Sanquin

08

Scientific Report

Blood and Beyond

Scientific Report 2008

Contents

Words of welcome

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In our 2008 scientific report you will find information on our organization and our policies towards research. An overview of all our research is described, with some key publications for further reference.

In 2007 we introduced the system of Principal Investigator (PI), in which a senior scientist is responsible for her or his research group with respect to personnel and funding. To become a PI, the experienced researcher must have proven that she or he is able to obtain funding, not only for her/himself, but also for a number of PhD students and technicians. Furthermore, there must be a proven record of independently authored scientific publications. A research department may harbor one or more PIs. In this 2008 report we have listed all PIs in alphabetical order with their staff, students and contact information.

We are pleased to continue the long-standing collaboration with the Academic Medical Center of the University of Amsterdam in the joint Sanquin – AMC Landsteiner Laboratory for Transfusion Medicine. A new long-term agreement was signed in December 2007, expanding the joint research program with epidemiology of blood transmitted infections, and hemostasis and thrombosis, while continuing research in the fields of immunology and hematology as well as strengthening translational research. In 2008 medical microbiologist Hans Zaaijer MD PhD joined Sanquin from the Academic Medical Center as head of the Department of Blood-borne Infections within the Landsteiner Laboratory.

In 2008 the research programs at our Blood Bank research departments were further strengthened. The department of Research and Development of Blood Bank South East Region concentrates on donor-related studies, with expertise from epidemiology and the social sciences, in order to have our donor policies scientifically based. Blood Bank South West Region concentrates on clinical studies in close collaboration with academic hospitals, especially Leiden University Medical Center, as well as the larger general hospitals in the Netherlands. Blood Bank North West Region is dedicated towards transfusion technology in close collaboration with Sanquin Research. In the Blood Bank North East Region the focus is on the post

authorization surveillance of blood components, in collaboration with the Groningen University Medical Center and regional hospitals in the North of the Netherlands.

Our collaboration with Utrecht University on coagulation and on transfusion technology assessment was continued, as was the collaboration with Leiden University Medical Center with respect to clinical research. Talks with the LUMC were started on the establishment of a joint center for clinical transfusion medicine.

In November 2008 the second internal Sanquin Research Day was organized. A number of senior staff members presented their research interest, and over 60 posters of the younger generation of researchers were presented and discussed. Three PhD award nominees gave lectures on their research projects. Bram van Raam won this year's PhD award. Runners up were Marloes Tijssen and Ruben Bierings.

Early 2008, a number of PhD students and post docs took the initiative for a quarterly meeting with a lecture and drinks for PhD students and post docs of all Sanquin locations. Subjects range from leadership to patents and to defending a thesis. This initiative is a welcome addition to our attempts to create a true coherent national research program, as young researchers from various departments and locations have the opportunity to meet informally.

Prof Lucien Aarden held the prestigious Van Loghem lecture at this year's Dutch Society for Immunology annual meeting, entitled 'Why do SLE patients make anti-DNA antibodies?'

A new state-of-the-art confocal microscope was added to the central equipment facility. Erik Mul, head of this facility, developed a microclimate cover for this microscope, for which he received the Dutch Creativity Award 2008. The supplier is interested in taking the cover into production, and the Dutch Cancer Institute already uses it.

Introduction

Introduction

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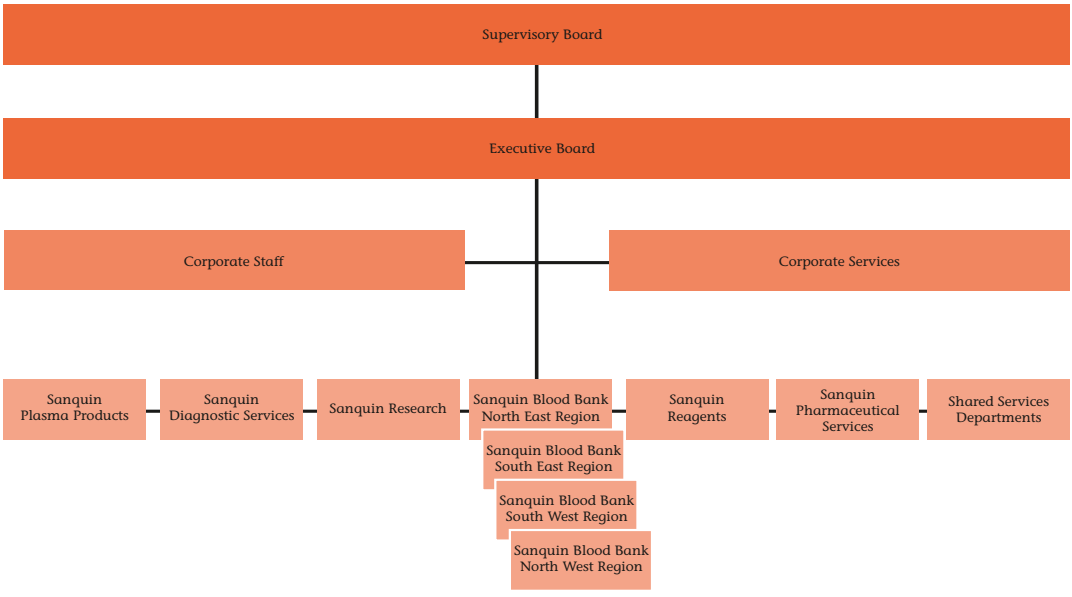
Sanquin Blood Supply Foundation

