Professorships Sanquin Staff

Prof Rob Aalberse PhD (Biological immunology, Subfaculty 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, Leiden University Medical Center)

Prof Ernest Briët MD PhD (Epidemiology of blood transfusion, Leiden University Medical Center and Medicine, Academic Medical Center, University of 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 Dick van Rhenen MD PhD (Blood transfusion medicine, Erasmus University Medical Center, University of Rotterdam)

Prof Dirk Roos PhD (Non-specific immunology, Academic Medical Center, University of Amsterdam)

Prof Hanneke Schuitemaker PhD (Virology, especially viro-pathogenesis of AIDS, Academic Medical Center, University of Amsterdam)

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

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In a collaborative European study (BloodGen) we validated the first prototype of a so-called 'blood group-chip'. A panel of selected DNA samples from donors with common and rare blood group phenotypes was tested. We also further extended a multiplex PCR methodology by which gene fragments carrying the polymorphic nucleotides specific for 29 blood group antigen systems are simultaneously amplified. We published a pilot study on the feasibility of a micro-array set up (applying ASO-probes spotted on glass) for blood group typing.

The humoral immune response against RhD has been studied at single cell level. Intriguingly we found that in the peripheral blood of hyperimmunized anti-D donors a large memory B-cell pool against the RhD antigen exists in an IgM^+ $CD27^-$ negative B-cell subset. We are presently characterizing this B-cell subset in more detail.

The IgG transport by FcRn (the main receptor responsible for the transport of IgG across the placenta) has been studied *in vitro*. Despite the differences in half life of IgG1 and IgG3 in plasma due to difference binding characteristics of these IgG's of FcRn, a similar transport rate was observed in vitro. Preliminary data indicate that the transport of IgG1 was found to be inhibited by the presence of IgG3 and vice versa.

A nation-wide study evaluating two prevention measurements that since July 1998 are part of the national prevention program pregnancy immunization, was completed. This program aims to reduce the risk of Rhesus-D-alloimmunization by administration of anti-D prophylaxis and to identify pregnancies at risk of hemolytic disease of the fetus and newborn (HDFN), to facilitate timely treatment. First, the routine screening for irregular erythrocyte antibodies (IEA) performed in the first trimester of pregnancy was evaluated by a case-control study and detailed analysis of the outcome. This study focussed on non-rhesus-D-IEA. It was concluded that yearly 4 cases need to be treated by intra-uterine blood transfusions to prevent fetal death resulting from non-rhesus-D-IEA HDN. In addition, each year, 12 children need to be treated with exchange or blood transfusions because of non-rhesus-D-IEA. Most cases with severe HDN are due to rhesus antibodies (mainly anti-c) and Kell antibodies. The study identifies possibilities to reduce the costs of the current

screening policy. Second, the efficacy of the antenatal anti-D-prophylaxis to RhD-negative women in their first pregnancy was evaluated. It was observed that early in a second pregnancy, antenatal anti-D prophylaxis in the foregoing pregnancy indeed reduced the incidence of RhD alloimmunization with the expected 50%. If antenatal anti-D-prophylaxis in the first pregnany results in a reduction of the incidence of anti-D formation also at the end of the second pregnancy will be investigated.
An economic evaluation of PCR-guided antenatal anti-D prophylaxis (only antenatal anti-D is give to women carrying a D-positive child) showed that this approach is cost-effective. In the European Network of Excellence (SAFE) the application of new technological approaches for non-invasive prenatal genotyping on fetal DNA present in maternal blood is further investigated.

Key publications

Beiboer SHW, 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 PCR and DNA microarray. Transfusion 2005; 45:667-79.

Bianchi DW, Avent ND, Costa JM, van der Schoot CE. Noninvasive prenatal diagnosis of fetal Rhesus D: ready for Prime(r) Time. Obstet Gynecol 2005; 106:841-4.

Molecular blood group polymorphisms

Principal investigator
Petra Maaskant-van Wijk PhD
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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 (poly)transfused patients with (multiple) antibodies.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 of paternal RHD zygosity is of clinical interest for RhD alloimmunized RhD negative pregnant women, as the risk of an affected child is 100% when the father is homozygous for RHD.The RFLP method was compared with 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. 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. For other (non-Rh-D) blood group antigens, we developed automated Pyrosequencing assays and validated these for HPA-1, -2, -3, -5 and –15 platelet antigens. Patients with thrombocytopenia sometimes develop HPA antibodies and benefit from the constant availability of an HPA typed donor pool for transfusions with HPA-matched blood products. Therefore blood banks should maintain a HLA and HPA-typed donor pool of apheresis-platelet donors. Donors registered in 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. For neonates with trombocytopenia HPA typed donor platelets are constantly available. 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 European Consortium, of which the Blood Bank South West Region is a subcontractor, 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. The prototype of this micro array was developed in 2004.

Key publications

Grootkerk-Tax, Martine GHM, et al. The highly variable RH locus in nonwhite persons hampers RHD zygosity determination but yields more insight into RH-related evolutionary events. Transfusion 2005; 45:327-37.

Tax, Martine GHM, et al. RHC and RHc genotyping in different ethnic groups, Transfusion; 2002; 42:634-44.

Qun X, et al. Systemic analysis and zygosity determination of the RHD gene in D-negative Chinese Han population reveals a novel D-negative RHD gene. Vox Sanquinis 2005; 88:35-40.

Beiboer, Sigrid HW, et al. Rapid genotyping of blood group antigens by multiplex polymerase chain reaction and DNA microarray hybridization. Transfusion 2005; 45:667-79.

Granulocyte activation

Recognition of microbes

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Research and Emma
Children's Hospital AMC
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Neutrophils can bind pathogens to their surface either by means of Pattern Recognition Receptors for microbial structures or by complement and Fc receptors that recognize complement fragments and antibodies that cover the microbes.
To study the function of pattern recognition receptors in neutrophils in more detail, we have adopted a culturing system with which we can generate mature human neutrophils from $CD34^+$ cells isolated from cord blood, bone marrow or peripheral blood. We have pursued the research concerning the Toll-like receptor (TLR) family of surface recognition proteins in this system. We are currently expressing different mutants of either TLRs or proteins implicated in intracellular transduction of signals from TLRs in $CD34^+$ cells, which we then differentiate towards mature neutrophils. The $CD34^+$ culturing system is also suitable for knock-down experiments by the use of retroviral or lentiviral vectors expressing shRNA sequences. We have elucidated the roles of different receptors involved in yeast recognition, including complement receptor 3, the beta-glucan receptor Dectin-1 and TLR2 (manuscript submitted). We found that each of these receptors has a specific and defined role in yeast uptake and subsequent cytokine production by neutrophils. CR3 was found to be the receptor responsible for yeast phagocytosis, whereas Dectin-1 and TLR2 are pivotal for the secretion of cytokines and the priming of neutrophils by yeasts, respectively. In addition, we are currently studying the expression and function of intracellular pattern recognition receptors, the so-called Caterpillar family of proteins. Preliminary results have shown that neurophils express various representatives of this protein family, either constitutively or in response to growth factor and/or cytokine stimulation. This research is carried out in close collaboration with the group of Prof Jurg Tschopp (Lausanne, Switzerland).
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 various pathogens (bacteria, yeast and fungal species). In the past year, we have tested the MBL-dependent opsonophagocytosis of *Staphylococcus*

aureus, Escherichia coli, Streptococcus pneumoniae (different strains), *Candida albicans, Cryptococcus neoformans* and *C zymosan* (derived from *Saccheromyces cerevisiae*), with several MBL-sufficient (A/A) or MBL-deficient (0/0) sera. We found a significant reduction in the phagocytosis of zymosan ($P=0.003$) and Candida albicans ($P<0.05$) after opsonization with the MBL-deficient compared to MBL-sufficient sera, whereas no significant difference was detected for the phagocytosis of the various bacteria tested. Micro-organisms incubated with heat-inactivated serum (30 min, 37°C) were not phagocytised, identical to micro-organisms not incubated with serum, indicating that complement fixation does play a role in the opsonization, albeit in case of the various bacteria, not via MBL. The opsonophagocytosis data were compared with the complement deposits on the opsonised micro-organisms by incubation with monoclonal antibodies directed against MBL, C3 and L-ficolin (another complement-activating lectin, like MBL) and measured in the FACS. MBL ($P<0.0001$ for all particles) and C3b ($P\leq0.02$) deposits on the micro-organisms were significantly higher after opsonization with MBL-sufficient sera than after opsonization with MBL-deficient sera. L-ficolin deposits on the micro-organisms were similar after opsonization with MBL-sufficient or MBL-deficient sera. However, the extent of deposited C3b was not correlated with the level of phagocytosis. Currently we are repeating these measurements in the presence of blocking anti-C1q monoclonal antibodies in the opsonization step, to measure specifically the lectin pathway of complement activation. With lysates of the opsonised micro-organisms, Western blots are currently performed to determine the deposition of MBL, ficolin, MASP1, MASP2, C3, C3bi, C4, IgG and IgM. This revealed that only the high oligomeric forms of MBL bind to the micro-organisms.

We are also involved in several clinical studies regarding MBL deficiency and susceptibility for immunology-related 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 these groups we measure MBL concentrations with a solid-phase ELISA and 6 single nucleotide polymorphisms (SNPs) in the MBL gene by Taqman allelic discrimination. 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 in Amsterdam, and the Serum Staten Institute in Copenhagen, Denmark. A total of 12 patients will be supplemented with MBL during their neutropenic period. Ten patients have already been included.

Another line of research concerns the impact of Fc-gamma receptor numbers and structure on the function of neutrophils. Variation in gene copy number (CNP) as well as single nucleotide polymorphisms (SNPs) driven by evolution are regarded as sources for inter-individual differences. We focus on the gene-to-function relationship of low affinity Fc-gamma receptor gene cluster, notably FcγR2 and FcγR3. By utilizing multiplex ligation-dependent probe amplification (MLPA), we investigate whether variation in the SNPs and CNPs within the FcγR2 and FcγR3 gene clusters predisposes to auto-immune diseases such as idiopathic thrombocytopenia (ITP) and Kawasaki Disease (KD). In relation to this notion, we also quantify mRNA as products of gene transcription by semi-quantitative RT-PCR on the Lightcycler. Previously, we reported that the ratio of FcγRIIa/FcγRIIb2 mRNA correlated to responsiveness of neutrophils to IgG-containing complexes. Furthermore, we showed that differences in FcγRIIa/FcγRIIb2 mRNA ratios observed within the healthy population were strongly associated to the 2.B4 promoter haplotype of FcγR2B. Currently, we are developing antibodies specific for FcγRIIa and FcγRIIb to determine the protein expression levels within these cells.

Key publications

Brouwer N, Dolman KM, van Zwieten R, Nieuwenhuys E, Hart M, Aarden LA, Roos D, Kuijpers TW. Mannan-binding lectin (MBL)-mediated opsonization is enhanced by the alternative pathway amplification loop. Molecular Immunology 2006; 43(13):2051-60.

Van Mirre et al. Neutrophil responsiveness to IgG determined by fixed ratios of mRNA levels for activating and inhibitory FcRII (CD32): stable over time and unaffected by cytokines. Blood, in press.

Frakking et al. The role of mannose-binding lectin (MBL) in pediatric oncology patients with febrile neutropenia. Eur J Cancer 2006, in press.

Frakking et al. High prevalence of mannose-binding lectin (MBL) deficiency in premature neonates. Clin Exp Immunol. 2006, in press.

Maffei G, Brouwer N, Dolman KM, van der Velden U, Roos D, Loos BG. Plasma Levels of Mannan-Binding Lectin in Relation to Periodontitis and Smoking. J Periodontol 2005; 76(11):1881-9.

Sprong et al. Deficient alternative complement pathway activation due to factor D deficiency by two novel mutations in the Complement Factor D gene in a family with meningococcal infections. Blood, in press.

NADPH oxidase

Principal investigators

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Dept Blood Cell Research
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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. The activation of the NADPH oxidase is controlled by several small Rho- or Ras-like GTPases, one of which may be Rap1A. In various cell types it has been found that cyclic AMP (cAMP) induces Rap1 activation via the guanidine exchange factor EPAC. However, in neutrophils cAMP does not induce Rap1A activation. Since cAMP is also a protein kinase A (PKA) effector and Rap1 can be phosphorylated by PKA, we investigated the effect of PKA stimulation/inhibition on the formyl-methyl-leucyl-phenylalanine (fMLP)-induced Rap1 activation. We found that pre-treatment of neutrophils with the PKA inhibitor H89 prolongs the fMLP-induced Rap1 activation, whereas stimulation of PKA by a cell-permeable cAMP analog inhibits the fMLP-induced Rap1 activation. We are currently investigating the mechanism of this PKA-mediated regulation of Rap1 activation.
Patients with a deficiency of the leukocyte NADPH oxidase suffer from chronic granulomatous disease (CGD). This can be caused by defects in any of 4 genes

encoding components of the leukocyte NADPH oxidase. One of these is the autosomal NCF1 gene encoding the p47^{phox} protein. Most (>97%) CGD patients without p47^{phox} (A470 CGD) are homozygotes for one particular mutation in NCF1, a GT deletion in exon 2. This is due to recombination events between NCF1 and its two pseudogenes (ψNCF1) that contain this GT deletion. We have previously set up a gene-scan method to establish the ratio of NCF1 genes and pseudogenes. With this method we now found in two families patients with the normal number of two intact NCF1 genes (and four ψNCF1 genes), and in six families we found patients with one intact NCF1 gene (and five ψNCF1 genes). All patients lacked p47^{phox} protein expression. These results indicate that other mutations were present in their NCF1 gene than the GT deletion. To identify these mutations, we designed PCR methods to specifically amplify the cDNA or parts of the genomic DNA from NCF1 but not from the ψNCF1 genes. We found point mutations in NCF1 in seven families, and a 2860-bp deletion in another family. In all families except two, the patients were compound heterozygotes for the GT deletion and one of these other mutations. Family members with either the GT deletion or one of these other mutations were identified as carriers. This knowledge was used in one of the families for prenatal diagnosis.

We are also involved in joint research with the group of Timo van den Berg (Free University, Amsterdam) concerning signal regulatory protein (SIRP)-alpha as a potent negative regulator of the phagocyte NADPH oxidase. This is supported by the following evidence. First, we have observed that granulocytes, monocytes and macrophages from mice that lack the SIRP-alpha cytoplasmic tail and therefore the capacity of the receptor to signal, have a significantly enhanced oxidative burst as compared to cells from normal mice. Consistently, we have found that overexpression of full-length SIRP-alpha in human myeloid PLB985 cells essentially abolishes their oxidative burst capacity. Our subsequent experiments have provided evidence that SIRP-alpha acts, at least in part, by a selective downregulation of gp91^{phox}, which constitutes the catalytic subunit of the phagocyte NADPH oxidase complex. SIRP-alpha signaling most probably involves the SIRP-alpha-associated tyrosine phosphatases SHP-1 and/or SHP-2, because negative regulation of the oxidative burst and gp91phox does not occur when either the entire SIRP-alpha cytoplasmic tail is

deleted, or when the tyrosine residues of the ITIM motifs that mediate SHP-1 and SHP-2 recruitment are mutated. Our observations also provide evidence for a second and probably much faster acting mechanism of NADPH oxidase regulation by CD47-SIRP-alpha interactions. This is based on the findings that antibodies that block SIRP-alpha-CD47 interactions promote the oxidative burst in either human or rodent phagocytes. Because these antibodies exert their effect within minutes after addition, we propose that they somehow act by promoting NADPH oxidase assembly instead of regulating expression levels of $gp91^{phox}$ or any other relevant protein.

During the standard screening of neutrophil functions in patients with recurrent infection, we found three patients characterized with a novel defect in NADPH-oxidase activation. Activation of the oxidase with fMLP after priming with platelet-activating protein (PAF) or other priming agents appeared to be strongly diminished, whereas phorbol myristate acetate (PMA)-induced activation of the oxidase was only slightly affected, and the serum-treated zymosan (STZ) response was unaffected. Every other fMLP-induced response was normal, indicating that the receptor for fMLP is present and functional, and suggesting that the defect is in the signal transduction. However, all signal transduction intermediates tested were unaffected. Measurement of oxidase activation on a single-cell basis with the NBT-slide test revealed cell subpopulations capable or incapable of superoxide production after fMLP stimulation, while all cells produced superoxide after PMA stimulation. The hypothesis that the cells incapable of superoxide production after fMLP stimulation represent not-fully-matured neutrophils that are normally not present in the circulation, is currently under investigation. One patient, in contrast to the other two, seems to have overcome the defect, because the fMLP-induced oxidase activation normalised over a period of two months.

Mutations in the SBDS gene have been shown to contribute to the Schwachman-Diamond syndrome, which is characterized by neutropenia and defective neutrophil migration. To date, little is known about the cellular and molecular functions of the SBDS protein and we therefore raised antibodies against recombinant SBDS protein. These antibodies were used to detect endogenous SBDS in the human promyelocytic

HL-60 and PLB cell lines, and our studies show a slight downregulation of SBDS expression during differentiation of these cells. Moreover, we have shown that endogenous SBDS as well as overexpressed GFP-tagged SBDS is localized in both the cytoplasm and the nucleus, suggesting a role for this protein in both cellular compartments. Our future studies will further investigate the expression levels and subcellular localization of SBDS in human $CD34^+$ cord blood stem cells and their differentiated neutrophilic progeny. Moreover, we will manipulate SBDS expression levels in both promyelocytic cell lines and human neutrophilic cells to investigate the effect of SBDS on proliferation, apoptosis, differentiation and migrational properties of these cells, to increase our understanding of the cellular function of this protein. In parallel, studies will be undertaken via a genetic and proteomic approach to unravel the molecular properties of SBDS.

In the past, we have published a unique case of familial glutathione reductase (GSR) deficiency in human blood cells, resulting in oxidative harm to these cells by products from the phagocyte NADPH oxidase. We have now identified the genetic defect underlying the absence of enzyme activity in this family. We found a homozygous deletion in the GSR-encoding gene, which – if transcribed and translated - would result in an enzyme lacking the C-terminal dimerization domain, i.e an inactive enzyme. Furthermore, we have found a second patient with GSR deficiency, and we have characterized her mutation as a combination of a nonsense and a missense mutation. In collaboration with the group of Dr Schirmer in Heidelberg we are expressing the recombinant mutant proteins and studying their properties.

Key publications

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Apoptosis

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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 the homeostasis of these cells as compared to other leukocytes. Neutrophil mitochondria maintain their membrane potential in the absence of normal respiration. Our data seem to indicate that the citric acid cycle in neutrophils is active in the absence of a fully functional respiratory chain. The mitochondrial membrane potential, maintained by the F0/F1-ATPase, is required for ATP production by the citric acid cycle. When glycolytic substrates are scarce, neutrophils rely on the extra ATP produced by their mitochondria, in addition to the ATP produced by glycolysis. Inhibition of the citric acid cycle increases the life span of neutrophils significantly. We hypothesize that culturing the neutrophil under hypoxic conditions will mimic these results and are currently investigating this process, which may have implications for neutrophil storage.