Sanquin Blood Supply Foundation comprises seven divisions and two business units. A three member Executive Board is responsible for the organization and reports to the Supervisory Board. A corporate staff office and a number of Corporate Services support the organization. All four Blood Bank divisions have their own department of Research and Education. At the Amsterdam premises we find Sanquin Plasma Products, Sanquin Diagnostic Services, Sanquin Research, and the business units Sanquin Reagents and Sanquin Pharmaceutical Services.



Principal investigators

As already mentioned above, Sanquin introduced the system of Principal investigator (PI). You will find more information on the following PI research groups:

Principal investigator	Department
Prof Rob Aalberse PhD	*Immunopathology, Sanquin Research*
Prof Lucien Aarden PhD	*Immunopathology, Sanquin Research*
Prof Anneke Brand MD PhD	*Research & Development, Sanquin Blood Bank South West Region*
Wim de Kort MD PhD	*Research & Development, Sanquin Blood Bank South East Region*
Dirk de Korte PhD	*Research & Development, Sanquin Blood Bank North West Region, and Blood Cell Research, Sanquin Research*
Janny de Wildt-Eggen PhD	*Research & Development, Sanquin Blood Bank North East Region*
Peter Hordijk PhD	*Molecular Cell Biology, Sanquin Research*
Prof Koen Mertens PhD	*Plasma Proteins, Sanquin Research*
Timo van den Berg PhD	*Blood Cell Research, Sanquin Research*
Cees van der Poel MD PhD	*Transfusion Technology Assessment, Sanquin Research and Julius Center, Utrecht University*
Prof C Ellen van der Schoot MD PhD	*Experimental Immunohematology, Sanquin Research*
Prof Hanneke Schuitemaker PhD	*Laboratory of Viral Immune Pathogenesis, Academic Medical Center, University of Amsterdam*
S Marieke van Ham PhD	*Immunopathology, Sanquin Research*
Prof Dick van Rhenen MD PhD	*Research & Development, Sanquin Blood Bank South West Region*
Arthur Verhoeven PhD	*Blood Cell Research, Sanquin Research*
Jan Voorberg PhD	*Plasma Proteins, Sanquin Research*
Hans Zaaijer MD PhD	*Blood-borne Infections, Sanquin Research*

Research themes	Principal Investigators														
	Aalberse	*Aarden*	*Brand / Van Rhenen*	*De Kort*	*De Korte*	*De Wildt*	*Hordijk*	*Mertens*	*Schuitemaker*	*Van den Berg*	*Van der Poel*	*Van der Schoot*	*Van Ham*	*Verhoeven*	*Voorberg*	*Zaaijer*
hematology			X				X			X		X				
hemostasis/thrombosis								X							X	
immunology	X	X	X										X			
blood-borne infections									X		X					X
quality, safety, effciency					X	X						X		X		
new therapies and evalution of clinical applications			X	X								X				
donor studies				X												

Research Programming Committee

The Research Programming Committee advises the Executive Board on strategic issues and on selection of projects funded from Sanquin's own resources. A yearly call for proposals on product and process development is issued, and projects are reviewed by international referees before selection to guarantee the quality of the research proposals. In 2008 further actions were continued to improve the quality of research proposals to be submitted to external funding agencies and charities by internal review meetings and procedures.

The Research Programming Committee consisted of five members representing four product/market combinations: Blood Banks (prof DJ van Rhenen MD PhD, HJC de Wit PharmD), Plasma Products (PFW Strengers MD), Diagnostic Services (R Baumgarten MD PhD) and Research (prof E Briët MD PhD), supported by an executive secretary (JW Smeenk MSc).

Scientific Advisory Board

The Scientific Advisory Board supervises the research quality system, advises the Sanquin Executive Board on all matters concerning strategy, (co-ordination of) research and research infrastructure, and checks annually whether Sanquin's research

program meets the framework of the policy plans. Furthermore, the Scientific Advisory Board assesses the quality of Sanquin's research, based on bibliometric analyses and reports of site visits.

On 31 December 2008 the Scientic Advisory Board 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)

Prof AF Cohen MD PhD (Center for Human Drug Research & Leiden University)

Prof RRP de Vries MD PhD (Leiden University)

Prof DE Grobbee MD PhD (Utrecht University)

Prof MM Levi MD PhD (University of Amsterdam)

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

JW Smeenk MSc, Executive secretary (Sanquin Corporate Staff)

Research Assessment

All research groups of Sanquin are visited by an international peer review committee once every five years. With the introduction of the Principal Investigator, it was decided to organize the site visit system based on research groups of the PIs.
In 2008, the Research group of Ellen van der Schoot was reviewed, as were the groups of Anneke Brand and Dick van Rhenen. The preliminary findings of both peer review committees were very positive on the quality of research. A number of recommendations on organizational issues and PhD training were given and are being taken into account in 2009. As in earlier years, the Peer Review Commitees were supported by an executive secretary from the independent agency Quality Assurance Netherlands Universities (QANU).

Academic affiliations

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 mainly housed within Sanquin premises.
At many Dutch universities, members of the 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 for laboratory technicians. Of course, Sanquin is also involved in training of specialists in blood transfusion medicine, other medical specialties, and training of nurses.
Sanquin has established a recognized training program for medical doctors specialising in transfusion medicine and donor care.
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. With the University of Groningen Medical Center, Sanquin Blood Bank North East Region runs a postgraduate masters program, under the heading of the Academic Institute for International Development of Transfusion Medicine (IDTM). Sanquin is a WHO Collaborating Organization for Transfusion Medicine.

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

Prof C Ellen van der Schoot MD PhD (Experimental Immunohematology, 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)

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. Researchers of Sanquin contribute to the research programs of the Center for Immunology Amsterdam (CIA) and the Center for Infection and Immunity Amsterdam (CINIMA).

Accreditation and quality assurance

Code of conduct

In 2006 the Sanquin Executive Board decided on a research code of conduct, that is based on various codes of conduct from Dutch Universities and the Royal Netherlands Academy of Arts and Sciences. Sanquin was awarded membership of LOWI – the national organization for scientific integrity – that acts as independent advisory body in case of a breach of scientific integrity by a Sanquin member of staff. An independent Ombudsman was already appointed in 2006.

Accreditation

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In 2008 a study focusing on quality of data management has taken place. The results of this study were presented to the board of Sanquin and to the management team of Sanquin Research.

The departments of Virus Safety Services, Clinical Monitoring and Blood Transfusion Technology were visited by the Dutch Accreditation Council (RvA) and the CCKL in April 2008. They extended their accreditation according to ISO 17025 and certification according to the CCKL 'Code of practice version four' with four years.

The laboratory for Stem Cell Transplantation held its certification to ISO 9001 and ISO 13485 as it was successfully visited by the Lloyds auditor. An audit team of JACIE (Joint Accreditation Committee ISCT & EBMT) and CCKL also inspected the laboratory for Stem Cell Transplantation and granted a certificate to the Standards for Haematopoietic Progenitor Cell Collection, Processing & Transplantation and the CCKL 'Code of practice version four'.

In December 2008 the Lloyds auditor granted an ISO 9001:2008 certificate to the department for Cryobiology.