In normal neutrophil apoptosis the whole cascade of pro-apoptotic caspases is eventually activated spontaneously. This results in the activation and translocation of pro-apoptotic Bcl-2 family members, such as Bid, to the mitochondria. Bid, together with Bax, then participates in the formation of the permeability transition pore (PTP) that is required for the release of pro-apoptotic proteins from the mitochondria. When upstream caspases are inhibited, however, Bid is no longer cleaved but it still translocates to the mitochondria. The nature and relevance of this effect is currently under investigation. After mitochondrial permeabilization, pro-apoptotic proteins are released. The best studied of these is cytochrome *c* that is required for caspase-9 activation. The cytochrome *c* levels in neutrophils are very low, although caspase-9 is activated normally. We are currently determining the amount of cytochrome *c* in neutrophils to investigate whether this amount alone suffices to activate caspase-9 or whether additional factors are required.

Finally, we are investigating the nature of the inflammasome in neutrophils. The inflammasome is a large protein complex in which pro-inflammatory caspases are activated during inflammation, leading to the activation of pro-IL-1β.

The inflammasome has been characterised in macrophages, which produce high levels of IL-1β during inflammation, but has not been studied in neutrophils. Neutrophils do express large quantities of an important adaptor protein of the inflammasome complex, called ASC. We have evidence that the pro-inflammatory caspase-1 is activated in neutrophils upon infection, and that these cells also express other major components of the inflammasome, even though they produce only minimal amounts of IL-1β. The role of the inflammasome in neutrophils during infection and the implications of caspase-1 activation for apoptosis are currently under investigation.

Key publication

Meischl et al. Ischemia induces nuclear NOX2 expression in cardiomyocytes and subsequently activates apoptosis. Apoptosis, in press.

Granulocyte transfusions

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Neutropenic and immunosuppressed individuals suffer severely from bacterial and fungal infections. Despite treatment with broad spectrum antibiotics/mycotics, sepsis remains a serious problem. Transfusion of polymorphonuclear (PMN) concentrates can be a promising tool in addition to exclusively pharmacological treatment. Donor stimulation with a combination of G-CSF and dexamethasone increases the number of neutrophils that can be collected for the granulocyte transfusion therapy. Clinical utility, however, is limited by the inability to store functional PMNs ex vivo (due to apoptosis and subsequential lost of their functions). Additionally, to avoid graft-versus-host disease, PMN concentrates have to be irradiated prior to transfusion, which can possibly induce cellular damage and functional loss.

During the first part of our study we investigated whether the G-CSF/dexamethasone treatment, followed by irradiation of concentrates, has an impact on neutrophil functions and survival, compared to PMN concentrates obtained from healthy untreated donors. Our results show that neutrophils obtained from G-CSF/dexamethasone-treated (GTX) donors do not differ from control cells in the standard

functional assays: chemotaxis, adhesion and respiratory burst. Surface expression of CD11b/CD18, CD32, CD63 and CD66b was comparable to the control; however, we did observe increased level of CD64, and a decrease in the expression of CD16 and CD62L, which was probably caused by the G-CSF treatment. Despite lower levels of CD62L, rolling and adhesion of neutrophils to human endothelial cells was normal.

The life-span of GTX neutrophils was prolonged in the *in vitro* cultures, which showed the opportunity of storage. PMN concentrates were stored for 24 hours, undiluted at 22°C without shaking. The total WBC count was maintained after 24 hours at levels of at least 95% of the previous count. Cell morphogly, observed on cytospins and EM pictures, showed no obvious differences from the t=0 control. Inducible respiratory burst activity, adhesion and chemotaxis were also maintained. Surface expression of CD11b/CD18 was slightly increased, which together with further decreased levels of CD62L can suggest that neutrophils become activated. That explanation was supported also by increased unstimulated adhesion and chemotaxis. The expression of other receptors remained unchanged.

From those results we conclude that that granulocyte concentrates can be effectively stored at 22°C with preservation of their basic functional activity. Nonetheless, further investigation of their bactericidal and fungicidal activity as well as production of inflammatory cytokines (e.g. IL-8) is necessary. Also the interaction of stored neutrophils with endothelial cells needs to be investigated.

Signaling in transendothelial migration

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The groups main research theme, leukocyte-endothelium interactions, has been continued successfully in the year of 2005. Two personal, prestigious grants were funded: a NWO-VENI grant for Paula van Hennik on the role of chemorepellents in stem cell homing, and a post-doc stipendium from the Netherlands Heart Foundation for Jaap van Buul. Jaap, who is currently finishing his post-doc training in the lab of Keith Burridge in the USA and will return to the department in summer 2006 to work on the role of RhoG in endothelial cells. In addition, a PhD project on Rac1 signaling in cell migration and chemorepulsion, funded by the AMC, has started near the end of 2005.

The research in the department deals with two main research lines (i) the control of hematopoietic (stem) cell migration and (ii) regulation of endothelial integrity. Both lines of research are closely related to a broad range of (patho-)physiological conditions, including (chronic) inflammation, hematopoietic stem cell homing and tumor cell metastasis. Common denominators in both research lines are the SDF1-CXCR4 axis, intracellular signaling through the Rho-like GTPases and the dynamics of the actin cytoskeleton.

The control of hematopoietic (stem) cell migration

Leukocyte chemotaxis is initiated by chemoattractants 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 after transplantation. In addition, SDF-1 is involved in migration of inflammatory and tumor cells.
Previous work has shown that CXCR4 can be rapidly internalized by activation or inhibition of specific signaling events. This internalization is controlled by the C-terminus and we have invested significantly over the past year to identify novel proteins that bind this domain. So far, this has been unsuccessful; ongoing experiments now include the role of CXCR4 phosphorylation in these interactions.
A complementary line of research focuses on the role of the chemorepellent Slit

proteins and their Robo receptors in chemotaxis. Slit interferes with SDF-1-mediated hematopoietic cell migration, through an as yet unknown mechanism. By using Robo and Slit isoform specific primer pairs in a Taqman-based RT-PCR, we made an extensive inventory of the mRNA and protein expression of a broad range of Robo receptors and Slit ligands in hematopoietic stem cells and different hematopoietic lineages. Interestingly, the results indicate that the Slit-Robo expression profile is cell lineage-specific. This suggests differentiation-regulated expression as well as different functional requirements for Robo and Slit at different stages of hematopoietic differentiation. Furthermore, the simultaneous expression of Robo receptors and Slit ligands may imply a novel autocrine stimulatory mechanism for this receptor-ligand pair. Finally, we established that Robo and Slit expression is dynamic and regulated by cytokines and chemoattractants in primary hematopoietic cells, both at the cell surface and at the mRNA level.
In the coming year we will perform functional studies to characterize the inhibitory effect of the various Slit ligands on directional migration of hematopoietic cells. In addition, we will determine whether the altered Robo and Slit expression profile in hematopoietic cells after incubation with cytokines or chemoattractants, has functional consequences for the migratory capacity of cells toward a chemotactic cue. A second part of this study is aimed at identifying the signaling events involved in Slit-Robo responses by searching for novel Robo1-interacting proteins. To this end, we will use a proteomics approach consisting of pull-down assays in hematopoietic cells with GST-fusion proteins of (mutants of) the intracellular tail of Robo1 followed by SDS-page and mass-spectrometry.

Within the context of a project, funded by the Netherlands Asthma Foundation, we further extended our work on the role of Epac1 in leukocyte chemotaxis. We found that activation of Epac by cAMP promotes SDF-1 induced chemotaxis and our most recent findings suggest that this pathway is relevant for co-operativity between different agonists, binding to G-protein-coupled receptors.
Novel research in this area has addressed the mechanism by which cAMP promotes endothelial integrity and cell migration. Recent data show that the cAMP-mediated activation of either the PKA pathway or the Epac1-Rap1 pathway leads to improved

endothelial barrier function. These pathways appear to act in a redundant fashion. An important approach in this project has been the use of an Epac1 knockdown (in collaboration with H Bos, Utrecht University) in combination with the analysis of endothelial integrity using the ECIS technology. The molecular mechanisms of Epac-driven cell-cell contact are currently under investigation.

Signaling through Rho like-GTPases is a key aspect of cell migration. We found that the Rac1 GTPase associates, through its C-terminal targeting domain, to the Rac/CDC42 activator beta-PIX. This interaction is mediated by the beta-PIX SH3 domain and a series of prolines in the Rac1 C-terminus. Importantly, binding of beta-PIX to Rac1 mediates Rac1 targeting to the membrane and to focal adhesions in the leading edge of migrating cells. In addition, beta-PIX is required for Rac1 activation. The mechanisms that control PIX-mediated targeting and activation of Rac1 are presently under investigation.
In addition to beta-PIX, the Rac1 C-terminus binds very efficiently to a nucleosome assembly protein, called SET. SET is an inhibitor of the protein phosphatase 2A and is primarily a nuclear protein. Remarkably however, Rac1 activity recruits SET to the plasma membrane. We are currently investigating what the functional relevance of this translocation of SET is in the context of Rac1 mediated signaling and cell motility.

Regulation of endothelial integrity

Endothelial integrity remains one of our key topics of interest. We have analyzed the mechanisms that control the internalization and breakdown of VE-cadherin as a mechanism to modulate endothelial integrity and migration. Current efforts focus on the role of the small GTPase RhoB and the PI-3K-Akt pathway in the regulation of endothelial cell motility.
We previously identified endothelial signaling through the Rac1 GTPase, triggered upon activation of VCAM-1, which results in a transient loss of cell-cell adhesion. In the context of a Netherlands Heart Foundation project, we analyzed proteins that specifically associate with the C-terminal domains of ICAM1 and VCAM1. This is done by pull down assays in combination with mass spectrometry (in collaboration with A Deelder, LUMC). We found a number of proteins that bind specifically to ICAM-1.

These are mainly adapter proteins that may bridge the transmembrane ICAM1 to the intracellular cytoskeleton. Their regulation and recruitment to sites of leukocyte adhesion as well as their functional relevance with respect to cytoskeletal dynamics and leukocyte transendothelial migration are currently under investigation.

Key publications

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Red cell research

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

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The 24-hour post transfusion recovery of transfused red blood cells, that have been stored, is the only *in vivo* parameter which is mentioned in the European and Sanquin guidelines. At least 75% of the transfused RBCs should still be present 24 hours after transfusion. The most common method for measuring this *in vivo*

recovery is by labeling the RBCs with 51chromium. In the Netherlands, however, it is not allowed to use this technique. In the past, we have developed a method for flow cytometric determination of the survival of transfused RBCs using differences in erythrocyte antigenic phenotype between donor and patient. This approach has also the potential to determine the long-term survival of transfused donor RBCs, and to compare the survival of different RBC populations in one patient.
In 2005 we started with an *in vivo* survival study, in collaboration with the Depts of Hematology and Transfusion Services of the Radboud University Medical Centre Nijmegen (RUNMC). The aim of this study was to determine the survival of RBCs from the currently used red cell concentrate (RCC) in the Netherlands and whether there are differences in survival between short and longer stored RBCs. Ten immuno-compromised patients received a transfusion with a short and a longer stored RCC (mean storage time 5 resp. 30 days). The mean 24-hr recovery of short stored RCC is 86.4% and of longer stored RCC 73.5%. These recoveries are significantly different from each other. Statistically, the 24-hr recovery of RCC with a short and a long shelf life comply with the European and Sanquin guidelines. However, only 4 out of 10 of the RCC with a long shelf-life versus 7 out of 10 of the RCC with a short shelf life have a 24-hr post transfusion survival which is 75% or more. After the first 24 hours of transfusion, the RBCs with a short and a long shelf life have an identical survival time. Further analysis will be performed.

RCC products that were therapeutically donated by patients with hereditary hemochromatosis (HH), either by whole blood or erythrocytapheresis collection, were stored under blood bank conditions up to 7 weeks. A comparison was made with stored whole blood- and apheresis-derived RCC from regular donors. Independent of their collection method, RCC of HH patients comply with the European guidelines for stored RCC during a shelf life of 7 weeks. There are no significant differences between RCC from HH patients and RCC from regular donors. However, there are significant differences in some storage-related changes between the RCC that were collected by erythrocytapheresis and the RCC that had been obtained by whole blood collection.

Proteomic analysis has been performed in collaboration with the Dept of Molecular Biology of the Radboud University Medical Centre (RUNMC) on RBCs and their vesicles at different storage times. Extensive analysis of the results is in process.

In collaboration with the research group of Salzer and Prohaska (Vienna), vesicles of stored RBCs (3, 13 and 49 days) were analysed for their quantity, size and protein content. Calcium- and storage-induced vesiculation might share some similar mechanisms (at least concerning the microvesicular fraction). However, the quantity of storage-induced vesicles increase with storage time and are enriched in stomatine. The ability to induce vesiculation by calcium decreases with storage-time. Calcium- and storage-induced nanovesicles differ from each other on immunoblot and need further investigation.
Fourier-transform Infrared Spectroscopy has been performed in collaboration with the department of Biochemistry of the RUNMC on weekly samples of stored RBCs. These measurements can give more information on global structure changes in e.g. proteins during storage
The 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 (GJCGM Bosman, Dept of Biochemistry, RUNMC; Prof WJ de Grip, Dept of Biochemistry, RUNMC; JM Werre, Dept of Transfusion Services, RUNMC; NPM Schaap, Dept of Hematology, RUNMC and FL Willekens, Rijnstate Hospital, Arnhem).

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Hemostasis and thrombosis

Biosynthesis of the factor VIII-von Willebrand factor complex

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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. To further address this issue we studied the effect of total hepatectomy on the circulating levels of factor VIII and its expression in extra-hepatic tissues. Total hepatectomy induced a two-fold increase in circulating factor VIII levels. Also circulating levels of VWF were elevated in anhepatic pigs. Analysis of extra-hepatic tissues did not reveal an increase in factor VIII mRNA levels. Immunohistochemistry of tissue sections revealed pronounced alterations in the cellular distribution of factor VIII in the spleen and kidney. Together our findings confirm the presence of extra-hepatic synthesis of factor VIII. Our data also suggest that the lack of synthesis of factor VIII by the liver can be adequately compensated by an increase in factor VIII production by other tissues, most notably spleen and kidneys.

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. We have previously established that agonists that raise intracellular concentrations of cAMP induce clustering of Weibel-Palade bodies to a perinuclear region. We have now explored this phenomenon in more detail. Clustering of Weibel-Palade bodies is dependent on an intact microtubule network. Transport of vesicles along microtubules is mediated by motor proteins. Plus-end movement towards the periphery of the cell is mediated by kinesins whereas minus-end movement along microtubules towards the perinuclearly located microtubule

organizing centre is mediated by dynein. Interfering with dynein-mediated transport abolished clustering of Weibel-Palade bodies whereas inhibition of kinesin-mediated transport did not affect clustering. Pharmacological studies revealed that protein kinase A directed clustering of Weibel-Palade bodies. Remarkably, incubation with the phosphatase inhibitor okadaic acid induced clustering of Weibel-Palade bodies in the absence of cAMP raising agonists. These results suggest that phosphorylation of an as yet unidentified target protein for protein kinase A mediates dynein-dependent clustering of Weibel-Palade bodies in endothelial cells. We hypothesize that clustering of Weibel-Palade bodies following agonist-induced stimulation provides a feed back mechanism to maintain adequate levels of recruitable hemostatic components and inflammatory mediators from intracellular storage pools.

Key publications

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Structure and function of enzyme-cofactor complexes

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The coagulation cascade comprises several serine proteases that act in combination with a non-enzymatic cofactor. During the past 10 years we are studying the mechanism by which activated factor IX (factor IXa) assembles with its cofactor, factor VIII. Factor IXa shares a typical cofactor binding site, the α-helix 162-171 (chymotrypsin numbering), with other vitamin K-dependent serine proteases from the coagulation cascade. For instance, the corresponding α-helix in factor VIIa contributes to binding of tissue factor, while the same helix in factor Xa interacts with activated factor V. We previously reported that the α-helix in factor IXa (residues 333-339) plays a predominant role in the rate enhancement of factor IXa

by factor VIII. Others have confirmed these findings, and at the same time suggested that this interaction drives factor IXa activity by the isolated factor VIII A2 domain. If the A2 domain alone stimulates factor IXa in the absence of the factor VIII A1/A3-C1-C2 moiety, is seems likely that the A2-domain interactive region in the serine protease domain of factor IXa comprises some catalytic switch that overcomes the low intrinsic activity of this enzyme. The factor IXa helix 333-339 comprises two residues, R333 and R338, that are part of an extended basic exosite that further contains K293, K341, K400 and K403. This exosite binds heparin, and also contributes to the interaction of factor IXa with low-density lipoprotein receptor-related protein (LRP). Surface Plasmon Resonance studies using recombinant factor IXa variants demonstrated that residues K293, K400 and K403 of the anionic exosite contribute to the assembly with the factor VIII A2 domain, while the anionic helix residues R333 and R338 predominantly determine the extent of catalytic rate enhancement. This questions the concept that this helix is the primary factor VIII-binding site, but explains why functional studies have previously identified this helix as being a factor VIII-sensitive structure element. We further addressed the mechanism by which the isolated A2 domain should stimulate factor IXa activity. We were able to confirm published findings employing highly purified A2 domain from plasma-derived factor VIII. Unexpectedly however, this activity was fully inhibited by monoclonal antibodies against the factor VIII light chain (domains A3-C1-C2). Apparently, rate enhancement by factor VIII A2 domain also involves a light chain component. Indeed, purified A2 domain contained traces (less than 1%) of light chain. We therefore speculate that at least part of the cofactor activity of the A2 domain reported in the literature originates from the reassociation of free A2 domain with residual A1/A3-C1-C2 dimer during the functional analysis. To what extent the A2 domain alone really has some factor IXa stimulatory effect therefore remains an open question. Our studies underscore that enzyme-cofactor assembly involves multiple domains on both factor IXa and factor VIII.

Key publications

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Circulating antibodies to blood coagulation factors

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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. We have previously used phage display to isolate human monoclonal antibodies directed towards factor VIII. Human antibodies were expressed as single chain variable domains (scFv). Using this approach a panel of scFv directed towards major inhibitor epitopes present in the A2, A3 and C2 domain have been isolated. In collaboration with Dr Ortel, scFv directed against the light chain of factor VIII have been used to identify amino acid regions that contribute to binding of factor VIII to phospholipids membranes. Crystallization-studies of the C2 domain of factor VIII have suggested that residues M2199, F2200, L2251, L2252, V2223, W2313 and V2314 at the tips of beta-hairpins and loops contribute to phospholipid membrane binding. In accordance with these findings scFv directed towards the C2 domain competed with binding of factor VIII light chain to phospholipid vesicles. Mutagenesis studies revealed that amino acid residue F2200 is required for high affinity binding of scFv, a finding consistent with involvement of this residue in binding of factor VIII to phospholipids membranes. Surprisingly, phospholipid vesicles also interfered with binding of the scFv binding to the A3 domain of factor VIII. These data suggest that similar to factor V the A3 domain of factor VIII may also contribute to binding to phospholipids. These results also suggest that inhibitory antibodies directed towards the A3 domain of factor VIII may compete for binding of factor VIII to phospholipids membranes.

Thrombotic thrombocytopenic purpura (TTP) is a micro-angiopathy that is related to an acquired or congenital deficiency of the von Willebrand factor (VWF) cleaving

protease ADAMTS-13. Functional absence of ADAMTS-13 results in the presence of ultra large VWF (UL-VWF) multimers in the circulation. These UL-VWF polymers mediate formation of platelet-rich thrombi in the microcirculation that cause thrombocytopenia and hemolytic anemia. In plasma of the majority of patients with acquired TTP circulating antibodies directed against ADAMTS-13 are present. We have developed an assay for the characterization of these antibodies using recombinant ADAMTS-13. Analysis of a panel of patients with acquired TTP revealed that variable levels of anti-ADAMTS-13 antibodies are present in plasma of different patients. For further characterization we prepared a series of recombinant fragments corresponding to different domains of ADAMTS-13. In all samples analyzed we observed antibodies directed towards the spacer domain of ADAMS-13. In one out of 7 patients we detected antibodies directed towards the TSP2-8 domains of ADAMTS-13. Our findings show that the 130 amino acid spacer domain comprises a major binding site for anti-ADAMTS-13 antibodies. This observation suggests that the spacer domain contributes to the VWF cleaving activity of ADAMTS-13. Based on our observations we speculate that the spacer domain of ADAMTS-13 is involved in binding to VWF. Antibodies binding to this site may prevent the binding of VWF and thereby interfere with the processing of UL-VWF multimers that originate from storage pools present in vascular endothelial cells.

Key publications

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Cellular receptors involved in clearance of factor VIII

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The identification of the mechanism involved in the clearance of coagulation factors remains a continuous challenge. Dysfunction of these mechanisms may cause elevated coagulation factor levels, and as such be a risk factor for developing venous thrombosis. In 1999 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. During the past few years we have explored the assembly of factor VIII with several other LDL-receptor family members, including very-low density lipoprotein receptor (VLDLR), low density lipoprotein receptor (LDLR) and megalin (LRP2). *In vitro*, these receptors are similar to LRP in that they all exhibit substantial binding to factor VIII.

Recently, we have been able to assess the *in vivo* relevance of these interactions using mouse models. Crucial in this approach was the availability of a mouse model of conditional hepatic LRP deficiency. This 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) resulted in increased factor VIII levels as well. Because this also occurred in mice that were lacking hepatic LRP, we concluded that other RAP-sensitive receptors than LRP contribute to the regulation of factor VIII levels in the circulation. This issue has been addressed using mice with multiple receptor deficiencies. By this approach we demonstrated that VLDLR, which is present on the endothelial cells of the vascular wall, does not contribute to factor VIII clearance to any appreciable extent. Studies using mice with combined LDLR and hepatic LRP deficiency, however, revealed that LDLR does contribute to factor VIII clearance, in particular in the absence of hepatic LRP. This was a surprising finding, because apart form LDL, no other ligands were known to be regulated by

LDLR *in vivo*. The clearance of LDL involves a dual receptor mechanism, in which LRP and the much smaller LDLR cooperate in the removal of LDL particles from plasma. We now conclude that the same holds for factor VIII.

The role of the LDL receptor family on factor VIII clearance is reflected by a 5-fold increase of factor VIII plasma levels in LDLR/LRP deficient mice. Similarly, factor VIII infusion studies demonstrated that factor VIII half-life was 5-fold prolonged in LDLR/LRP deficient mice. Prolongation of factor VIII half-life could be beneficial for factor VIII replacement therapy of patients with hemophilia A. Therefore, the molecular mechanism that drives the assembly of factor VIII with endocytic receptors continues to be a target of our studies. LRP binding generally involves electrostatic interactions of positively-charged patches that are exposed at the ligand surface. The factor VIII molecule comprises several of such areas, in particular within its light chain. In view of the notion that von Willebrand factor (VWF) protects factor VIII from premature clearance, it seems likely that part of the putative LRP-interactive sites in factor VIII are buried in the factor VIII–VWF complex. On the other hand, however, it seems conceivable that specific LRP-interactive sites are exposed that drive the clearance of factor VIII from its complex with VWF.

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.

Bovenschen N, Mertens K, Hu L, Havekes LM, van Vlijmen BJ. LDL receptor cooperates with LDL receptor-related protein in regulating plasma levels of coagulation factor VIII in vivo. Blood 2005; 106:906-12.

Inflammation and sepsis

Immunoglobulins

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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).

Dimers in intravenous immunoglobulin

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 substantially 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. Interactions with Fc fragments were investigated in other types of immunoassays. The results confirmed that immunoglobulins of the IgG4 isotype may interact with other immunoglobulins in a way reminiscent of Rheumatoid Factor. In some assays this interaction is restricted to IgG4-IgG4 interactions, in other assay formats IgG4 was found to interact with solid-phase-coupled IgG1 as well as IgG4.

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. This suggests that the process, which involves breaking disulphide bonds as well as strong hydrophobic interactions, is catalyzed *in vivo*. In close collaboration with Genmab, two IgG1/IgG4 sets of chimeric mouse/human monoclonal antibodies to two soluble, non-crossreactive monoclonal antigens have been prepared. Mixtures of these antibodies are being tested for exchange of half-molecules, both *in vivo* (in a mouse model) and *in vitro*.

Role and specificity of IgM in ischemia-reperfusion

The role and specificity of IgM in ischemia-reperfusion is being investigated in close collaboration with the Dept of Experimental Surgery at the AMC. The experiments are performed in an intestinal mouse model.

Key publications

Aalberse RC, Schuurman J. IgG4 breaking the rules. Immunology 2002; 105:9-19.

Diemel et al. Characterization of immunoglobulin G fragments in liquid intravenous immunoglobulin products. Transfusion 2005; 45(10):1601-9.

Inflammation

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The Inflammation Research group focuses on the classical and lectin pathway of complement activation and on development and clinical evaluation of complement inhibitors. It was found that covalent fixation of activated C4 and C3 to C1q occurred during classical pathway activation and not during activation of other complement pathways. 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 and plasma levels appeared to correlate with disease activity. A quantitative assay to measure C4 fixation by the MBL route was established as well as new methods to measure protein levels and biological activities of C4A and C4B.

Key publications

Wouters D, Wiessenberg HD, Hart M, Bruins P, Voskuyl AE, Daha MR, Hack CE. Complexes between C1q and C3 or C4: novel and specific markers for classical complement pathway activation. J Immunol Meth 2005; 298:35-45.

Wouters D, Voskuyl AE, Molenaar ET, Dijkmans, BAC, Hack CE. Evaluation of classical complement pathway activation in rheumatoid arthritis. Measurement of C1q-C4 complexes as novel activation products. Arthritis Rheum 2006; 54:1143-50.

Immune regulation

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In this research line we focus on the regulation of immunological cascades that involve proteases by protease inhibitors. A major inhibitor of classical complement pathway is C1-inhibitor (C1-Inh), a serine protease inhibitor (serpin). The work on the structure and function of the C1-Inh was continued. Based on previous results of a study of a genetic deficiency of C1-Inh a novel recombinant mutant of C1-Inh 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. Next, the mutants were purified in large quantities for *in vivo* studies. Currently, we are studying the clearance rate and pharmacokinetics of recombinant C1-Inh infused in rabbits in comparison to plasma purified C1-Inh. In addition, we are investigating the effect of the mutations in the recombinant C1-Inh on the pharmacodynamics of the protein in circulation. Finally, we have started to study whether and by which mechanisms C1-Inh has any cell-protective effects in a model simulates the mechanical damage that venous cells experience when used in cardiac bypass constructions. This part of the project is performed in collaboration with prof H Niessen from the Dept of Pathology at the VU Medical Center.

Next to the extracellular serpin C1-Inh we continued research on Fahsin, the novel protease inhibitor of human neutrophil elastase, cathepsin G and proteinase 3. We had previously generated recombinant Fahsin in yeast and demonstrated that it was functionally active as an reversible inhibitor of human neutrophil elastase and insensitive to chemical and biological oxidation. In the past year, we optimised the Fahsin production and purification method and adjusted it to suit large scale preparation under cGMP conditions. The batches that were generated were subjected to *in vitro* toxicity studies and were demonstrated to be non-toxic. In

addition, we demonstated that Fahsin is able *in vitro* to inhibit elastase activity in sulcus material obtained from patients that suffer from periodontitis. These data now allow that Fahsin can be used to test *in vivo* efficacy in a clinical setting in periodontitis patients.

Finally, we are investigating the function of intracellular serpins by studying the role of granzymes and granzyme-inhibiting serpins in the innate and adaptive immune system. The activity of granzyme B is regulated by the human intracellular serpin SERPINB9. Last year, we demonstrated that mast cells express both granzyme B, perforin and SERPINB9 and that GrB and perforin produced by the mast cell line HMC-1 are active in hemolytic assays. These findings pointed to a novel cytolytic mechanism for human mast cells in host defense and/or tumor rejection. This year, we demonstrated that the different types of mast cells release GrB when triggered with their specific physiological stimulus. Next to GrB, cytotoxic T cells and natural killer cells produce Granzyme A (GrA). GrA seems to induce target cell apoptosis via a different cellular pathway than GrB. We started to generate recombinant GrA in the yeast *Pichia pastoris*. Recombinant GrA could be expressed and purified and was demonstrated to be actively able to cleave a specific substrate. Currently, we are investigating the mechanisms by which GrA induces cell death. Finally, we are exploring if we can identify intracellular GrA substrates as well as GrA inhibitors.

Key publications

De Bruin EC, Roem D, Bulder I, Dieker M, Voerman G, and Hack CE. Production, purification and characterisation of recombinant Fahsin, a novel antistasin-type proteinase inhibitor. FEMS Yeast Res 2005; 5:1069-77.

Bladergroen BA, Strik MC, Wolbink AM, Wouters D, Broekhuizen R, Kummer JA and Hack CE. The granzyme B inhibitor proteinase inhibitor 9 (PI9) is expressed by human mast cells. Eur J Immunol 2005; 35:1175-83.

Immunology

Antigen presentation

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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 MHC class II molecules in normal and malignant B cells in cooperation with the Depts of Hematology and Pathology of VU 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 that regulate the composition of the antigenic peptide repertoire. Last year, we demonstrated that aberrations in the expression of these proteins in malignant B cells correlate with disease status in B cell chronic lymphocytic leukemia (B-CLL). In addition, we have identified aberrancies in the T cell compartment of these patients. This year, we demonstrated that in B-CLL the T cell compartment is shifted from a naive status towards and effector phenotype. This points an antigen driven process. In addition, we demonstrated that the observed T cell deviations correlate to the previously observed aberrancies in the antigen presentation pathway of the malignant B cells. Next, we have generated 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. We demonstrated that particulate antigens and bacteria that are recognized by the B cell receptor (BCR) are rapidly internalized and induce antigen presentation. Thus, B cells behave as professional phagocytes when triggered via their BCR. We are currently investigating the mechanisms of phagocytosis and the consequences for bacterial survival and the generation of a specific humoral immune response.

We have continued our 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. Last year, we set up the methods to generate both immature

and mature DCs from monocytes and the assays to monitor DC effector function. In collaboration with the Blood Bank North West Region we now developed a leukocytapheresis method using the MCS+ (Haemonetics®) to obtain enough monocytes for DC preparation. Moreover, we are evaluating the use of the ElutraTM for closed system purification of monocytes from the above mentioned leukocytapheresis product and to developing culture conditions of DCs in bags in order to be able to generate DCs in a closed system. We evaluated the effect of the presence of granulocytes in the monocyte preparation on the efficacy of generation of DCs, as granulocytes and monocytes are collected in the same fraction from the ElutraTM. We found that presence of 20-30% granulocytes in the monocyte preparation had no major influence on the generation of DCs. As the DCs that are currently applied in the clinics are suboptimal for the induction of effective anti-tumor response, we developed a new maturation-cocktail via which effective Th1 inducing DCs can be generated (essential for effective anti-tumor responses). The newly matured DCs are still capable to migrate, another important requirement for an effective DC product. A patent application has been filed to protect this finding for future application in cellular immunotherapy programmes of Sanquin.

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 we are currently generating a new type of MHC class II tetramers.

Key publications

Zwart W, Griekspoor A, Kuijl C, Marsman M, van Rheenen J, Janssen H, Calafat J, van Ham M, Janssen L, van Lith M, Jalink K and Neefjes J. Spatial Separation of HLA-DM/HLA-DR Interactions within MIIC and Phagosome-Induced Immune Escape. Immunity 2005; 22:221.

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Immunomodulation of blood transfusions in transplantation tolerance

Principal investigator

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Aim of the study is by unraveling the transfusion effect to define requirements of blood transfusions intended for induction of allogenic tolerance. In particular donor selection and *in vitro* treatment/modification of donor APC's prior to transfusion is central in this study.

In a retrospective study in a group van kidney-pancreas transplant patients (1996-2002), administration of pre-transplantation one-DR shared transfusions had resulted in significant less rejection episodes compared to non-transfused patients. This effect was however abolished in case patients that had received preemptive treatment with ATG. 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. Currently from 30 prospective kidney-pancreas patients blood is withdrawn prior, and 2 and 10 weeks after transfusion. PBMCs are cryopreserved and will be evaluated for enumaration of Tregs, primary T cell responses, allo-antibodies and donor chimerism. Because the hypothesis is that Tregs maintaining tolerance are induced by indirect T cell stimulation, a model is sought to measure indirect antigen stimulation *in vitro* using (overlapping) peptides and allogeneic cell lysates. However It turns out that indirect T cell stimulation test as published in the literature are of questionable quality and lack appropriate control for direct stimulation and irrelevant peptides.
This research project is a collaboration with FHJ Claas and DL Roelen, Leiden University Medical Center.

Key publication

Waanders MM, Roelen DL, Brand A, Claas FHJ. The putative mechanism of the immunomodulating effect of HLA-DR shared allogeneic blood transfusions on the alloimmune response. Transfus Med Rev 2005; 19:281-7.

Allergy

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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 Allergy Research Group has relocated to the Academic Medical Center, where research will be continued in the Dept of Experimental Immunology. In its last half year at Sanquin, the group continued to focus on the humoral immune response against inhalant and food allergens. Most research activities have been carried out in the frame of EU-funded projects on the one hand and contract research for allergen manufacturers on the other hand. On June 1st a large EU project called EuroPrevall started which aims at performing large cross-sectional and longitudinal studies (birth cohorts aiming at 12.000 newborns in 8 European countries) to establish the prevalence, risk factors and socio-economic impact of food allergy in Europe. At the same time improved in vitro diagnostic tests for food allergy are being developed using recombinant allergens and protein micro-arrays. Development of novel strategies for immunotherapy has mainly focused on the introduction of recombinant allergens and novel adjuvants driving Tregs.

In 2005 the Allergy Research group moved to the Academic Medical Centre of the University of Amsterdam.

Key publications

Van Ree R, Dorpema JW, Vieths S. Allergy Vaccines: A Need for Standardisation in Mass Units of Major Allergen. Pharmeuropa Bio 2005; 1:27-30.

Akkerdaas JH, Schocker F, Vieths S, Versteeg S, Zuidmeer L, Hefle SL, Aalberse RC, Richter K, Ferreira F, van Ree R. Cloning of oleosin, a putative new hazelnut allergen, using a hazelnut cDNA library. Mol Nutr Food Res 2006; 50(1):18-23.

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Blood transmitted infections

Virological aspects of AIDS pathogenesis

Sensitivity of primary R5 HTV-1 to inhibition by RANTES correlates with sensitivity to small-molecule R5 inhibitors

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In approximately 50% of HIV-1 subtype B-infected individuals, progression to AIDS is preceded by the emergence of CXCR4-using (X4) variants, whereas the rest progress to AIDS in the presence of CCR5-using (R5) variants only. In a previous study, we showed that during disease progression in the presence of R5 variants only, HIV-1 variants emerge with a decreased sensitivity to inhibition by RANTES, a natural ligand of CCR5 that inhibits cellular entry of R5 variants. This observation was of potential clinical relevance as HIV-1 small-molecule R5 entry inhibitors are a new class of drugs that, in analogy to RANTES, target the binding and subsequent entry of HIV into the target cell. Here we show that R5 HIV-1 sensitivity to RANTES correlates with sensitivity to the R5 small-molecule inhibitor AD101. HIV-1 small-molecule entry inhibitors are a new class of drugs that target the binding and subsequent entry of HIV into the target cell. Furthermore, we found that R5 variants obtained from individuals who later developed X4 variants were less sensitive to AD101 inhibition compared with R5 variants obtained from individuals who never developed X4 variants. These results may have implications for the evaluation of R5 inhibitors in future clinical trials.

Key publication

Koning FA, Koevoets C, van der Vorst TJ, Schuitemaker H. Sensitivity of primary R5 HTV-1 to inhibition by RANTES correlates with sensitivity to small-molecule R5 inhibitors. Antivir Ther. 2005; 10(2):231-7.

Low-level CD4$^+$ T cell activation is associated with low susceptibility to HIV-1 infection.

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Some individuals have remained HIV seronegative despite high risk sexual behaviour. In five out of six high risk seronegative homosexual men and in five out of five individuals 7.8 to 1.6 years prior to seroconversion, we detected HIV-1 proviral DNA at very low levels in sequential peripheral blood mononuclear cell samples. These

data indicate a high prevalence of low-level HIV-1 DNA in exposed seronegative individuals. This proviral DNA could either reflect transmission of replication incompetent virus or dead-end infection of initially replication competent virus. In a subsequent study we analysed host factors associated with protection from productive infection in the same group of high risk seronegative individuals. So far, different features have been associated with low susceptibility to HIV type 1 (HIV-1) infection in exposed seronegative individuals. These include genetic make-up such as homozygosity for the CCR5-D32 allele and the presence of HIV-specific CTLs. We studied immune activation and immune responsiveness in relation to HIV-1 susceptibility in 42 high-risk seronegative (HRSN) participants of the Amsterdam Cohort Studies and 54 men from the same cohort who were seronegative at the moment of analysis but later became HIV seropositive. HRSN had higher naive (CD45RO CD27) CD4 and CD8 T cell numbers and lower percentages of activated (HLADR CD38, CD70) CD4 and proliferating (Ki67) CD4 and CD8 T cells, irrespective of previous episodes of sexually transmittable infections. Furthermore, whole blood cultures from HRSN showed lower lymphoproliferative responses than healthy laboratory controls. These data suggest that low levels of immune activation and low T cell responsiveness may contribute to low HIV susceptibility.

Key publications

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GB virus C coinfection and HIV-1 disease progression: The Amsterdam Cohort Study

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The effect that GB virus C (GBV-C) coinfection has on human immunodeficiency virus type 1 (HIV-1) disease progression is controversial and therefore was studied in 326 homosexual men from the prospective Amsterdam Cohort Studies who had an accurately estimated date of HIV-1 seroconversion and were followed up for a

median period of 8 years. METHODS: A first plasma sample, obtained shortly after HIV-1 seroconversion, and a last plasma sample, obtained before 1996, were tested for GBV-C RNA and envelope protein-2 antibodies. The effect that GBV-C has on HIV-1 disease progression was studied by use of time-dependent Cox proportional-hazards models with adjustment for baseline variables and time-updated HIV-1 RNA and $CD4^+$ cell count. RESULTS: Men who lost GBV-C RNA between collection of the first sample and collection of the last sample had a nearly 3-fold-higher risk of HIV-1 disease progression than did men who had never had GBV-C RNA. This effect became much smaller after adjustment for time-updated CD4+ cell count. CONCLUSION: Rather than a positive effect of GBV-C RNA presence, a negative effect of GBV-C RNA loss on HIV-1 disease progression was found, which disappeared after adjustment for time-updated $CD4^+$ cell count. We therefore hypothesize that GBV-C RNA persistence depends on the presence of a sufficient number of $CD4^+$ cells- -and that the $CD4^+$ cell decrease associated with HIV-1 disease progression is a cause, not a consequence, of GBV-C RNA loss.