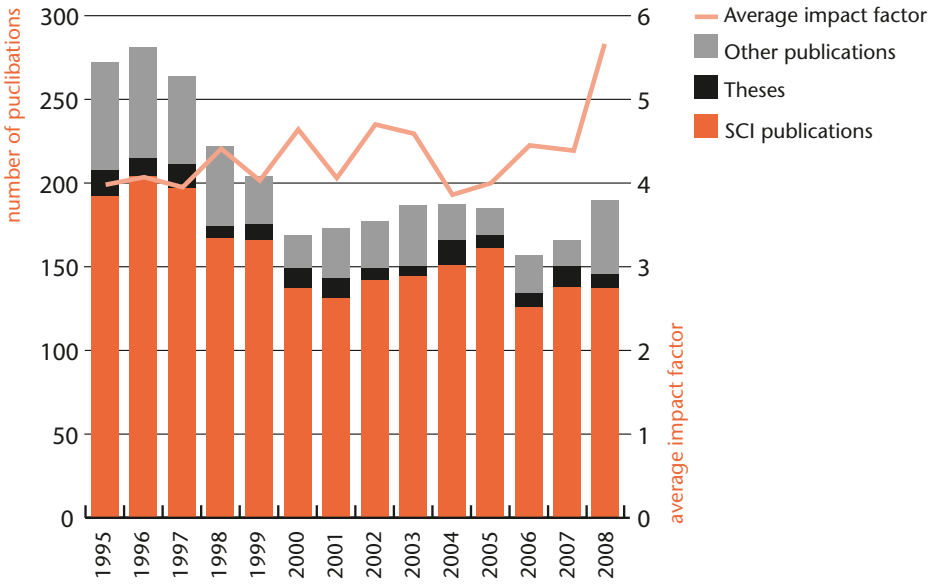
Publications

The number of papers in peer reviewed journals is similar to previous years. The average impact factor is higher, 5.6. The number of citations in the five years after publishing (2003) was 3018 for 136 papers, an average of 22.2 citations per paper.

Scientific publications

Year	*Total number*	*SCI publications*	*Theses*	*Average impact factor*
1993	226	179	12	
1994	230	185	11	
1995	272	192	16	4.0
1996	281	204	11	4.1
1997	264	197	14	4.0
1998	222	167	7	4.4
1999	204	166	9	4.0
2000	169	137	12	4.7
2001	173	131	12	4.1
2002	177	142	7	4.7
2003	187	144	6	4.6
2004	187	151	15	3.9
2005	185	161	8	4.0
2006	157	126	8	4.5
2007	166	138	12	4.4
2008	185	137	8	5.6

Scientific publications and average impact factor



Articles* published in 1995 through 2003 annual reports cited** in five full years after publication

Publications from year	*Total citations*	*Number of SCI publications*	*Average number of citations per publication*
1995	3215	192	16.7
1996	3057	204	15.0
1997	2962	197	15.0
1998	3448	167	20.6
1999	2910	166	17.5
2000	2699	137	19.7
2001	2220	131	16.9
2002	3042	142	21.4
2003	3018	136	22.2

* Only SCI publications are included

** Excluding self citations

Articles* published in 1994 through 2003 annual reports cited** in five full years after publication

Publications from year	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008
1994	871	686	552									
1995	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			
2001						349	510	513	434	414		
2002							498	667	735	594	548	
2003								447	693	631	584	663

* Only SCI publications are included

** Excluding self citations

Funding

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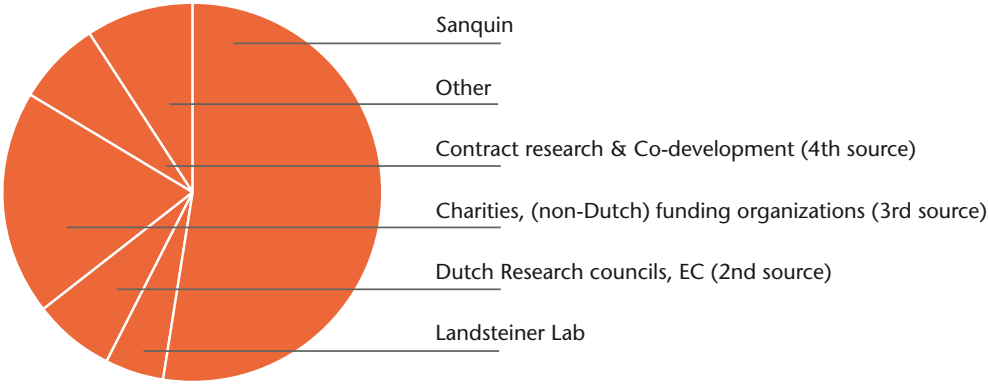
In 2008 Sanquin researchers were again successful in obtaining external funding (see page 154 for an overview of our sponsors). As European Research funds become more and more important, Sanquin invests in external consulting services to assist our staff in forming consortia and writing proposals for the seventh Framework Program, and to assist in administrative and organizational matters.
After a review on quality by external experts and relevance to Sanquin's mission by the Research Programming Committee, twelve research projects were funded from Sanquin resources for product and process development for cellular products. Unfortunately over ten good proposals could not be funded, due to lack of resources. The available funds for product and process development within the organization are expected to grow slightly in the years to come.