Key publication

Van der Bij AK, Kloosterboer N, Prins M, Boeser-Nunnink B, Geskus RB, Lange JM, Coutinho RA, Schuitemaker H. GB virus C coinfection and HIV-1 disease progression: The Amsterdam Cohort Study. J Infect Dis 2005; 191(5):678-85.

Natural controlled HIV infection: preserved HIV-specific immunity despite undetectable replication competent virus

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Long-term non-progressive HIV infection, characterized by low but detectable viral load and stable CD4 counts in the absence of antiviral therapy, is observed in about 5% of HIV-infected patients. Here we identified four therapy naive individuals who are strongly seropositive for HIV-1 but who lack evidence of detectable HIV p24 antigen, plasma RNA, and proviral DNA in routine diagnostic testing. With an ultrasensitive PCR, we established that frequencies of pol proviral DNA sequences were as low as 0.2-0.5 copies/10(6) PBMC. HIV could not be isolated using up to 30x10(6) patient PBMC. One individual was heterozygous for CCR5 Delta32, but CCR5 expression on $CD4^+$ T cells was normal to high in all four individuals. *In vitro*

R5 and X4 HIV-1 susceptibility of CD8-depleted PBMC of all study subjects was significantly lower than the susceptibility of CD8-depleted PBMC of healthy blood donors. All individuals expressed protective HLA-B*58s alleles and showed evidence of HIV-specific cellular immunity either by staining with HLA-B*57 tetramers folded with an HIV RT or gag peptide or after stimulation with HIV-1 p24 gag, RT, or nef peptides in ELIspot analysis. HIV-specific $CD4^+$ T helper cells were demonstrated by proliferation of $CD4^+$ T cells and intracellular staining for IL-2 and IFNgamma after stimulation with an HIV-gag peptide pool. Sera of all individuals showed antibody-mediated neutralization of both R5 and X4 HIV-1 variants. These data implicate that very low-level antigen exposure is sufficient for sustained HIV-specific immunity and suggest the possibility of a multi-factorial control of HIV infection.

Key publication

Kloosterboer N, Groeneveld PH, Jansen CA, van der Vorst TJ, Koning F, Winkel CN, Duits AJ, Miedema F, van Baarle D, van Rij RP, Brinkman K, Schuitemaker H. Natural controlled HIV infection: preserved HIV-specific immunity despite undetectable replication competent virus. Virology 2005; 339(1):70-80.

Determinants of in vitro HIV-1 susceptibility

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The natural course of HIV-1 infection is widely variable with extremes of disease progression within 2 years or continuous asymptomatic infection for more than 15 years. Moreover, certain people are relatively resistant to HIV-1 infection despite high levels of sexual risk behaviour. *In vitro*, the ability of HIV-1 to replicate on $CD4^+$ T cells or macrophages varies considerably from donor to donor and cells from some individuals resistant to HIV-1 infection or disease progression *in vivo* have also shown reduced HIV-1 susceptibility *in vitro*. In the majority of cases, the underlying mechanism responsible for the variable outcome of exposure to HIV-1 is not known.

The overall goal of our research is to identify the host factors responsible for this restriction. To address this goal we are quantifying *in vitro* HIV-1 susceptibility of cells from a large panel of blood donors. In order to carefully quantify *in vitro* HIV-1 susceptibility, we first developed an assay that reproducibly scores HIV-1

replication in purified $CD4^+$ T cells and monocyte-derived macrophages. To this end, we optimized existing procedures and developed new protocols. These involved: purification of $CD4^+$ T cells and $CD14^+$ monocytes from small volume blood donations using magnetic beads, stock generation of a model virus clone by transfection and characterization of this stock by infecting both primary cells and cell lines, and determination of optimal infection conditions for $CD4^+$ T cells and monocyte-derived macrophages. Once optimized, the susceptibility assay can now be used to test the robustness of the assay by performing the assay on cells from repeat donations from the same donor. Thereafter, we will use the assay to type *in vitro* HIV-1 susceptibility for 400 healthy blood donors. Highly susceptible and highly resistant donors will then be used in a high-throughput genotyping assay to identify single nucleotide polymorphisms associated with *in vitro* HIV-1 susceptibility.

Trim5α mediated inhibition of HIV-1 replication

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As a natural defense against retroviruses, human and non-human primate cells express inhibitory factors that interfere with virus replication at an early step after virus entry. One of these inhibitory factors has recently been identified to be the cytoplasmic body component Trim5α. Although the mechanism by which Trim5α interferes with HIV-1 infection is still unknown, the viral determinant involved in this restriction is located in the cyclophilin A (CyPA) binding region of the viral capsid protein. Interestingly, we previously observed that mutations in the cyclophilin A (CypA) binding region of the capsid protein makes HIV-1 resistant to Trim5α.
In our present study, we analyzed whether amino acid changes in the CyPA binding region of the capsid protein that are associated with resistance against Trim5α, occur during HIV-1 infection and whether Trim5α resistant variants play a role in disease progression. We observed that Trim5α escape mutants emerge predominantly in X4 progressor (17%) relatively late in infection. Emergence of Trim5α was associated with a prolonged asymptomatic stage and late development of X4 variants.
This suggests that Trim5α might play a role in control of viral burden during the asymptomatic stage. However, an increase in disease progression (based on CD4 counts below 200/ul) was observed late in infection suggesting an accelerated disease progression after the emergence of Trim5α resistant variants.

Quality, safety and efficiency

Pathogen detection and inactivation

Pathogen inactivation in platelet concentrates by UV-C light

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In collaboration with CAF/DCF (Brussels), we study the possibility to use UV-C illumination (as delivered by a lab scale apparatus developed by CAF) for pathogen inactivation in platelet concentrates. 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. The best balance between these opposite effects were obtained by having 10% residual plasma, which can be achieved in blood bank practice by adjustment of the centrifugation procedure of the starting material, i.e. whole blood derived buffy coats.

As most important limitation of the UV-C illumination, it was noted that HIV was the most insensitive virus among the different viruses tested. We therefore investigated whether higher doses of UV-C could be used with concomitant filtering of other wavelenghts, hypothesizing that UV-C light itself (255 nm) did not result in platelet damage. Although 3 log kill of HIV is achievable under these conditions, platelet damage was not reduced by eliminating UV-B and higher wavelengths and became unacceptable high. Filtering, therefore, does not offer a solution for the relative insensitivity of the UV-C procedure.

It can be envisaged that UV-C might still be an option for pathogen inactivation in platelet concentrates, considering the fact that blood donations are tested for possible HIV contamination. We therefore tested the effect of UV-C treatment on its effectiveness to kill different strains of bacteria (in 10% plasma). We selected a Coagulase Negative Staphylococcus strain (frequently found during screening of platelet concentrates), a Coagulase positive Staphylococcus strain (*Staphylococcus aureus*), a spore former (*Bacillus cereus*) and a Gram negative strain (*Escherichia coli*). The latter three, although relatively rarely found during screening, are most often associated with severe transfusion transmitted bacterial infections. *Staphylococcus aureus, Staphylococcus epidermidis* and *Escherichia coli* showed a 4 log reduction at a relatively low dose (125 J/m^2), increasing to at least 5.5 log reduction at a dose of 500 J/m^2. *Bacillus cereus* was relatively insensitive to UV-C, with 2 log reduction at

125 J/m^2, increasing to 3 log at 500 J/m^2. This is probably caused by the presence of spores in the bacteria culture, which will survive UV-C. We will investigate this in more detail with cultures depleted or enriched for spores (in collaboration with Th Montag-Lessing, Paul Ehrlich Institute, Germany). We concluded that UV-C seems to be valuable for inactivation of bacteria at doses with very limited damage to the platelets (250–500 J/m^2).

Key publications

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.

Li J, Lockerbie O, de Korte D, Rice J, McLean R, Goodrich RP. Evaluation of platelet mitochondria integrity after treatment with Mirasol pathogen reduction technology. Transfusion 2005; 45:920-926.

Platelet pathogen reduction

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The Blood Bank South West Region participated in a European study on the effect of S-59 platelet pathogen reduction 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 were promising. Other international studies, however, gave conflicting results. For this reason a national study to investigate the performance of S-59 platelets in clinical practice in collaboration with the Dutch Hemato-oncological Research Federation (HOVON) will start in a short term.

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 detection system for use in validation studies

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The introduction of donor screening, testing of donations for viral markers and the use of viral inactivation and elimination steps, have greatly improved the safety records of plasma derived products. Of concern are newly emerging infectious diseases for which such systems are not yet fully implemented, such as prion diseases, for which the best known human disease is Creutzfeldt-Jakob Disease (CJD) and its variant form (vCJD). The goal of our research is to develop an *in vitro* model system to evaluate the potential of various manufacturing steps used in the production of plasma derived products to remove prions.

Several cell lines have been used by others for *in vitro* culture of prions including neuronal cell lines such as N2a, GT1-1, ATT20, and SMB and various fibroblast cell lines such as 3T3 and L929. Most experiments in this study were performed using SMB and SMB-P/S cells, i.e. chronically infected scrapie mouse brain cells and pentosan-cured scrapie mouse brain cells, respectively. We previously developed an Eliblot as an alternative prion detection method for the more conventional Western blot, as it would allow higher sample throughput. Cell lysates were prepared and tested with Eliblot and Western blot in parallel. The critical step was the proteinase K digestion step that is required to discriminate between prion positive and negative cells and the amount of proteinase K per total amount of protein should be carefully controlled. The total amount of protein was determined with the BCA assay (Pierce) or indirectly by counting the number of cells. While both Eliblot and Western Blot protocols are still undergoing improvements, a conservative estimate is that the Eliblot was at least as sensitive as the Western blot. The minimal amount of protein resulting in a positive result corresponded to 10^5 proteinase K digested SMB cells. After successfully setting up the Eliblot for read-out of prion infected cells, we can now initiate *in vitro* prion infection experiments and evaluate the prion replication kinetics and optimal assay conditions for validation studies.

Prion removal by filtration of erythrocytes

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Although the risk for contamination of red cell concentrates (RCC) with prions is unknown, several companies are developing filters for prion reduction of blood components. The prion filter for RCC recently developed by Pall (LAPRF), was combined with whole blood leukodepletion in a Fresenius whole blood system and tested for the *in vitro* quality of RCC during storage for up to 42 days.
Whole blood was collected in standard Fresenius in line systems with integrated whole blood filter. The whole blood was either leukoreduced after overnight storage at 22°C (group I; n=8) or within 4 h (group II; n=8) and subsequently separated into plasma and RCC in SAGM. For group I, the RCC units were immediately filtered over the prion filter (Composafe Pr) and for group II the RCC were filtered over the prion filter after overnight storage at 4°C. After filtration over the prion filter, all RCC were stored at 4°C and sampled at various time points during storage.
The CompoSafe Pr filter had no effect on the total amount of protein in the supernatant of the RCC, but removed factor IX from the supernatant (before 0.14 IE/ml, after < 0.01 IE/ml) which has been indicated as a pseudomarker for prion removal. The RCC in both groups contained 54 ± 4 g Hb (mean±SD), with a combined loss due to the whole blood and RCC filtrations, including losses in tubes and bags by transfer, of about 20 g Hb. The CompoSafe Pr filtration causes an additional loss compared to whole blood filtration of about 7 g Hb. The filtration over the CompoSafe Pr filter induced a slight increase of hemolysis (about 0.03% increase), but this did not result in a more rapid increase during subsequent storage: after 42 days 0.26% hemolysis for group I and 0.16% for group II was detected. For group I at day 42, 1.3% of the red cells were positive for AnnexinV (representing PS exposure), whereas for group II this was 0.9% (comparable to standard leukodepleted RCC in SAGM). The amount of ATP was predicted to be above 2.7µmol/g Hb at day 35, with about 15 mM glucose remaining at day 42.

We concluded that despite the double filtration step in the total procedure (one to leukoreduce the whole blood and one to remove prions from RCC), the remaining RCC met the European requirements for Hb content. The amount of Hb in the final RCC is similar to that in leukodepleted RCC prepared from whole blood after buffy

coat depletion. The *in vitro* quality after 42 days was similar to those of standard leukodepleted RCC, prepared from whole blood after buffy coat depletion.

16S rDNA PCR

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Based on real-time PCR technology a broad-range 16S rDNA PCR assay was developed and optimized in 2003 in collaboration with the VUmc Dept of Microbiology (Head Prof van den Broucke-Grauls).
This project was terminated in November 2004, the PhD student finalized the publications in 2005 and will defend her thesis in 2006. A new grant application has been accepted by Sanquin in 2005 to further explore applications for this interesting assay.

Key publications

Mohammadi T, Reesink HW, Vandenbroucke-Grauls CMJE, Savelkoul PHM. Removal of contaminating DNA from commercial nucleic acid extraction kit reagents. J Microbiol Med 2005; 61(2):285-8.

Mohammadi T, Pietersz RNI, Vandenbroucke-Grauls CMJE, Savelkoul PHM, Reesink HW. Detection of bacteria in platelet concentrates: comparison of broad-range real-time 16S rDNA polymerase chain reaction and automated culturing. Transfusion 2005; 45:731-6.

Mohammadi T, Reesink HW, Pietersz RNI, Vandenbroucke-Grauls CMJE, Savelkoul PHM. Amplified-fragment length polymorphism analysis of Propionibacterium isolates implicated in contamination of blood products. Brit J Haem 2005; 131:403-9.

Mohammadi T, Pietersz RNI, Scholtalbers LAH, Vandenbroucke-Grauls CMJE, Savelkoul PHM, Reesink HW. Optimal sampling time after preparation of platelet concentrates for detection of bacterial contamination by quantitative real-time polymerase chain reaction. Vox Sang 2005; 89:208-14.

Impedance measurement

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The project of impedance measurement for detection of bacteria in platelet concentrates granted by the European Commission in the Fifth Framework of demonstration projects does not have shown much progress. Sanquin Blood Bank North West Region is the 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 the Blood Bank. In 2005 the newly developed sensors and the reading equipment were first tried out in Israel. In November the equipment was installed in Amsterdam and pilot experiments were performed. Unfortunately the results of unspiked and spiked PC were not easy to read or interpreted. Discussions how to improve the calculations and interpretation of the results are ongoing.

Role of virus preparation in validation studies

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The introduction of donor screening, testing of donations for viral markers, and the use of viral inactivation and elimination steps, have greatly improved the safety records of plasma derived products. The practice of validating processes for their capacity to inactivate a range of non-enveloped and enveloped viruses provides additional confidence. Regulatory guidelines state that the viruses for validation should closely resemble viruses which may contaminate the products and should represent as wide a range of physico-chemical properties as possible. To that end, certain relevant viruses and -in their absence- model viruses have been proposed for validation studies. In addition, the specific laboratory virus strains and preparations used as spiking material will influence the outcome of validation studies. Biological properties, such as viral membrane constituents, degree of aggregation, and purity, are likely to affect the response to various treatments. The methods used to generate virus preparations are likely to affect these biological properties and thus the outcome of validation studies.

We previously observed that the herpes model virus pseudorabies virus (PSR) was partly inactivated already in the untreated plasma product as compared to the original virus inoculum. Literature review suggested that anti-α-galactocyl antibodies

present in human plasma may recognize the virus preparation grown on the porcine cell line PD5, which expresses α-galactocyl. To determine if this is indeed the case, we decided to generate PSR virus stocks on the monkey cell line VERO, which does not express α-galactocyl, and subsequently to compare the two virus preparations in various assays. No loss in virus titer was observed upon 0.45µm filtration, but filtration through a 0.22µm filter resulted in a 2-log decrease of PSR-VERO infectivity, while only a 1-log decrease in PSR-PD5 infectivity was observed. This suggests that virus properties may indeed change when PSR is produced on VERO cells. This was subsequently confirmed in various assays comparing the stability and response to inactivating treatments: PSR-VERO was slightly more resistant to drying, solvent detergent treatment, and pasteurization. Next, we will determine the effect of human plasma on the PSR-VERO titer to determine the level of resistance to anti-α-galactocyl antibodies. In conclusion, our data suggest that the methods used to generate virus preparations can indeed influence the response to various treatments. This is an important factor to consider when designing validation studies.

Validation of disinfection procedures

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Transmission of blood-borne viruses, like human immunodeficiency virus (HIV), hepatitis B virus, and hepatitis C virus (HCV) in clinical and laboratory settings is a major risk concern. Next to preventive measures like wearing protective coats and gloves, cleaning of non-disposable materials and surfaces is essential. In a previous publication it was shown that a high concentration alcohol mixture (80% ethanol and 5% isopropanol) was very effective in reducing lipid-enveloped (LE) viruses like bovine viral diarrhoea virus (BVDV, a specific model for HCV), HIV (a relevant virus), and pseudorabies virus (PRV, a general model for HBV). In a setting where 5% virus was spiked in the alcohol mixture after 20 seconds of treatment already a reduction value of > 6.0 $\log_{10}$ was found. For non-lipid-enveloped (NLE) viruses like canine parvovirus (CPV, a specific model for human parvovirus B19) and hepatitis A virus (HAV, a relevant virus), no reduction for CPV and limited reduction (approximately 2 $\log_{10}$) for HAV was found.

In the above experiments, the disinfection procedures were performed in a so-

called 'wet setting', i.e. performed in the disinfecting agent. However, in clinical and laboratory settings the viruses also can be dried onto the surface and in this setting the viruses may be less accessible to the cleaning agents. We are currently investigating the effect of drying of viruses, the effect of cleaning agents and the effect of matrices. Preliminary results for LE viruses show partial loss of infectivity after drying, however even after 7 days infectious virus was still present. For NLE viruses no loss of infectivity was observed after drying. When viruses were dried onto a surface, the effect of cleaning reagents was less pronounced and longer incubation times were needed. Eventually, after 10 minutes of incubation, the high concentration alcohol mixture resulted in 4 $\log_{10}$ reduction or more for the LE viruses. After pre-treatment with aqua dest before addition of the high concentration alcohol mixture, faster inactivation was observed, suggesting that dissolving the dried virus was crucial. Also disinfection with 0.1N sodium hydroxide or 0.1% hypochlorite did not result in rapid inactivation, after 1 minute of treatment infectious virus could still be found for LE and NLE viruses. Again, additional inactivation was observed when the incubation time was prolonged. This series of experiments will be finalized by testing the effect of drying virus in a matrix of blood. In the presence of high protein contents (i.e. blood), the stability of virus is assumed to be even higher.

Key publication

Van Engelenburg FAC et al. The virucidal spectrum of a high concentration alcohol mixture. Journal of Hospital infection 2002; 51:121-5.

Improving materials and methods for blood bank processing

Assessment of Compocool II butane-1,4-diol cooling plates

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Collected whole blood is placed immediately under cooling plates filled with butane 1,4 diol to induce a uniform decrease of temperature in all collected units from about 34°C to about 22°C. Hereafter the whole blood units can be stored at ambient temperature until they are centrifuged up to 24 h after collection.

New, vertical, butane-1,4-diol plates and special containers with a lid that can be closed have been developed to meet new requirements for transportation and environment. We first investigated the temperature decrease in a paired experiment comparing old and new cooling plates placed in its respective crates. The temperature decrease conformed to the requirements. The new plates in the new containers cooled the whole blood units slightly but significantly faster than the old plates. Next whole blood units were separated into blood components following overnight storage with old and new plates. Leuko-reduced red cells in SAGM were prepared and stored for up to 6 weeks measuring *in vitro* parameters such as blood cell composition, pH, glucose, lactate, ATP and hemolysis. Results will be ready in February 2006.

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Apheresis monocytes for dendritic cell culturing

As part of a Sanquin project (principal investigator Jaap Jan Zwaginga MD PhD, Sanquin Research) apheresis techniques were developed to obtain a monoscyte-rich component. MCS+ equipment from Haemonetics was used and initially the program to harvest peripheral blood stem cells was applied and fine-tuned to obtain a monocyte-rich blood component in an acceptable collection time. Contamination with granulocytes and platelets preferably should be below 5% each. The product was further processed using the ELUTRA equipment from Gambro to purify and enrich the monocyte fraction. Next the product was handed over to Sanquin Research where the cells were cultured to become dendritic cells under sterile and GMP conditions. The first results showed that it is possible to obtain a product with very few granulocytes and a high number of monocytes and further handle it under sterile and GMP conditions. However, to optimize the procedure a new apheresis disposable had to be developed and the apheresis product was too 'clean' for the ELUTRA, i.e. contained too few lymphocytes to properly load the ELUTRA. Therefore, the apheresis disposable was adjusted and recently 7 procedures have been performed. The results will be evaluated, next preliminary product specifications will be made for the apheresis product and the method will then be validated.

Apheresis Granulocytes from G-SCF stimulated related donors

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Request from hospitals have resulted in a protocol to harvest granulocytes via apheresis following G-CSF (without or with hydrocortisone) stimulation of related donors. The blood bank support and perform the apheresis procedure, which is performed in the hospital under responsibility of physician in charge of the patient, usually children. In 2005 about 39 procedures have been performed. The mean volume was 294 mL, with a mean number of granulocytes of $5.4x10^{10}$/unit. On average every two to three days granulocytes were transfused, for two to three weeks during the aplastic phase of the bone marrow. In most cases this supportive therapy was successful in fighting the (fungal) infections. From these data we will embody an granulocyte apheresis protocol and product specifications. These can be used for further product improvement and validation.

Bacterial contamination of platelet concentrates

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Since the end of 2001, all platelet concentrates (PC) in the Netherlands are cultured for bacterial contamination. Diversion of the first blood volume during collection of whole blood units became obligatory nationwide in the Netherlands starting July 1, 2004. Screening results over the years 2002-2005 were evaluated.
Prior to the implementation of diversion, a total of 127.979 PC units were tested, with 1155 initial positive signals (0.90%). After implementation of diversion, a total of 104.978 PC units were tested, with 474 initial positive signals (0.45%). Although most types of microorganisms showed a significant decrease, remarkably the percentage BacT/Alert positive cultures from which no micro-organism could be cultured, showed a tendency to increase. No difference was found between the total degree of contamination for PC units in plasma or additive solution, neither before nor after the introduction of diversion.

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 2006; 46:476-85.

Validation and development research program

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Validation and implementation of new machines and new tests were hot items. Another item was the comparison of various hematology analyzers for platelet counting. As result of this study a Sysmex 2000i was purchased for the R&D department.

A start was made with contract research for testing leukoreduction filters on whole blood and red cell concentrates and in the hospitals.
A post authorization surveillance (PAS) for monitoring platelet increments after platelet transfusion was started in three hospitals. This PAS will be continued in 2006.

Two national send arounds were organized: (I) for counting platelets in platelet concentrates and (II) for counting leukocytes in plasma, red cell concentrates and platelet concentrates. These send arounds will be repeated yearly, from now on.
Furthermore, a study was started to study the quality of platelet concentrates in various platelet processing systems. As result of this study, we will continue processing platelet concentrates in systems of Fresenius. Investigations with the tested systems will continue in 2006.
Finally, we participated in a national platelet activation working party. As result of this working party the CD62p test and the Annexin V test were uniformed within Sanquin.

Key publication

van der Meer PF, Gullikkson H, Aubuchon JP, Prowse C, Richter E, de Wildt-Eggen J. Biomedical Excellence for Safer Transfusion (BEST) Collaborative. Interruption of agitation of platelet concentrates: effects on *in vitro* parameters. Vox Sang 2005; 88:227-34.

Improving materials and methods for blood bank components

Cryopreservation of red blood cells

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Research on the cryopreservation of red blood cells has mainly focused on improving the quality of the red cells during post-thaw storage. The use of a closed system to add and remove glycerol, will allow the storage time after thawing of previously frozen red blood cells to be longer than 24 h. Previous studies had indicated that the additive solution AS-3 maintained the integrity of thawed red cells much better than SAG-M. 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 due to the lower pH of AS-3, which results in a lower intracellular pH and a lower rate of glycolysis.

In order to increase the intracellular pH after thawing, a washing solution with higher pH (PBS pH 7.4) was used instead of the glucose-containing washing solutions normally used (with a pH of 6.0). This modification resulted in a higher intracellular pH and better maintenance of the ATP content during post-thaw storage. Storage of the cells in AS-3 with a higher pH (pH 7.6) resulted in an even higher internal pH and a better maintenance of the ATP content. At this elevated pH, the production of lactate was increased, indicating higher glycolytic activity. Since there is a correlation between ATP content and *in vivo* survival of red cells, it is important to maintain ATP levels during storage. The use of a washing solution with higher pH for deglycerolization is an easy way to achieve this.

Erythrocyte storage solutions

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During preparation and storage of red cell concentrates (RCC), levels of 2,3-DPG levels fall rapidly. Recently, we developed an additive solution allowing maintenance of high 2,3-DPG levels without concurrent ATP decline. Our new solution is based on the 'chloride-shift' principle demonstrated 10 years ago, resulting in a more alkaline cytosol favouring 2,3-DPG formation. Early results indicated large variation in the effects on 2,3-DPG when our new solution (called PAGGG-M) was used to replace the standard SAGM solution. Because this could be caused by donor

variation or by variation in time between collection and RCC preparation, we investigated these two variables in more detail.
When prepared after 4 h storage as whole blood, the initial value of 2,3-DPG was about 15 µmol/g Hb in whole blood and in RCC, whereas after 24 h storage this value had declined to 7 µmol/g Hb (4 different donors). During storage for 14 days in PAGGG-M, the RCC prepared after 4 h showed an increase in 2,3-DPG from 15 to 26 µmol/g Hb, whereas in the RCC prepared after 24 h this only increased from 7 to 11 µmol/g Hb. Upon storage in PAGGS-M (a standard additive solution, used as control), units prepared after 4 h showed a decrease to 10 µmol/g Hb and units prepared after 24 h to 2 µmol/g Hb. For all RCC, the ATP level remained unchanged during the first two weeks. During storage as whole blood, the intracellular pH was 7.17 after 4 h and 7.03 after 24 h. This initial difference in whole blood was also observed in the RCC after preparation.
We concluded that the variable response with respect to 2,3-DPG levels with our experimental additive solution was not caused by donor variation. The effect was dependent on the time between collection and component preparation. Apparently, the decrease in intracellular pH during whole blood storage has a long-lasting effect on the response to an alkaline storage medium.

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 storage

Platelet metabolism during storage

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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. During 2005, two main subjects were studied:

Role of AMP-dependent kinase in platelet apoptosis

One of the properties of platelets is the ability, upon activation, to expose phosphatidylserine (PS), providing a negative surface necessary for the docking of the blood coagulation complexes. Many events underlying PS exposure are still unknown despite intensive research over the last 20 years. The involvement of a phosphorylation event is suggested and we therefore considered the AMP-dependent kinase (AMPK) as a candidate involved in the cascade leading to PS exposure.

Under substrate-deprived conditions, AMPK becomes phosphorylated (reflecting activation), but in the presence of mitochondrial substrates (pyruvate, acetate), AMPK was phosphorylated to a lesser extent. Hardly any phosphorylation was found when glucose was present. The analysis of the nucleotide profiles showed a good correlation between phosphorylated AMPK and AMP/ATP ratios. Interestingly, PS exposure showed a strong correlation with AMP/ATP ratios and the phosphorylation of AMPK. We conclude therefore, that in human platelets activation of AMPK strongly correlates with PS exposure, suggesting a role for AMPK in the induction of PS exposure.

Effect of extracellular pH on platelet concentrates

During storage of platelets, among other changes (known as 'platelet storage lesion'), a gradual decrease of pH as a result of lactate production takes place. So far, it is unknown if the pH decrease as such induces the 'platelet storage lesion'. Therefore, we decided to apply various pH values to platelets, resuspended in additive solution (to avoid plasma effects) and investigated several biochemical parameters during storage. The experiments were repeated in 30% plasma/70% additive solution to test if the results could be applied to blood bank conditions. An applied pH between 6.8 and 7.2 (at 37°C) was found to be optimal, with at lower or higher pH values increased rates of glycolysis and CD62P expression. The applied pH values were well maintained during storage for up to 6 days, except

for the low pH values as all glucose was converted to lactate. The increase of PS exposure was similar at all pH values tested during the first 3 days. At day 6, the PS exposure was clearly increased for those samples with low pH in which the glucose was depleted. The JC-1 signal (reflecting mitochondrial membrane potential) was relatively constant over the first 3 days, independent from applied pH, and collapsed on day 6 only in the samples without glucose. In the presence of plasma, platelets were able to partially counteract the applied pH. An applied pH of 6.15 resulted in a pH around 6.7 within hours, which was in contrast with washed platelets, in which the pH only increased up to 6.4. Platelets incubated in the presence of plasma were clearly much better (higher pH, lower percentage CD62P and PS positive cells, higher JC-1 signal) than in the absence of plasma over the whole pH range.

We conclude that human platelets during storage at pH < 6.8 and > 7.15 do change with respect to some, but not all characteristics of the 'platelet storage lesion'. The enhancing effect of low pH on glycolysis represents a potentially deleterious forward loop during storage in plasma or plasma/additive solution mixture. An interesting finding is that at low pH an acceleration of the glycolysis was measured (in contrast with literature data).

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.

Storage of leuko-reduced apheresis platelets processed with TRIMA® and MCS+ equipment and stored in plasma up to 7 days at 20 - 24°C with gentle agitation

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In 2004 we investigated single donor leuko-reduced apheresis platelets obtained with equipment and materials from Gambro (TRIMA®). We investigated 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 2005 in a second study we investigated leuko-reduced apheresis platelets processed with MCS+ from Haemonetics. Results also conformed to requirements. However in both the results of the TRIMA and from the MCS+ it was observed that requirements such as platelet concentration had a narrow band width, underlining the importance of validation of each apheresis equipment and procedure. A report has been written and will be discussed with the Medical Advisory Committee of Sanquin in the beginning of 2006. The application to prolong storage of leuko-reduced apheresis platelets to 7 days will only regard TRIMA and MCS+.

Storage of leuko-reduced apheresis platelets in plasma for pediatric transfusion

Principal Investigator
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Leuko-reduced apheresis platelets in plasma are divided into 4 units for pediatric transfusion. The whole apheresis unit is stored in a platelet storage bag with a volume between 1 and 1,5 L depending on the manufacurer. The split units are stored in small (600 mL) bags in plasma up to 5 days. On the market are 600 mL bags made from polyolefin (see publication), one manufacturer recently made 600 mL bags of another gas permeable material plasticized with butyl-trihexyl-citrate (BTHC). In a paired experiment we investigated leuko-reduced apheresis platelets stored in plasma up to 7 days in both types of bags. The results will be summarized in January 2006.

Key publication

van der Meer PF, Vrielink H, Pietersz RNI. Preparation and storage of white blood cell-reduced split apheresis platelet concentrates for pediatric use. Transfusion 2005; 45:223-7.

'Hibernation' project

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Glucose deprivation and metabolic suppression by addition of antimycin A can be used to preserve platelet function during prolonged storage at 4°C. The experiments to prove this were done in test tubes in 2003 and 2004 and in 2005 we investigated whether a 'translation' of these findings to storage of platelets in plastic bags was possible. To imitate the test tube circumstances we resuspended platelets in Hepes Tyrode buffer without and with 5 mM glucose, incubated them for 4 h at 22°C to consume the remaining glucose and induce glucose deprivation and next at 4°C without agitation in 600 mL bags. Controls were suspended in Hepes Tyrode with

glucose and stored at 22°C with agitation or at 4°C without agitation. Metabolic suppression was observed in the platelets stored in the glucose-free medium at 4°C and following 'waking up' with 20 mM glucose TRAP-induced aggregation was recovered, depending on the total storage time at 4°C. In the controls the platelet responsiveness was significantly lower. When the metabolic suppressed platelets were stored in gas-impermeable bags, the results were even better. A paper is in preparation and will be submitted to Transfusion in 2006.
The study is performed in collaboration with the Dept of Trombosis and Hemostasis of the Utrecht Medical Centre (Head Prof JW Akkerman).

Key publication

Badlou BA, IJseldijk MJW, Smid WM, Akkerman JWN. Prolonged platelet preservation by transient metabolic suppression. Transfusion 2005; 45:214-22.

A non-radioactive method for survival studies of transfused platelets

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We explored the feasibility to track minor platelet populations by fluorescent-labeled monospecific monoclonal HLA-class I antibodies. Using a panel of informative monoclonals we determined sensitivity and specificity of these antibodies under several circumstances, relevant to design robust test-reagentia. In the past year 2005 we tested a large panel of Moabs and selected 8 different Moabs fulfilling our criteria. These 8 moabs were 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 different transfused platelet populations simultaneously. The selected hybridomas (provided by dr A Mulder, Dept of Immune Hematology and Blood transfusion, Leiden University Medical Center) are subsequently produced in large quantities. After several weeks of culture Ig is purified from the supernatant and labeled with Alexa Fluor-488 dye, which is spectrally similar to fluorescein. These processed, labeled and stored culture supernatants are currently tested for suitability for platelet tracking *in vivo* after platelet transfusion. As part of an NIH-granted study conducted by Dr Slighter (Seattle), the moabs will be tested in selected patients and compared with the radio-active method as 'gold standard' for

platelet survival as part of a clinical trial. Intention is to apply for further financial support to perform a clinical study on the suitability for this method to compare platelet products of presumed different quality.

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.

Biochemical and biophysical changes in platelets during storage

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We have already shown that ADP receptor function *in vivo* is protected from desensitization by so-called nucleotidases, present *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.
In 2005 we have conducted a small clinical trial in which we compared responsiveness of transfused platelets from PASII versus plasma stored platelets. Transfused platelets were tested for thrombin generative and adhesive capacity. There was no difference between PASII and plasma stored platelets when tested in these assays. However, after pre-stimulation with ADP or collagen PASII stored transfused platelets responded less than plasma stored transfused platelets. In the coming year we will evaluate this phenomenon on large scale in the Sanquin-HOVON study. We will evaluate the predictive value of these tests in for risk of bleeding during a thrombocytopenic period.
Furthermore, we have studied the shedding of platelet microparticles under minimal activation conditions (such as during storage under blood bank conditions). This microparticle formation was independent on autocrine activation, but relied on the integrin αIIbβ3 (GPIIbIIIa) activity. Upon outside-in signalling via integrins, the actin cytoskeleton is destabilized, resulting in the shedding of procoagulant platelet microparticles.

Key publications

Keuren JF, Cauwenberghs S, Heeremans J, de Kort W, Heemskerk JW, Curvers J. Platelet ADP response deteriorates in synthetic storage media. Transfusion 2006; 46(2):204-12.

Cauwenberghs S, Feijge M, Hageman G, Hoylaerts M, Akkerman JW, Curvers J, Heemskerk J. Plasma ecto-nucleotidases prevent desensitization of purinergic receptors in stored platelets: importance for platelet activity during thrombus formation. Transfusion, in press.

New functional whole blood assays to assess platelet function under flow

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Objective was to quantify microparticle (MP) formation in stored platelet products at different time intervals. We have succesfully set up a flow cytometric assay to quantify MP numbers and determine their cellular origin. We have compared our method to an ELISA technique (developed at Sanquin BCR, Amsterdam) based on CD36 expression on MP surfaces and found a good correlation (r=0.9) between both measurements.

We have further investigated the mechanisms through which storage induced MP might support blood coagulation. We determined cellular origin and surface expression of tissue factor (TF), procoagulant phosphatidylserine (PtdSer) and glycoprotein (GP) Ibα. The influence of MP on initiation and propagation of coagulation were examined in factor Xa and thrombin generation assays using MP free plasma and compared with that of synthetic phospholipids. About 75% of MP were platelet-derived and their number significantly increased during storage of platelet concentrates. About 10% of the MP expressed functionally active TF. However, TF-driven thrombin generation was only found in plasma in which tissue factor pathway inhibitor (TFPI) was neutralized, suggesting that MP associated TF in stored platelet concentrates is of minor importance in initiating hemostasis. It was further established that 60% of all MP expressed PtdSer. In comparison with synthetic procoagulant phospholipids, the maximal rate of thrombin formation in TF-activated plasma was 15-fold higher when platelet free plasma was titrated with MP. This difference could be attributed to the ability of MP to propagate thrombin generation by thrombin-activated factor XI. Collectively, this points at a role of microparticles in supporting hemostasis by enhancement of the propagation phase of blood coagulation.

In the Xa generation assay we also found that MP bear active factor V (FVa) on their surfaces, that is able to promote Xa generation in the absence of added FV.

However, APC-catalyzed inactivation of MP-bound FVa resulted in a significant lower loss of FVa activity (62%) than found on synthetic vesicles (95%). On MP from platelet products, the velocity of Arg506 and Arg306 cleavage by APC is reduced when compared to synthetic lipids. We still have to confirm this notion by western blot analysis (in cooperation with the division of plasma proteins). We hypothesize that there might be a difference in the binding of FVa on different surfaces e.g. synthetic vs platelet (derived) surfaces, rendering it more resistant to cleavage by APC. This is subject for further study. The APC resistence of factor Va bound to storage induced MP adds up to the other clot-promoting actions of MP and may have a beneficial clinical effect in patients who need hemostatic support (and lack sufficient platelets).

Key publication

Keuren JFW, Magdeleyns EJP, Govers-Riemslag JWP, Lindhout T and Curvers J. Effects of storage induced platelet microparticles on the initiation and propagation phase of blood coagulation. British Journal of Haematology, in press.

New therapies and evaluation of clinical applications

New cellular therapies

Cellular therapy research

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Projects in this research line are focused on the development of new cellular therapies. that a) can facilitate the hematopoietic stem cell transplantation and b) can facilitate tissue regeneration.