Funding Research projects 2008 (direct costst only)



Valorization

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Sanquin Research is both within and outside our own organization a sought-for partner in co-development and contract research activities, be it in the area of therapeutic or diagnostic product development, testing of devices or process innovation. Our first focus with regards to valorization of our know-how are the stakeholders of Plasma Products, Diagnostic Services and the business units of Reagents and Pharmaceutical Services. Biotech, pharmaceutical and diagnostic companies also know how to value Sanquin's in-depth and long existing expertise in the areas of bloodtransfusion, immunology, blood coagulation, hematology, hemostasis & thrombosis, and the translational mindset of our researchers. Income generated from cooperation with companies and from out-licensing of patents/ hybridoma's generate additional funding for research. On page 154 you will find an overview of commercial parties with whom Sanquin Research collaborated through the years. On page 153 an overview of out-licensed and available hybridoma's and published patents is shown.

The Life Sciences Centre Amsterdam, comprising of all the Technology Transfer Offices of the knowledge centers of Amsterdam, i.e. Sanquin, Academic Medical Center, University of Amsterdam, Vrije Universiteit Medical Center, Free University, Dutch Cancer Research Institute (NKI) and Swammerdam Institute for Life Sciences, University of Amsterdam, is established to strengthen their valorization efforts. Combining their expertise with respect to best-practices in business development, jointly participating in meetings like the BIO USA and BIO EU, and organizing the so called 'Industry Days', enabling scouts to get in contact with our researchers and get an overview of our facilities, bio-banks and patent portfolio's, puts Amsterdam's assets in the spot light.

Research groups

Prof Rob C Aalberse PhD

Immunochemistry

Post docs

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TH den Bleker

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Immunoglobulins

Structural and functional properties of human IgG4

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Human IgG4 has been found to exchange half-molecules with other IgG4 molecules 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, but is observed both *in vivo* (in a mouse model) and *in vitro* (in the presence of glutathione as catalyst). Key structural features were identified using a panel of IgG4/IgG1 mutants in collaboration with Genmab.

Another peculiar property of IgG4 is its reported ability to bind to other IgG molecules via Fc interactions. These interactions were shown to be related to the exchange reaction and revealed structural aspects of IgG4 underlying both phenomena, in particular the involvement of the CH3 domains. IgG4 binds to all human IgG subclasses if directly immobilized. Binding to IgG4, but not IgG1, is observed in case IgG4 is coupled to a solid phase via antigen binding. These Fc interactions may enhance antigen binding *in vivo*.

IgG4 stands out from other human IgG subclasses not only in structural features, but also in its appearance during a Th2-driven immune response. Whereas IgG1 antibodies are readily formed upon antigenic challenge, IgG4 antibody titers rise only slowly upon persistent antigenic stimulation. However, the IgG4 response dominates in the end. The underlying mechanisms that control switch to and proliferation of IgG4-producing B cells are only partially understood. We initiated research aimed at unravelling these mechanisms. We have been able to quantify IgG4-positive B cells in blood using FACS analysis and purify IgG4 B cells with MACS using the subclass-specific antibodies produced by Sanquin. In line with the relatively low serum levels of IgG4 (3-4% of total IgG), the number of IgG4 positive B cells is correspondingly low. Tools are currently being developed to investigate features that might explain why the immune regulation of IgG4 differs from other isotypes. 1) developmental stages of the IgG4 B cell; 2) how and when the isotype switch is induced; and 3) at which stage the cell becomes an IgG-secreting cell.

Key publications

Rispens T, Ooievaar-De Heer P, Vermeulen E, Schuurman J, van der Neut Kolfschoten M, Aalberse RC. Human IgG4 binds to IgG4 and conformationally altered IgG1 via Fc-Fc interactions. J Immunol 2009; 182:4275.

Aalberse RC, Stapel SO, Schuurman J, Rispens T. Immunoglobulin G4: an odd antibody. Clin Exp Allergy 2009; 39:469.