Mesenchymal stem cells are known in this light to support the efficacy of stem cell transplantation. In this regard we investigated the effect of MSC on endothelial cells. The supernatant of MSCs was shown to alter the endothelial growth pattern and to induce the expression of CD90 in endothelial cells, which can function as an adhesion molecule at least for myeloid progenitor cells and maybe for stem cells also. The other focus of our cellular therapy research deals with the potential of blood or bone marrow derived cells to form the blood vessel lining endothelium. It is now getting more and more clear that the so far called endothelial progenitor cells actually consist out very rare immature bone marrow derived stem cells with high proliferative potential and more mature monocytic cells from peripheral blood that can exhibit some endothelial mimicry which might play a role in facilitating the growth of the immature EPC and possibly help in collateral vessel formation. We have demonstrated that monocytic cells are responsible for the outgrowth in the so-called Endocult assay, a colony assay for Endothelial colony forming cells (CFU-EC). T-cells seemed to facilitate the monocytic colony formation by a still unknown paracrine factor. To screen for the revascularizing potential of these cells we made the ischemic hind limb model operational at our department. Sequential LDPI (Laser Doppler Perfusion Imaging) is used to monitor the normalization of blood flow after ischemia induction. While infusion of monocytes increased the recovery of the blood flow in this model, we are now investigating if modulating the phenotype of the monocytes by other inflammatory (e.g. T-) cells, TNF, angiogenic growth factors and even MSC supernatant can favourable influence this mechanism.

We previously showed that stem cells can be expanded *ex vivo* into megakaryocytes by the combination of thrombopoietin (Tpo) and IL-1. These cultures of expanded

cells might still be the used to shorten the thrombocytopenic period after autologous stem cell transplantation. In animal studies we have now clearly shown a short term platelet repopulating effect via these expanded stem cell products. In concluding experiments we hope to find a simple way (e.g. by cultures at 39°C) to even increase the efficacy of this platelet repopulating therapy. The *ex vivo* megakaryopoiesis studies have also led to better understanding of mutations in the thrombopoietin (the Mpl) receptor. By cloning and transfection of mutated genes that code for the Mpl receptor in test cells, we have now determined different mechanisms (signaling defects and protein expression defects) that are responsible for the lack of megakaryocytopoiesis in patients with these mutations. Finally we were able by comparing the RNA (the transcriptome) of very pure megakaryocytic cultures with that in erythroblast cultures to define genes that are specifically active in 'blood platelets'. These gene lists are an important basis for a large Euopean Integrated project (Bloodomics), that aims to correlate small variations (so-called SNP's) in those genes to a changed risk for cardiovascular disease. Our department will when promising risk-associated genes are characterized, use so-called gene silencing techniques (siRNA) in megakaryocyte cultures to see what the function is of these often new gene (variations). While experiments so far indicate that the siRNA approach is feasible, platelet formation from megakaryocyte cultures is still a problem. It would be of large importance if *in vitro* platelet production (e.g. by introducing endothelial cells in the equation) can be made possible.

Within the laboratory for Stem Cell Transplantation (SCL) most processes involve the processing of cellular products for conventional autologous and allogeneic transplantation of adults and children. However, in recent years the SCL became closely associated with several clinical trials; at first, the Hebe trial in collaboration with ICIN (inter university cardiology institute) whereby patients receive enriched peripheral blood or bone marrow derived stem cells after a myocardial infarct. Secondly, the monocyte trial in collaboration with the Dept of Rheumatology Academic Medical Centre; for this project monocytes are isolated from peripheral blood of patients suffering from rheumatoid arthritis. Furthermore, a new project on dendritic cells has been started in collaboration with Marieke van Ham (Sanquin

Research, Dept of Immunopathalogy). Carlijn Voermans, head of the Stem Cell laboratory, worked throughout 2005 as a postdoctoral fellow in the laboratory of Dr T Reya (Duke University Medical Center, Dept of Pharmacology and Cancer Biology) financially supported by Talent stipend from NWO. She worked on two projects involving the molecular mechanisms of hematopoietic stem cell regeneration. In the first part of the study it was shown that following injury (in response to the chemotherapeutic agent cyclophosphamide (Cy) and the growth factor granulocyte colony stimulating factor (G)), the bone marrow microenvironment has an enhanced ability to support HSCs *in vitro*. These findings suggest that activation of Wnt signaling is an important element of hematopoietic regeneration, and provide novel insight into the integration of HSCs and their micro-environment during injury repair. Furthermore, genome wide changes in gene expression in HSCs in response to Cy/G treatment were analyzed. To test whether this screen allowed identification of novel candidates that regulate HSC function, the role of transforming growth factor beta inducible gene-h3 (βig-h3), a gene highly upregulated after Cy/G treatment was studied.

Key publications

Hunting CB, Noort WA, Zwaginga JJ. Circulating endothelial (progenitor) cells reflect the state of the endothelium: vascular injury, repair and neovascularization. Vox Sang 2005; 88(1):1-9.

Rattis FM, Voermans C, Reya T. Wnt signaling in the stem cell niche. Curr Opin Hematol 2004; 11(2):88-94.

Factors affecting proliferation and differentiation of stem and progenitor cells

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This project aims to understand in more detail the expression, regulation and function of genes controlling proliferation and differentiation of megakaryocyte (MK) 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 the small GTPase Rac during MK progenitor expansion and differentiation.

Role of mTOR during megakaryopoiesis

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 tumour suppressor complex TSC1/2. As the regulation of cell number and cell 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 downstream effectors of mTOR, p70 S6K and 4E-BP are phosphorylated by Tpo in a rapamycin- and LY294002-sensitive manner. Treatment of $CD34^+$ cells and primary MKs with rapamycin inhibits Tpo-induced cell cycling and blocks cells in G_0/G_1. Furthermore, rapamycin markedly inhibits the clonogenic growth of MK progenitors with high proliferative capacity, but does not reduce the formation of small MK colonies. Addition of rapamycin to MK suspension cultures reduces the number of MK cells, but inhibition does not significantly affect expression of MK-specific glycoprotein's IIb/IIIa and Ib, nuclear polyploidization levels or cell survival. The results demonstrate that the mTOR pathway is activated by Tpo and plays a critical role in regulating proliferation of MK progenitors, without affecting differentiation or cell survival.

Role for Rac during megakaryocyte migration and platelet formation.

During thrombopoiesis, megakaryocyte (MK) progenitor cells proliferate, differentiate and ultimately form mature MKs from which platelets are shed. These processes are accompanied by major changes in morphology and in organization of the actin cytoskeleton. Within the Rac subfamily of GTPases, Rac1 and Rac2 have been shown to play distinct roles in stem cells, neutrophil functions and in B-cells. In this study, we examined the role of Rac GTPases during megakaryopoiesis using

the highly specific, reversible small-molecule inhibitor of Rac activation NSC23766. Experiments were performed using $CD34^+$ cells (purified from mobilized peripheral blood) and primary human MK cells (obtained from suspension cultures). Inhibition of Rac markedly reduced the clonogenic growth of MK progenitors, however, the effect of Rac inhibition on MK proliferation in suspension cultures containing Tpo and SCF was smaller. Addition of NSC23766 to suspension cultures did not inhibit MK differentiation as measured by expression of the glycoprotein's CD41 and CD42 after 7 days. In contrast, in suspension cultures containing Epo and SCF erythroid differentiation was markedly affected by Rac inhibition as measured by expression of glycophorin A. These data indicate a diverging role for Rac in erythroid versus MK differentiation. As Rac GTPases control actin polymerization and cell motility, we examined the effect of Rac inhibition on actin polymerization and migration of MK cells. In primary MK cells, SDF-1 induced actin polymerization was rapid and transient, reaching maximal levels 15-30 seconds after stimulation. Pre-incubation with NSC23766 resulted in abrogation of actin polymerization and a strong reduction in cells migrating to an SDF-1 gradient. Furthermore, Rac activity was found to be critical for platelet formation from mature MKs. Concluding, these data demonstrate that Rac signaling pathways are required at different stages of megakaryopoiesis; Rac is required for clonogenic growth of MK progenitors, but not for differentiation to MK cells. In addition, Rac signaling plays an essential role in MK actin polymerization, migration and platelet shedding.

The research described above is performed in close collaboration with the Hematology Research Laboratory, University Medical Centre Groningen (head Prof E Vellenga).

Key publications

Drayer AL, Olthof SG, Vellenga E. Mammalian target of rapamycin is required for thrombopoietin-induced proliferation of megakaryocyte progenitors. Stem Cells 2006; 24:105-14.

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; 23:240–51.

Cord blood

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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. For this purpose three options (possibly combined) are promising: *ex vivo* expansion, co-transplantation with mesenchymal stem cells and multiple donor transplantation. In 2005 we focused on *ex vivo* expansion and co-transplantation with mesenchymal stem cells (MSC) to evaluate improvement of transplantation results with cord blood in the NOD-SCID mouse model.

With regard to *ex vivo* expansion we developed an *in vitro* proliferation protocol of cord blood stem cells based on trombopoietin (mpl-ligand, Tpo) as single growth/differentiation factor. Tpo not only triggers proliferation of the stem cells but also differentiation towards megakaryocyte precursor cells. We demonstrated that transplantation of NOD/SCID mice with *ex vivo* Tpo expanded cells resulted in earlier platelet recovery in the NOD/SCID mice model without loss of engraftment potential or multilineage differentiation capacity, indicating that no loss of pluripotent stem cells had occurred during expansion. In addition, we have analyzed in the NOD/SCID mice model, the effect of co-transplantation with MSC on platelet recovery and total bone marrow engraftment. We observed no further acceleration of platelet recovery, however, the MSC can increase the total number of platelets in the long term. Moreover, higher levels of bone marrow engraftment have been observed when MSC were co-transplanted with Tpo *ex-vivo* expanded CB cells. Because recent clinical studies have shown that certain *ex vivo* expansion protocols may have a detrimental effect on graft versus host disease (GvHD), it is thus essential to investigate the immunological profile of *ex vivo* Tpo expanded cord blood mononuclear cells. Moreover, since a cord blood donor can not be requested for post-transplant immunotherapy (DLI) in case of relapse, the graft versus leukemia capacities of the cells will be explored as well. In 2005, we have started to investigate the effect of *ex vivo* Tpo expansion on the immunological profile of the graft. Preliminary experiments show that *ex vivo* Tpo expansion, reduce the number of reactive T-cells in the graft. Moreover, it seems that the reactivity of the recipient towards the graft (e.g. rejection of the graft) is also lower, although further experiments are needed to

confirm these results and to study the consequences for graft versus leukemia.
In addition to *in vivo* engraftment efficacy and immunology, we have investigated the feasibility to cryopreserve *ex vivo* expanded cord blood $CD34^+$ cells. This would not only facilitate the transplantation logistics, but more important this would increase the safety of the transplant enabling control for bacterial contamination during *ex vivo* culture. Our first results show that *ex vivo* Tpo expansion of cord blood $CD34^+$ cells before cryopreservation is feasible and appears not to have an adverse effect on the clonogenic pluripotent potential of the cells.
Furthermore we investigated the role of nucleated red blood cells (NRBC) in cord blood. We observed a strong correlation between NRBC and $CD34^+$ stem cell numbers, what may account for the described relation between NRBC and progenitor growth capacity of the cord blood.

This research project is a close collaboration with Drs Fibbe and Goulmy of the Dept of Immunohematology and Blood Transfusion, LUMC, Leiden.

Key publications

Van Hensbergen Y, Schipper LF, Brand A, Slot MC, Welling M, Nauta AJ, Fibbe WE. Ex vivo culture of human CD34+ cord blood cells with thrombopoietin (TPO) accelerates platelet engraftment in an NOD/SCID mouse model. Submitted.

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

Schipper LF, Brand A, Reniers NC, Melief CJ, Willemze R, Fibbe WE. Differential maturation of megakaryocyte progenitor cells from cord blood and mobilized peripheral blood. Exp Hematol 2003; 31:324-30.

Schipper LF, Brand A, Reniers NC, Melief CJ, Willemze R, Fibbe WE. Effects of thrombopoietin on the proliferation and differentiation of primitive and mature hemopoietic progenitor cells in cord blood. Br J Haematol 1998; 101:425-35.

Mommaas B, Kamp JA, van Halteren AGS, Enczmann J, Wernet P, Kögler G, Mutis T, Brand A, Goulmy EAGJ. Cord blood comprises antigen-experienced T cells specific for maternal minor histocompatibility antigen HA-1. Blood 2005; 105:1823-27.

Research on cellular blood products

The collection of autologous blood products by double erythrocytapheresis; A cost effectiveness study

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It is the custom to collect blood for autologous donations by standard whole blood phlebotomy just like procedures in regular donors, however for autologous donors it is more critical to obtain the ordered number of units from themselves. Double-erythrocytapheresis has the potential to collect only erythrocytes while directly compensating for loss of plasma in half the number of sessions than current phlebotomy practice.

To explore this more efficient/effective type of autologous blood collection we studied in a concurrent comparison 52 consecutive autologous donations collected by double-erythrocytapheresis with the Haemonetics MCS + machine in period between April 2003 to June 2005 and 49 autologous donors/patients collected in the same period by classical whole blood donation. Comparisons were made as to number of required procedures, collected units of packed cells, transfused units of packed cells to the autologous patient/donor and donor and technical complications between double erythrocytapheresis and whole blood donations. From the 52 patients in the whole blood group 4 were excluded because of: bad veins (2), fear of procedure (1), medical reasons (1). From the 49 patients in the erythrocytapheresis group 3 were excluded because of: bad veins (1), fear of procedure (1), medical reasons (1). As to the comparability of the two groups, the erythrocytaferesisis group was significantly older and had a higher systolic blood pressures, larger estimated circulating blood volume as well as a higher initial Hb at time of referral. Both donor/patient groups were comparable with respect to the indications for operation. The percentage of successfully collected products by either method was comparable (90% versus 91%). In the erythrocytapheresis group 110 units were requested: 100

were collected in 50 erythrocytapheresis procedures. In the whole blood group 113 units were requested: 102 were collected in 102 whole blood procedures.
The risk of donor/medical complications between erythrocytapheresis and whole blood estimated by odds ratio was 1.21 (95%CI 0.70–2.10). All these complications fell in the category light (hematoma, dizziness, mild citrate reactions). The percentage of technical errors was higher in the erythrocytapheresis group, 4% (with 80% in the initial phase of the project) versus 1% in the whole blood group. Though donors collected by erythrocytapheresis had significant lower Hb levels pre-operation (8.0 mmol/l) when compare to whole blood donors (8.8 mmol/l)($P < 0.001$), less autologous transfusions were used in the erythrocytapheresis group.
The results of this study warrant the following conclusions.
Double-erythrocytapheresis seems as safe and effective as whole blood collections to collect autologous blood.

- Double-erythrocytapheresis results in a 50% gain in efficiency in required blood collection procedures, since half of the procedures were required for the requested units.
- Double-erythrocytapheresis is < 5% more expensive than whole blood collection for blood banks.
- From a public health as well as donor perspective, double-erythrocytapheresis seems more efficient than whole blood collection (since it reduces donor visits to the blood bank).

Other aspects: An explorative storage study, collaboration with D de Korte indicated that ATP levels may diminish up to 10% faster in erythrocytes derived by double-product erythrocytaferesis compared with erythrocytes obtained by classical blood collection, although this difference was not statistically significant, given the small underpowered size of the study.

Therapeutic erythrocytapheresis as treatment for hemochromatosis patients

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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. Since the start of the project the catchment-area for inclusion of patients was extended by greater collaboration with the gastroenterologists and hepatologists from the regional hospitals as established at a presentation of the project (October 2004) at their yearly scientific meeting. To allow inclusion of more patients' 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.

A total of 12 patients eligible and willing to be randomized were included in the trial and treated. As a direct spin-off from the start of the project, the Dept of Internal Medicine of the University Hospital of Maastricht in collaboration with our department started a special out-patient clinic for hemochromatosis patients.

Also, in collaboration with the Dept of Internal Medicine of the University Hospital of Maastricht, we have started a 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. A Maastricht University hospital based study on incidence and prevalence of hemochromatosis in the Sanquin South East Region will in due time allow to estimate the expected maximal yearly workload required from the blood bank to provide assistance in the therapy of iron overloaded patients.

In collaboration with the Dept of Clinical Chemistry of the University Hospital of Nijmegen (Dr Swinkels, Prof J Marx) we have followed hepcidine levels for and after a single treatment procedure in some of the patients. The results are not available yet.

Reduction of blood transfusion in orthopedic surgery

Principle investigator
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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 re-infusions, large multi-arm and multi-centre studies are needed. The basic requirement for such studies is a strict transfusion protocol. We showed in a randomised controlled study in three orthopedic centres that a uniform transfusion trigger is feasible and can be applied among hospitals. A multi-centre study was designed on integrated blood sparing approaches (Optimal Blood Management (Transfusie Op Maat study – TOMaat –). Because of the complex design the study started as a feasibility study in 2004, supported by Sanquin. From May 2004 to December 2005 two participating hospitals (Leiden University Medical Centre and Albert Schweitzer Hospital, Dordrecht) included 455 patients (inclusion percentage of 85%) showing that the design is feasible. In July 2005 a grant from the Dutch Medical Research Cuncil was received as co-sponsoring to enable the TOMaat study for the entire 5 years (until January 2009).

Key publication

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 orthopedic surgery? Vox Sanquinis 2004; 87 (Suppl.3):52 (abstract).

Clinical effects of the peri-operative use of, by-filtration leukocyte reduced, red cell transfusions

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The peri-operative use of blood products has been researched in cardiac surgery setting, in vascular surgery, orthopedic surgery, and oncologic surgery. In an international effort we are now creating a combined database from RCT's investigating the effects of by-filtration leukocyte reduced transfusions in cardiac surgery patients. In a collaborative effort, the authors of the RCTs (Brand, Bilgin, Boshkov, Wallis & van de Watering) and the independent statistician (EC Vamvakas), have compared and discussed the initially collected data elements. We agreed on the data elements that are to be entered in the newly combined database. These data are now being abstracted and (re)coded. In collaboration with Groningen

University Institute for Drug Exploration GUIDE/GRIP, cost-effect analyses were performed on the use of by-filtration leukocyte reduced red cell transfusions in the cardiac surgery patients that participated in our two-centre RCT.
In 2005, the collection of follow-up data for analysis of long term survival after gastrointestinal (GI) oncology surgery was started among the patients of the TacTicS-trial. Additional clinical variables (specific oncologic) are now also collected to further complete the dataset, and facilitate long-term survival analyses.

Key publications

Van Hilten JA, van de Watering LMG, van Bockel JH et al. De effecten van leukocytenverwijdering uit rodebloedcelconcentraten door filtratie. NTvH 2005; 4:140-7.

Bilgin YM, van de Watering LMG, Eijsman L, Versteegh MIM, van Oers MHJ,.Brand A. Bloedtransfusies en postoperatieve complicaties na hartklepchirurgie. Hart bulletin, jaargang 36, nr.4, aug.2005.