Van der Neut Kolfschoten M, Schuurman J, Losen M, Bleeker WK, Martínez-Martínez P, Vermeulen E, den Bleker TH, Wiegman L, Vink T, Aarden LA, De Baets MH, van de Winkel JG, Aalberse RC, Parren PW. Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. Science 2007; 317:1554.

Pro- and anti-inflammatory fractions in intravenous immunoglobulin (IVIg)

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 purpura (ITP), Kawasaki syndrome and Guillain-Barre.

In applications other than replacement therapy, the mechanisms of action are largely uncertain. Possibilities are a.o. effects due to scavenging of complement activation products, blockade of Fc receptors, effects of IgG dimers and effects of specific antibodies (for example: cytokine neutralization).

The stability of the IgG dimers present in IVIg under different physical conditions was investigated by size-exclusion chromatography and sodium dodecyl sulfate (SDS) electrophoresis. Most dimers dissociate rapidly under conditions mimicking those in patients after administering IVIg. Of the remaining dimers, one type will dissociate upon SDS denaturation and comprise dimers in dynamic equilibrium with monomers as well as dimers that are stable upon dilution. Another type is SDS-resistant. This fraction was quantified using semi-quantitative SDS-PAGE to be 0.2%.

Treatment of conditions such as ITP require high doses of IVIG. It is reported that only the fraction of IgG molecules containing sialic acid is responsible for its anti-inflammatory action. These findings are based mainly on an arthritis mouse model. In cooperation with the Sanquin division of Plasma products, IVIG was enriched for sialic acid (SA). The SA-enriched and -depleted IVIG is currently being tested in a mouse model for ITP.

Monomeric precursors for aggregation of IgG are difficult to detect. Usually, hydrophobic fluorescent probes such as 1-anilino-8-naphthalenesulfonate are used that may detect exposed hydrophobic surfaces as a result of partial unfolding. We extented this approach by detecting binding of such probes using isothermal titration calorimetry. In addition to fluorescent probes, non-fluorescent probes, including peptides, can be used to probe native or non-native configurations.

Key publication

Rispens T, Lakemond CMM, Derksen NIL, Aalberse RC. Detection of conformational changes in immunoglobulin G using isothermal titration calorimetry with low-molecular-weight probes. Anal Biochem 2008; 380:303.

Prof Lucien A Aarden PhD

Autoimmune Diseases

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GJ Wolbink MD PhD

D Wouters PhD

SS Zeerleder MD PhD

PhD students

W Bos

F Stephan

P van Schouwenburg

Technical staff

MC Brouwer

I Bulder

ER de Groot

MHL Hart

JM Klaasse Bos

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Inflammation

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The Inflammation Research group focuses on complement activation and on preclinical, *in vitro* testing of new drugs.

Complement activation

To study the C4 polymorphism in various diseases we developed novel assays both on genetic and protein level. We have set up an MLPA assay to determine gene copy numbers of the C4A and C4B isotypes as well as ELISAs to measure protein levels and functional assays for protein activity. After applying these assays in healthy volunteers, we are now studying a large SLE patient cohort. Furthermore we analyze the properties of anti-C1q antibodies in SLE sera and we are investigating the properties of alternative pathway activation and regulation on red blood cells.

C1 Inhibitor

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In 2008 the work on C1-Inh from PI Marieke van Ham was integrated in the Research on Inflammation. We investigated possible new fields of clinical application for C1-Inh in close collaboration with Sanquin Plasma Products. In addition, in collaboration with Roel Bennink MD PhD (Dept Nuclear Medicine, AMC) we demonstrated that the clearance of radiolabeled recombinant C1-Inh (produced in yeast) from the circulation is regulated by the liver and is much faster than that of plasma-purified C1-Inh. Thus, plasma-derived C1-Inh is the product of choice for treatment with C1-Inh.

Key publications

Wouters D, van Schouwenburg P, van der Horst A, de Boer M, Schooneman D, Kuijpers TW, Aarden LA, Hamann D. High-throughput analysis of the C4 polymorphism by a combination of MLPA and isotype-specific ELISA's. Mol Immunol 2009; 46:592-600.

Wouters D, Brouwer MC, Daha MR, Hack CE. Studies on the haemolytic activity of circulating C1q-C3/C4 complexes. Mol Immunol 2008; 45:1893-9.

Wouters D, Wagenaar-Bos I, van Ham M, Zeerleder S. C1 inhibitor: just a serine protease inhibitor? New and old considerations on therapeutic applications of C1 inhibitor. Expert Opin Biol Ther 2008; 8(8):1225-40. Review

In vitro systems for preclinical testing of drugs

Next to complement we are working on *in vitro* systems for preclinical testing of drugs. We analyze the effect of these drugs in whole blood assays, in TLR-transfected cells and in mononuclear cell (MNC) cultures. Testing a variety of therapeutic plasma proteins we have seen nice correlation with pyrogenicity as measured in the rabbit pyrogen test and in the LAL test. The monocyte activation test is now developed to the extent that the same assay can be used worldwide. We have isolated MNC's from four blood donations, mixed them, aliquoted them in 400 ampoules and frozen them in liquid nitrogen. Each ampoule is sufficient to test IL-6 induction in MNC by therapeutic proteins in a 96-wells microtiter plate. The method is robust and more sensitive than the LAL test or the rabbit pyrogen test.

Key publication

Kikkert R, de Groot ER, Aarden LA. Cytokine induction by pyrogens: comparison of whole blood, mononuclear cells, and TLR-transfectants. J Immunol Methods 2008; 336:45-55.

Auto-immune diseases

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The Autoimmune Diseases research group aims to identify mechanisms that underlie the formation of auto-antibodies. Our hypothesis is that impaired clearance of dead cells leads to an increased risk for the formation of auto-antibodies against nuclear antigens which in their turn may lead to systemic lupus erythematosus (SLE). Clearance of dead cells is facilitated by a large number of plasma proteins. We noticed that incubation of apoptotic cells with plasma leads to rapid removal of nucleosomes from the dead cells. In the absence of serum this takes days but with as little as 5% serum the removal of nucleosomes is completed in 30 minutes. The responsible plasma protein is the serine protease hyaluronic acid-binding protein-2 also called Factor VII-activating protein (FSAP). We have developed monoclonal antibodies to FSAP. Those antibodies are now used to affinity purify this protein from human plasma. Furthermore we have developed a sensitive quantitative Elisa to measure FSAP levels in biological fluids such as plasma. Two of the monoclonal antibodies directed to the light chain of FSAP inhibit the nucleosome releasing activity of serum confirming the role of FSAP in removal of nucleosomes from dead cells. Activation of FSAP by apoptotic cells leads to formation of a covalent complex with another plasma protein. This other protein was identified as α2-antiplasmin. Using a monoclonal antibody specific for α2-anti-plasmin and a labeled monoclonal antibody to FSAP these complexes can be measured in plasma. Also complexes between FSAP and C1-inhibitor can be found in plasma incubated with apoptotic cells. Plasma's of healthy controls are negative but in patients with sepsis these complexes can be found. Necrotic cell death leads to release of the nuclear protein HMGB1. Released HMGB1 acts as an endogenous danger signal and seems to be important in a variety of inflammatory conditions. Because decent assays for HMGB1 protein levels are lacking we try to develop monoclonal antibodies to the protein. Differences between species are extremely small. Mouse and human HMGB1 differ

in only two amino acids and human and horse HMGB1 are 100% identical. We have now purified horse HMGB1 to homogeneity and immunized mice and chickens. Until now obtained antibody titers are extremely low and 3 fusions have yielded no monoclonal antibodies.

As model for antibody formation in auto-immune conditions, antibody formation to the TNF inhibitor drugs infliximab, adalimumab and etanercept in patients with RA was investigated. In cooperation with the Dept of Rheumatology of the Vrije Universiteit Medical Center, the Academic Medical Center, the Slotervaart Ziekenhuis and the Jan van Breemen Institute circulating levels of these drugs as well as antibodies to these drugs were determined. Within a year, 50% of patients treated with infliximab develop antibodies. For adalimumab this figure is about 20% whereas none of the 200 patients treated with etanercept developed antibodies. Antibody formation is clearly T-cell dependent in that antibodies were of IgG1 and IgG4 isotype. Antibodies are specific for the idiotype of the respective antibody and they neutralize the TNF binding capacity. However clinical relevance seems mainly related to enhanced clearance of the drug. Antibody formation results in clinical inefficacy; hence such measurements are important to guide treatment. Presently we have expanded the assay repertoire to therapeutic monoclonal antibodies with other specificities such as rituximab (anti-CD20), trastuzumab (anti-her2), omalizumab (anti-IgE) and natalizumab (anti-α4integrin).