Van Hulst M, YBilgin YM, Lan de Watering LMG, de Vries R, van Oers MHJ, Brand A, and Postma MJ. Cost-effectiveness of leucocyte-depleted erythrocyte transfusion in cardiac valve surgery. Transfusion Medicine 2005; 15:209-17.

Leo van de Watering and Anneke Brand. Independent association of massive blood loss with mortality in cardiac surgery. Transfusion 2005; 45:1235-6.

Transfusion triggers and effects

WOMB study: Well being of Obstetric patients on Minimal Blood transfusions

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Postpartum hemorrhage (PPH) is one of the top five causes of maternal mortality in developed and developing countries. The most important treatment of PPH is red blood cell (RBC) transfusion. The decision whether to prescribe RBC transfusion is mostly based on postpartum hemoglobin (Hb) values. RBC transfusion should be aimed to reduce morbidity and especially to improve Health Related Quality of Life (HRQoL). The goal of the WOMB study is to assess the effect of RBC transfusion on

HRQoL and to confirm the role of HRQoL in deciding whether RBC transfusion is necessary.

The WOMB study is a multicenter trial in patients with PPH, where a restrictive RBC transfusion policy will be compared with a more liberal RBC transfusion policy. Primary outcome in this study is fatigue measured with the MFI questionnaire. Inclusion criteria are: 1) 12-24 h after VD or CS; 2) 3.0 ≤Hb≤ 5.0 mmol/l; 3) blood loss ≥ 1000mL; 4) age≥ 18 years; 5) no anemic symptoms. Patients will be randomised for a RBC transfusion or not. The total follow-up period is 6 weeks. HRQoL will be measured at T=0 (12-24 hours postpartum) 3 days, 1, 3 and 6 weeks postpartum. At T=0 and 6 weeks postpartum Hb value will be measured as well as a screening on irregular antibodies. For the patients who receive a RBC transfusion, the effect of the RBC transfusion will be measured with the Hb value, Hct, platelet and leukocyte count, and the temperature of the patient before and after the RBC transfusion. The sample size is 400 patients: 200 patients after a VD (where 100 patients receive a RBC transfusion and 100 patients not) and 200 patients after a CS (also 100 patients with en 100 patients without a RBC transfusion).
The study started in May 2004 and is at the moment ongoing in 10 hospitals in The Netherlands.

Key publications

Jansen AJG, Essink-Bot ML, Hop WCJ, Beckers EAM, van Rhenen DJ. Transfusietriggers en Kwaliteit van Leven. Nederlands Tijdschrift voor Klinische Chemie 2003; 28:280-4.

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

Donor studies, epidemiology and cost effectiveness

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Transfusion Technology Assessment

While blood products for transfusion are presently very safe, more technology becomes available for even safer products. As the donations that are processed into blood products are derived from humans, they remain exposed to (emerging) infectious diseases. Blood transfusions can never be 100% safe. This creates opportunities for 'marketing of fear' to force implementation of new technologies. Therefore there is an increasing call for standards on blood safety efficacy. In collaboration with the Dept for Medical Technology Assessment (MTA) of the Julius Center of Utrecht University, Sanquin Research created a research group on Transfusion Technology Assessment (TTA). This group aims to perform independent risk assessments and health economic evaluations 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.

Amongst the contamination risks of blood transfusions, bacterial contamination of platelet transfusions is the most important. To become acquainted with the literature, a study was performed on the costs and effects of pathogen reduction and bacterial screening of platelets, which was accepted for publication by 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 should be preferred over platelets derived of 5 whole blood donations. 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. This study will be submitted for publication, together with further modeling studies on vCJD and risks of blood transfusions.

Commissioned by the Council of Europe, indicators of 'The Collection, Testing and Use of Blood Products in Europe' of 2002 through 2004 were evaluated and reported. The Survey is now a leading annual publication on blood transfusion indicators.

Commissioned by the International Plasma Fractionation Association, modeling studies on viral safety of plasma products were performed and discussed in an international working group.

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 2005 the collaboration of 4 academic hospitals was obtained to further optimize the required databases. Participation of a relevant and representational sample of hospitals in The Netherlands is essential and will be further elaborated. In addition to in-hospital blood recipient profiles, the survival of these patients is of importance. In 2005 the formal collaboration of the Netherlands Statistics Office was obtained for this purpose and in its pilot phase the 10 year survival probability of transfusion patients from one academic hospital was evaluated. 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. It appears feasible to obtain sufficient reliable information on blood recipient profiles in this manner.

Key publication

Janssen MP, van der Poel CL, Buskens E, Bonneux L, Bonsel GJ, van Hout BA. Costs and benefits of bacterial culturing and pathogen reduction in the Netherlands, Transfusion, in press.

Red cell alloimmunization

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The epidemiology of red cell alloimmunization is unknown. In collaboration with 19 regional and academic hospital in the Sanquin South West Region we studied the occurrence of clinically relevant erythrocyte antibodies in various transfusion populations: polytransfused hematolo-oncology patients, surgical patients with a few transfusion events and pregnant females treated with intra-uterine transfusions because Rh-HDN. In addition the incidence of alloimmunization before and after universal leukocyte reduction of red cell transfusions was investigated. During a 4 year survey, implicating almost 500.000 units of red cells transfused , we found a prevalence of 0.13% alloantibodies per unit transfused. Responders showed

a striking high 20% risk of producing additional antibodies onto a subsequent transfusion event.

Key publications

Schonewille Henk, Brand Anneke. Alloimmunization to red blood cell antgens after universal leucodepletion. A regional multicenter retrospective study. Br J Haematol 2005; 129:151-6.

Schonewille H, van de Watering LMG, Loomans DS, Brand A. Red cell antibodies after transfusion. Factors influencing incidence and specificity. Transfusion 2006; 46:250-6.

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Quality Assessment and Improvement Program

In 2005 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.

In 2005 a survey was carried out under the donors of the Sanquin Blood Bank North East Region (SBNO) regarding their opinion on the complaint procedure.
Also a regional survey was carried out under the personel of the SBNO regarding their opinion of the regional Sanquin Blood Bank newsletter NoNonsens.
On behalf of the Board of Directors and Corporate Staff a study was carried out under the complete file of active donors in the Netherlands regarding their general opinion on blood donation (donor satisfaction survey).
A repeat customer satisfaction survey under the hospital laboratories was prepared and carried out. Reports will be effectuated in 2006.
A customer satisfaction survey regarding ICT-CS service and products under Sanquin personel was prepared. This survey should for the first time within Sanquin make use of internet facilities to complete the questionnaire.
Advisory tasks were carried out to support a survey prepared by Dr J van Hilten, Sanquin Blood Bank South West Region, location Leiden (opinion and image of artificial blood and products).

The systematic recruitment of new blood donors

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In the past decade the number of Dutch blood donors has declined steadily; 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 these studies have recently been published. The next step was to perform a content analysis of the brochures of the blood bank. Analysis revealed that the factors that influence the decision to donate blood are currently not addressed in the brochures. The content of the brochures is currently being modified and experimentally tested. The study on the determinants of blood donation also revealed people who do not donate, are not acquainted with blood donors. We also know that most people, who do donate, once started donating due to contact with other blood donors. We therefore investigated whether blood donors are prepared to recruit non-donors. Results showed that two third of the donors under study are willing to participate in a donor-recruits-donor campaign. This project is performed in close cooperation with Maastricht University.

Key publications

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 2005; 45(6):945-955.

Lemmens KPH, Ruiter, RAC, Abraham C, Veldhuizen IJT, Schaalma HP. Psychological antecedents of blood donation motivation among non donors: Testing an extended Theory of planned behaviour. In preparation.

Seasonality of Hb and donor deferral

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Indications exist that deferral rates in blood donors are highest in summer. However, a detailed quantitative analysis is not available. We investigated the association between Hb-values, deferral rates and daily temperatures in a large dataset of blood donors. The study population consisted of both plasma and whole blood donors registered in the donor file in the South East Region of the Netherlands. Individual Hb-levels and other examination data between January 2002 and December 2004 were extracted from the donor file. Data on daily maximum temperatures were obtained and related to Hb-levels and Hb-deferrals. Results are reported separately for plasma and whole blood donors and for men and women. Data was available from 106,398 whole blood donors and 6,983 plasma donors, resulting in data of more than 600,000 examinations. Hb levels decreased with increasing daily temperature. Highest deferral rates were observed for the summer months, which was consistent over the several groups and over the three years. The highest Hb-deferral of 11.1% was observed for female whole blood donors on days with a maximum temperature of 25 degrees and above. In all four donor categories a gradual increase with temperature was observed with 1.7 to 2.2 times higher deferral rates on ho We observed a clear seasonal pattern in Hb-levels and in the percentage Hb-deferrals. The observed seasonal effect could not be explained by differences in donor characteristics. Our observations might have practical implications for donor management.

Key publication

Hoekstra T, Veldhuizen I, Van Noord P, de Kort W. Seasonal influences on Hb levels and deferral rates in whole blood and plasma donors. Submitted for publication.

Donor cohort studies

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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. In 2005 we tested the questionnaire in a pilot study among 600 donors (response rate 70%). Based on the pilot results, the questionnaire was slightly modified. In the questionnaire the following topics are addressed: demography, lifestyle factors (e.g. smoking, alcohol use), nutrition, medical history, medication use, physical activity, donor motivation, donation experiences, and for women, questions on menstruation patterns, menopause, hormone use and contraceptives. At the moment we are preparing the start of the cohort study. Data gathering will commence after the summer. Every fortnight from September 2006, 1,500 to 2,000 questionnaires will be sent out. Data gathered 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).

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- New therapies and evaluation of clinical applications
  - New cellular therapies
    - Cellular therapy research 81

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    - Regulation of endothelial integrity 36

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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)

Marketing authorization for Nanogam® was obtained and the work to implement Sanquin Plasma Products as production site was completed. Suitability studies for the use of paste II from third parties for the manufacturing of Nanogam® were started. In close collaboration with Sanquin Research, 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. A characterization program was developed to study impurities profiles of the intermediate products. Clinical studies in The Netherlands and USA with this virus safe Cetor® were started 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 2006. In close collaboration with Sanquin Virus Safety Services prion removal studies are started. Development work on a manufacturing process for a third generation liquid IVIG product was started. It is aimed for a process with a higher process yield.

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.

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The strategy of the CAF-DCF Product Support Division focuses on both the efficacy of plasma derivatives and their safety as regards pathogens and environmental pollutants. With the aim to develop a science-based quality assurance programme for focusing on therapeutic proteins and their excipients in plasma or concentrates, the division develops both immunological methods (e.g. total and strain- specific anti-pneumococcal antibodies in IVIG, epitope identification) and biochemical techniques (such as intrinsic and extrinsic protein fluorescence and exploits them in industrial applications. Besides studies on UVC irradiation as a new virus inactivation technology developed by DCF-CAF R&D, we have run ex vivo experiments on a new potential immunomodulation application in association with a treatment using intravenous immunoglobulins at the patient's bedside. Research is still ongoing on B19 erythrovirus (formerly parvovirus B19). The aim is to distinguish in donors its infectivity (multiplication) from its detection by nucleic acid amplification techniques (NAT). Only selected points are reported here.
The Product Support Division is also dedicated to providing expert scientific and technical advice and to offering state-of-the-art analytic methods meeting the latest regulatory requirements.

B19 infectivity

Erythrovirus (formerly parvovirus) B19, a very resistant human pathogen, is found in Belgian donors with a prevalence of 1/10,000. A strategy for reducing potential B19 transmission by plasma derivatives is based on NAT screening of mini-pools of 576 donations, identifying the positive donations (more than 5000 IU B19/ml), and discarding them. Measuring B19 infectivity in general, and particularly the residual infectivity present after parvovirus screening, neutralization by plasma immunoglobulins, or inactivation, remains a major public health challenge.

Does quantification of virus DNA or transcription provide a real measure of erythrovirus B19 infectivity, in particular of virus found in plasma pools?
A virus can be considered infective when its genome is expressed, the virion is multiplied, and the progeny viruses are themselves infectious. Starting from a small number of virions, an infective virus can propagate in the infected host and

favor transmission from one individual to another. Detection methods should thus measure the amount of infectious B19 and reflect to what extent it is inactivated or neutralised in the presence of antibodies.
The use of methods based on quantification of mRNA species by RT-PCR in infected permissive or semi-permissive cells (such as KU812FEp6, UT7) can lead to overestimating viral infectivity, as no protein synthesis occurs and also no amplification of viral DNA and thus no progeny. We have measured the production of infectious viral progeny in different cell lines under different oxygenation conditions close to physiological conditions of infection. Starting with a low multiplicity of infection (MOI) comparable to that occurring at the start of a natural infection, we found a few international units (IU) of B19 to be sufficient to produce, within 48 hours, an amount of progeny reaching 107 IU/ml. This is accompanied by an increase in the number of annexin-positive cells, annexin being a marker of apoptosis. This virus production is greatly reduced by antibodies raised against the B19 receptor (anti-antigen P). The progeny remains infectious through several viral cycles on successive subcultures of fresh cells. This contrasts with UVC-inactivated viruses: although they still produce DNA, they do not yield infective virions. Our test is readily adaptable to routine use (it can be automated easily).

Minimal infectious dose and genome equivalents for B19 genotype 1 and for a B19 variant found in a plasma pool
To determine the minimal infectious dose, a plasma determined to be positive for B19 genotype 1 by nested-PCR (primers in NS gene) and negative for anti-B19 antibodies was sequentially diluted and tested on two cell culture systems: the erythroid cell line KU 812F under hypoxic conditions and hepatocarcinoma cells under normoxic conditions. The smallest infectious dose in both systems was found to be about 1 IU. A plasmid containing the gene encoding the nonstructural protein NS (plasmid Blubac-NS) was constructed and used to estimate that 1 infectious IU is equivalent to 100 genomes or 100 particles, a dose similar to that determined for canine parvovirus. This allows a more reliable risk assessment for B19. In contrast, a variant B19 recently detected in a plasma pool was shown to have reduced infectivity. Undetected by B19 Real-Time PCR (Roche) (primers in VP), this variant

was detected by our in-house nested-PCR method (primers in NS), capable of detecting B19 genotypes A6 and V9. As Parvoviruses have a high rate of nucleotide substitution (10-4 substitution/site/year), screening of plasma pools should be performed with a NAT technique using primers located in highly conserved regions such as the viral nonstructural protein NS.

Specific anti-B19 antibodies and B19 infectivity in B19-DNA-positive donors and risk of transmission by blood products

Seventeen donors with an initial B19 level higher than 105 IU/ml were monitored for 1 year. Every donor was interviewed for clinical symptoms. Samples were collected and screened for B19-DNA and specific IgM and IgG antibodies. Two different serological ELISAs were used to identify anti-B19 specific to linear and conformational capsid epitopes. An infectivity test based on an adherent hepatocarcinoma cell line was used in parallel to determine B19 infectivity. No good correlation was found between clinical symptoms and viral load. In about one-half of the donors, the B19-DNA load decreased rapidly by more than 5 logs during the first 12 weeks and then stabilized at a low background level for 1 year. In the other donors (where the initial B19-DNA load was lower), a slow 2-3-log reduction was measured. All donors actively developed anti-VP antibodies. Despite the presence of abundant anti-B19 IgG antibodies, B19 infectivity persisted in several cases.

Intravenous immunoglobulins and immunomodulation using UVC

Level of total and individual pneumococcal antibodies in intravenous immunoglobulin concentrates (IVIG)

Streptococcus pneumoniae is a leading cause of morbidity and mortality in both developed and undeveloped countries. Antibodies against capsular polysaccharides protect against disease, probably by inducing complement-mediated opsonophagocytic activity. Controlled studies indicate that the prophylactic use of IVIG is of benefit in patients with hypogammaglobulinemia. A simple, specific, and reproducible ELISA has been developed to quantify specific antibodies against 23 strain-specific capsular polysaccharides in the starting plasma pools and the

IVIG. This CE-marked ELISA is sold by Zentech, Belgium under the name 'ELIZEN'. According to ICH guidelines a complete validation program has been carried out. The test could be performed according to WHO guidelines after absorption with polysaccharide C or/and polysaccharide 22F. First studies using different-sized pools were performed to determine the best standard containing anti-pneumococcal serotype antibodies with no impact of when or where the donations are collected. The results showed that it is a plasma pool containing at least 5,000 donations. Results on 20 standards issued from different pool batches produced over 10 years showed a variation of 2.7%. To evaluate the validity of measuring antibodies against the 23 most prevalent serotypes, a study was undertaken. It involved setting up 13 independent ELISA tests with one coating serotype antigen. The results of ELIZEN and of the 13 independent ELISAs were highly reproducible for both starting plasma pools and IVIG concentrates. The more abundant antibodies were specific to serotypes 10A and 14 (the most frequently isolated clinical strains in Belgium), followed by 3, 19F, 8, 1, 12F, and 18C. Interestingly, their individual concentrations reach the neutralization level required to inhibit the growth of pathogenic strains in patients.

Improved efficacy of UV-irradiated lymphocytes by intravenous immunoglobulin
UV light has a marked capacity to modulate the immune response and has found many clinical applications (photochemotherapy 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 controling pathogenic mechanisms such as sepsis and inflammatory diseases.
We previously reported that UVC irradiation induces lymphocyte apoptosis and subsequent phagocytosis by monocytes/macrophages, a step that preferentially activates IL-1Ra. This adds a new pathway of IL-1Ra activation to those previously described, including those involving agents such as LPS, IVIG, or GM-CSF. Interestingly, intravenous immunoglobulins was found to enhance UV-induced IL1-Ra production by monocytes/macrophages, mostly when high doses were used.

These studies were performed in collaboration with the, Laboratoires de Virologie Moléculaireand d'Immunologie Expérimentale, Université Libre de Bruxelles Brussels, Belgium, the Institute for Transfusion Medicine and Immunohematology, German Red Cross, Johann Wolfgang Goethe University Frankfurt, Germany and the CNRS, Institut de Biologie de Lille, and Institut Pasteur Lille, France.

Patent granted

US Patent n° 6,866,848 B2 granted 15/3/2005 and entitled 'Antigenic polypeptide sequence of Factor VIII, fragments and/or epitopes there of'.

Key publication

Craciun LI, Di Giambattista M, Schandené L, RLaub R, Goldman M, Dupont E. Anti-inflammatory effects of UVC irradiated lymphocytes: induction of IL-1Ra upon phagocytosis by monocyte/macrophages. Clin Immunol 2005; 114(3):320-6.

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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 newly developed or authorized plasma products. A new product of human normal immunoglobulin for intravenous use was authorized in Finland and, via a Mutual Recognition Procedure in the Netherlands and other European countries under the name Nanogam®.
The Medical Department cooperates closely with clinical investigators in the Netherlands and abroad for development and performance of the trials, i.e. with the Dutch Inter-University Working Party on the Study of Immune Deficiencies and the Hemophilia Treatment Centers.

The Medical Department is 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 passively based on received reports, or actively by performing post authorization safety studies (PASS) in ad random patient groups. Periodic Safety Update Reports (PSUR's) are prepared to report the data on pharmacovigilance to the authorities. PSUR's have been prepared for Cealb®/Albumine5%/G.P.O. and Aafact® for a review period of 5 years, as part of re-registration requirements and for Nonafact® for a one year review period.
PSUR's have been prepared for Amofil and for Albumin SPR for a review period of 5 years, and clinical expert statements on Amofil and Albumin SPR, as requirements for re-registration. PSUR's for Nanogam® were prepared, also together with Sanquin Oy in Finland, for the second and third half-a-year review periods.

The Medical Department provides 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 2005

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. In 2005 the observation period was completed for most patients. The last patients out will be in December 2006.

Nanogam®

The clinical trial 'Kinetics, efficacy and safety of IVIG-L (human normal immunoglobulin for intravenous use) in patients with hypogammaglobulinemia' generated data on efficacy and safety over a longer period of time. The study was closed in October 2004 and the clinical study report KB97003B was finalized in 2005.

A multi-centre placebo-controlled cross-over clinical study, to demonstrate efficacy and safety of Nanogam® in patients with CIDP (chronic inflammatory demyelinating polyradiculoneuropathy), is in preparation. Also a multi-centre controlled, cross-over clinical study to compare the use of Nanogam with the use of antibiotics in order to prevent and treat recurrent (upper respiratory tract) infections in patients with IgG-subclass deficiency or a deficient anti-polysaccharide antibody response, in cooperation with the Dutch Inter University Working Party, 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' was completed 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 was 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 report of the study will be finalized in 2006.

MBL

A clinical trial with MBL (Mannan Binding Lectin, a product from Statens Serum Institut (SSI), Copenhagen, Denmark), entitled 'Phase II study on Mannan Binding Lectin (MBL) substitution in MBL-deficient children with chemotherapy-induced neutropenia', has been going. 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 Center in Rotterdam, has been added to the study. The study will be closed in April/ May 2006. The clinical study report is in preparation.

C1-esterase inhibitor

Since 1997, Sanquin has marketing authorization for Cetor®, a highly purified C1-esterase inhibitor concentrate, for use in the prophylaxis and acute treatment of hereditary and acquired 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 progress in collaboration with the Academic Medical Centre Amsterdam, Erasmus Medical Center Rotterdam, University Medical Center Groningen, University Medical Center St Radboud Nijmegen and Haga Hospital The Hague. This study will have to demonstrate (pharmacokinetic) equivalence between the current Cetor® product and nanofiltrated Cetor®. A study report on the pharmacokinetic part, part A, of the study is in preparation. Furthermore, it will have to be demonstrated that the efficacy of the nanofiltrated Cetor® in the prophylaxis and acute treatment of angioedema, part B and C of the study, is equivalent to the

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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 2005 consisted of ongoing projects in the fields of blood grouping and immunology reagents.

The following development projects were continued:

(I) latex-based assays to quantify human IgG subclass species in blood for the Immage (Beckman Coulter).
(II) assays to detect free, human immunoglobulin light chains (kappa, lambda) in serum and urine.
(III) an assay to detect and quantify anti-myelin antibodies in blood of MS (Multiple Sclerosis) patients.
(IV) a blood donor bloodgrouping chip based on DNA genotyping of red cel antigens.
(V) a fully automated intrument for Cellbind gelcard testing (Magister).
(VI) a new procedure using UV-cleavable peptides to be used with class I MHC tetramer molecules.
(VII) Elispot assays to determine the frequency of cytokine secretion cells.
(VIII) an instrument to automate the filling, closing, labeling and packaging of Cellbind cards.

Red blood cells for test panels

At the begin of 2005, Sanquin Reagents stopped the supply of human blood bags from the USA. Blood from selected Sanquin donors is used in stead.

Products and patents

New products
The following new products were commercially introduced in 2005:
(I) various Elispot reagents and peptide MHC class I tetramer reagents.
(II) Cellbind test ery's and Pelicontrol reagents.
(III) various white label reagents (as spin-off form Sanquin Research).

MACS research reagents
Medio 2005, Sanquin Reagents discontinued the distribution of MACS products in the Netherlands. The new subsidary Miltenyi Benelux took over the marketing and sales activities.

Patents
The European patent covering the Cellbind gelcard principle was published.
Ongoing patent applications: 1) the UV-exchange of peptides in MHC class I molecules and 2) specific DNA sequences that are use in the bloodgrouping chip.

Quality system
Two ISO certificates were renewed in 2005 (ISO 9001 & ISO 13485).
In addition, ISO 10002 has been implemented: this norm relates to improved complaint handling & customer satisfaction.

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Sanquin Diagnostic Services offers routine and specialized testing in the field of blood-related diseases and immune-mediated disorders. The blood sample testing is carried out in Amsterdam and is available for all Dutch Health Care Institutions and other interested companies. The division aims to work according to the highest quality standards in order to function as a diagnostic reference centre in the fields mentioned above, in national as well as in international settings. With its fully certified laboratories, Sanquin Diagnostic Services can provide a vast array of both routine and tailor-made diagnostic tests. Sanquin Diagnostic Services is committed to continuously develop and introduce new diagnostic tests. New tests are often developed and validated in house, in R&D projects, most of which are carried out in close cooperation with Sanquin Research.

Developments in 2005

In 2005, a large number of new tests and services has been introduced:

- large-scale human platelet antigen (HPA) genotyping;
- IgG subclass determination in Neonatal Alloimmune Thrombocytopenic Purpura (NAITP);
- Automated Cyclic Citrullinated Peptide (CCP) determination;
- Antibodies against Soluble Liver Antigen/Liver Pancreas antigen SLA/LP;
- Serum levels of monoclonal therapeutics (Infliximab, Adalimumab and Enbrel);
- Serum levels of antibodies to therapeutics (Infliximab, Adalimumab and Enbrel);
- Multiplex Ligation-dependent Probe Amplification (MPLA) technology related to alpha-thalassemia;
- Functional determination of ADAMTS13 and autoantibodies against ADAMTS13;
- Development of an assay to detect the Marburg mutation in factor-VII-activating protease (FSAP);
- Semi-automated DNA isolation for HLA typing;
- HLA 'high-resolution' Sequenced Based Typing (SBT) for allogeneic bone marrow transplantation.

In close collaboration with all involved departments, a specialized committee of the Sanquin Diagnostic Services Division, dedicated to innovation of diagnostic services

(called DC-I), has been active in 2005 in the following fields:
- Use of array technologies for diagnostic purposes;
- Complement research, focused on C1-esterase inhibitor, MBL and C4 pheno- and genotyping;
- Extension of assays available for myositis;
- Development of a single nucleotide polymorphism (SNP) platform.

Contacts with external partners on possible collaboration in the field of high-throughput SNP identification and Cell-track systems have been initialized.
In the following paragraphs, the above-mentioned developments will be described in more detail, ordered according to department.

Immunocytology

Sanquin Diagnostic Services is involved in a study on Acute Lymphocytic Leukemia (ALL10) in which children with ALL are treated on the basis of minimal residual disease (MRD) values at the end of induction therapy. In high-risk patients, therapy is intensified, in low-risk patients, it is diminished. To use MRD values for this purpose, the assay has to fulfil very strict criteria on specificity. These criteria have been formulated in co-operation with the Dept of Immunology, Erasmus University Medical Centre, Rotterdam (prof JJM van Dongen) and the 'European Study Group on MRD detection', in which Sanquin Diagnostic Services actively participates.
In co-operation with the Leiden University Medical Center (LUMC), a flow cytometric Endothelial Progenitor Cell Assay has been developed. This method is currently used in the HEBE-trial, a clinical trial in which autologous mononuclear (blood/bone marrow) cells are infused into coronary arteries shortly after a myocardial infarction, to promote revascularization.
A JAK2 mutation occurs in 97% of Polycythemia Vera patients and in 20–50% of other myeloproliferative syndromes. Sanquin Diagnostic Services is among the first to have developed a diagnostic test for this mutation.
For diagnostic purposes, a new flow cytometer (FACS-Canto) has been introduced and validated. Furthermore, 4-colour immunofluorescence is now used for all assays.

Immunohematology

Methods for prenatal blood group testing and Rhesus phenotyping in foetal DNA obtained from maternal plasma, developed by Sanquin Diagnostic Services, are now fully validated and operational. National introduction now depends on approval by the College Voor Zorgverzekeringen (CVZ) and inclusion of the methods in the antenatal anti-Rh(D) prophylaxis program.
Improvements were realised in several diagnostic methods: the Kleihauer test for detection of cells with fetal hemoglobin has been optimised by FACS analysis, and methods for quantification of antibody titres and IgG subclass determination as part of the diagnostic procedures around Neonatal Alloimmune Thrombocytopenia have been developed.

Autoimmune Diseases

During 2005, a number of diagnostic tests has been renewed:
For the detection of antibodies to TSH Receptor the (human) TRAK assay has been introduced and validated. ANCA assays have been optimised by introduction of pure myeloperoxidase (MPO) (Calbiochem) as antigen, while at the same time ANCA slides from INOVA and Euroimmun have been evaluated. For detection of antibodies to Extractable Nuclear Antigens (ENA), the UniCAP 100 and Lineblot ANA Profile 3 have been evaluated. To improve myositis diagnostics, the Euroimmun myositis blot has been evaluated. In autoimmune hepatitis, confirmation assays for antibodies to mitochondria and liver and kidney microsomes (LKM) have been added to the already available assays and detection of antibodies to SLA/LP has been introduced. Furthermore, for the detection of antibodies to Cyclic Citrullinated Peptide (CCP) in rheumatoid arthritis, automated detection was introduced to replace the classical ELISA.

Immunochemistry

Assays for monoclonal therapeutics (such as Infliximab and Adalumumab) as well as assays for antibodies to these therapeutics were developed, validated and introduced for routine diagnostic purposes. Assays for other biologicals, amongst which Enbrel, are under development. Contacts with pharmaceutical companies in the field of

biologicals that are currently in medical trials may lead to further exploration of this area. The field of registered therapeutic monoclonal antibodies is growing rapidly, and quite a number of monoclonals are under development for future clinical use. Other assays, amongst which determination of CH50, AP50 and MBL, were automated during 2005.
Complement research was stimulated by the appointment of a post doc in this field.

Blood cell chemistry
For the characterization of alpha thalassemias a multiplex PCR method was developed to serve as an alternative for the currently used RFLP technique.
Screening for defects in granulocyte function has been improved:
- putative defects in the granulocyte oxidase system are searched for with a broader panel of cell stimuli;
- the chemotactic activity of granulocytes is now determined with more chemo-attractants;
- a screening test to determine the adherence of granulocytes to surfaces induced by various stimuli has been developed and validated.

Blood Coagulation
To assist in the rapid diagnosis of thrombotic thrombocytopenic purpura (TTP), functional tests of ADAMTS13, the enzyme that is deficient in this syndrome, have been evaluated and implemented. In addition, assays of autoantibodies directed against ADAMTS13 were evaluated and introduced. The implementation of these assays has been accomplished in close collaboration with the national working party on TTP.

Infectious Diseases (blood donor screening)/Viral Diagnostics
Automated screening systems for nucleic acid detection (NAT) of hepatitis B and C virus (HBV, HCV) and human immune deficiency virus (HIV) were studied to determine improvement of prevention of transmission of these viruses by blood components and plasma derivatives. Special attention was paid to HBV transmission, genetic variation of HBV and HCV among blood donors in relation to detection

and level of mechanization of test systems. It was concluded that implementation of NAT for HBV will only be effective when performed in small test pools (<8 donations or individuals). This can only be realised with a high level of automation. It was also found that the distribution of genetic variants for HBV and HCV among blood donors was related to risk factors like intravenous drug abuse, being born in countries in which HBV or HCV are endemic or having received a blood transfusion in the past.

It was necessary to improve the screening system for Parvovirus B19 to meet the safety requirements for plasma derivatives. Recently, new molecular variants of the virus were published. The screening system used until now did not recognize two molecular variants. Therefore the nucleic acid detection of Parvovirus B19 was improved to detect these new variants. Analyses of plasma for fractionation, equivalent to more than 2 million donations, revealed that levels of these two variants that would render plasma unsuitable for manufacturing were not found. The improved test was introduced for screening of blood donations intended for manufacturing plasma.

A study on West Nile Virus (WNV) infections among blood donors in The Netherlands was finalised. No evidence of WNV infection among donors was found.

Screening for HCV and HIV by NAT has been validated and introduced for post-mortem and living tissue donors in co-operation with Bio Implant Services (Leiden).

Furthermore, for plasma pool screening by Sanquin Blood Banks, a new computer system (PMS = Pool screening Management System) has been developed and will be introduced in the beginning of 2006 in the Sanquin screening laboratories.

Currently, research projects regarding anti-parvo antibodies, bacterial screening with NAT and malaria detection are being carried out. At the moment, the Parvovirus is considered absent in a person if this person has anti-parvo antibodies in two consecutive tests (with a period of 6 months in between). However, it has been shown that one can have antibodies and still be a carrier of parvovirus. This will be studied in more detail in 2006.

Currently, there is a period of quarantine for donors from malaria-infected regions. As more donors travel to malaria-infected regions for holidays, this is gradually becoming a serious problem.

HLA diagnostics

In collaboration with the Dept of Immunohematology, a semi-automatic DNA isolation technique (Magna-pure) has been introduced and successfully validated. The use of Luminex technology for HLA antibody screening is currently investigated. Validation of this technology will take place in 2006.

New platforms

An evaluation was carried out regarding putatively interesting new platforms for diagnostic services. As a tentative conclusion, the Illumina bead array technology will be studied in more detail.
MLPA (= multiplex ligation-dependent probe amplification) seems to be a very good method for quantification of gene copy numbers. This approach will be tested for complement C4 genotyping.

TRIX

In close collaboration with clinical chemists from The Netherlands, a computer-based register for irregular erythrocyte antibodies has been developed by Sanquin Diagnostic Services. This register (Tranfusion Register on Irregular antibodies and X (cross) test problems) with be installed on a nation-wide basis, to help prevent transfusion reactions on the basis of available data on previously detected IEA in recipients of donor blood. This will apply both to IEA that have become undetectable because of lowered titres and to IEA detected in another hospital.

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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. Other QC services such as protein characterization, stability test programs, formulation studies and process validation (for demonstrating 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

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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. As part of a blood-product producing organization VSS is intimately familiar with blood safety issues.

Virus validation studies

We have a broad experience in validation of various process steps, including the more delicate ones, such as 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). An Endorsement of Compliance with the OECD principles of GLP based on assessments performed according to the Netherlands GLP Compliance Monitoring Programme and according to Directive 2004/9/EC was granted in 2005. 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 the product under investigation is achieved.

Virus systems available

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

- 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 diarrhoea 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
- TGEV (Transmissible gastroenteritis virus), a specific model virus for SARS (severe acute respiratory syndrome)
- VSV (Vesicular stomatitis virus), a general model for lipid enveloped RNA viruses

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Sanquin Consulting Services of the Sanquin Blood Supply Foundation provides guidance and advice services to restricted economy countries.
Objectives are (i) to support restricted economy countries in developing safe, efficacious and sustainable blood supply systems based on current quality principles, (ii) to provide modular training programs on transfusion medicine for restricted economy countries focused on the managerial and quality aspects of the transfusion chain, and (iii) to extend and strengthen the training and consultative potential within the Sanquin organization.

Organization and structure

Sanquin Consulting Services is part of the corporate staff of Sanquin. The office is located in Groningen. International projects are executed in countries with developing economies involve in the fieldwork (advice, training and consultations). Consultants who participate in the projects are recruited from Sanquin divisions. About 40 Sanquin employees are registered, of whom so far 10 have been involved in active expert consultation activities abroad.
The expertise is focused on managerial aspects, guidance and advice to create and implement national organizational structures and comprehensive quality systems.

At the end of 2004 a substantial five year project in Africa has started in four countries. It is expected that this project will be a main part of the activities of SCS during the years to come, although the acquisition of new projects remains a priority for the long term sustainability of the activities.

To improve the quality of the projects SCS co-operates with external organizations as well as with Sanquin divisions.
During the last years SCS started to build up an office oriented quality system and management based on ISO 9001/2000 principles. An ISO certificate was awarded by the end of 2004.

Public relations and acquisition

Visibility both within the organization as well as internationally, is a necessary

requisite for acquisition that will safeguard the sustainability for the future:
Visibility started with a web page at the Sanquin website and the publication and of an annual report. Recently a communication plan was prepared by a group of management development trainees of Sanquin that will lead to improvement of the visibility. Elements of focus are improvement of access on the website, brochure and public relations strategy.

In an international environment SCS co-operates with a number of external organizations. In 2005 the following co-operations existed:

Sanquin Bloodbank is collaborating Centre for WHO for training and quality courses. The managing director of SCS is contact person for WHO.

Further an agreement with AABB to share the leadership of Sanquin Consulting Services (SCS) and Consulting Services Division (CSD) was continued and a new draft Memorandum of Understanding was proposed.

The Academic institute for International Development of Transfusion Medicine (IDTM) is the third party we co-operate with. This institute develops a postgraduate Master curriculum and course for Management of Transfusion Medicine. The course is modular and includes a 12 months e-learning part (9 modules) allowing fellows to study in their own environment combined with a 6 months (4 modules) tutorial and practical part to be completed in the Netherlands. The graduation is based on a thesis project. Finally a Masters degree from the University of Groningen Faculty of Medical Sciences will formally be provided.

Activities and projects
Sanquin Consulting Services, the WHO Collaborating Centre and the Academic Institute IDTM have been involved in a variety of activities and projects:
For more details, please contact us for the annual report of SCS and IDTM.

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, private 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
Adenbrooks Hospital
American Red Cross
Amcell Corporation
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 Corporation
Chiron corporation
Crucell
Diaclone
DSM Biologics
Finnish Red Cross
Fresenius HemoCare
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Genmab
GlaxoSmithKline
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HAL/Madaus
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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 s.a.
Zentral Laborator Bern

Other sources of funding
Ministry of Economic Affairs (WBSO)

Publications

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

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PhD theses

Erwan Piriou

31 March 2005

Viro-immunology of EBV-HIV co-infection

University of Amsterdam

Promotores: Prof F Miedema and Prof MHJ van Oers

Corine Bronke

24 March 2005

Cytomegalovirus-specific T-cell dynamics in HIV infection

University of Amsterdam

Promotor: Prof F Miedema

Atoosa Familian

15 April 2005

Studies on pentraxin mediated complement activation

Free University Amsterdam

Promotores: Prof CE Hack and Prof BAC Dijkmans

Christine Jansen

13 May 2005

Analysis of HIV-specific T-cell immunity in AIDS-pathogenesis

University of Utrecht

Promotor: Prof F Miedema

Edwin van Mirre

20 October 2005

Novel insights in Human Fcγ-receptors and IgG: on genes, transcripts and functional interactions

University of Amsterdam

Promotores: Prof CE Hack and Prof TW Kuijpers

Paula da Costa Martins

26 October 2005

Biology of monocyte interactions with the endothelium - the platelet factor -

University of Amsterdam

Promotor: Prof D Roos

Caroline Fribourg

28 October 2005

Catalytic function of coagulation factor IX

University of Utrecht

Promotor: Prof K Mertens

Jaap Akkerdaas

2 November 2005

Identification and Quantification of Hazelnut Allergens

University of Amsterdam

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Index on key words

Colophon

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