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Clinical studies on transfusion related efficacy and complications

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In 2008 five randomized clinical trials, three with red cells, one with platelet products and one with plasmaproducts, were ongoing. One study was finished.

ToMaat study

The study on optimal blood management in orthopedic surgery comparing allogeneic transfusions, erythropoietin, cell saver and woundblood (ToMaat).
Rationale: yearly almost 40,000 mostly elderly patients require orthopedic surgery of lower extremities. Several approaches to reduce allogeneic transfusions have been published but no studies are available that compared the various options in one study. The ToMaat study completed accrual with more than 2500 included patients in October 2008. The final analysis is expected in September 2009.

The Match study

A second study of red cells addresses the question for which patient population pre-emptive matching for clinically relevant erythrocyte antigens (Rh, K, Fy, Jk, S) is cost-effective.
Rationale: alloimmunization against red blood cell antigens is the most frequently reported adverse effect of transfusion, with frequencies ranging from < 1% to more than 70% depending on the population under study and study design. We studied several transfused patient populations and found for all transfusion indications, surgical and non-surgical, that once a patient had formed an RBC antibody the risk of additional antibodies upon a subsequent transfusion event exceeds 20%. The profile of patients at risk for primary immunization is however yet unknown. Prior to the implementation of new technologies using large-scale donor RBC genotyping, evaluation is needed for which patients extensive matching is efficient and may be cost-effective. In the Match study, a randomized controlled multi-center trial, potential transfusion patients are stratified on the basis of a first transfusion event or at high alloimmunization risk (patient has produced an RBC antibody after prior transfusion). Randomization in both strata involves routine T&S selected RBC versus extended (RH, K, Fy^a, Jk^a and S) matched red cell transfusions. After transfusion

patients are followed over time for RBC immunization. End 2008 three (2 university) hospitals started with patient recruitment.

Womb (Well being of Obstetric patients on Minimal Blood transfusions) study

In the Womb study women with post-partum anemia are randomized to a restrictive and liberal transfusion trigger to compare post-partum fatigue and functioning.
Rationale: Circa 3% of women after clinical delivery receives one or more blood transfusions. It is widely assumed that post-partum anemia leads to severe fatigue impairing breast-feeding and function. In this study patients, stratified for mode of delivery, are randomized to a liberal and restrictive transfusion trigger and up to 6 weeks scored at regular intervals on validated scales for fatigue and functioning.
It is expected that in 2009 the required 400 patient accrual will be completed.

Triplate study

In this study (in collaboration with the Dutch hemato-oncology research network, HOVON) efficacy of three platelet products stored up to 7 days are compared: plasma stored platelets, PAS stored platelets and pathogen-inactivated (PI) platelets.
At the end of 2008, 70% of the required patients have been included.
Rationale: Pathogen inactivation aims to reduce bacterial (and other microbial) contamination of platelet products with the advantage of prolonging platelet shelf-life without sterility risks. Both PASses and PI may diminish platelet function and viability. This question is addressed in a randomized study in hemato-oncological patients with the primary endpoint post-transfusion increment and secondary bleeding incidence.

Fiber (fibrin glue in CABG surgery) study

Randomized study in CABG (coronary artery bypass surgery) investigating whether fibrin glue reduces transfusion needs and indication for re-thoracotomy.
Rationale: Peri-operative blood transfusions for cardiac surgery are dose-dependently associated with post-operative infections and organ failure. It is unknown whether this is a causal relationship.
The Fiber study investigates whether the use of fibrin glue reduces transfusions and associated postoperative complications. The study will end 2011.

Autologous placental blood erythrocytes for preterm infants

In 2008 we finished the analysis of a randomized study evaluating the use of autologous placental blood erythrocytes for preterm infants. The study showed that for very low birth weight infants (24-27 weeks of gestation) not sufficient red cells could be collected and for infants born after 30-32 weeks of gestation the collected and stored placental red cells were generally not needed. Only for infants born after a gestational age of 28-30 weeks autologous cord blood erythrocytes can largely replace the need for allogeneic erythrocytes. The study also revealed that placental red cells can be stored up to 3 weeks. The use of placental red cells may offer an attractive source of transfusions for countries lacking a volunteer donor blood supply.

Key publications

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Observational and translational clinical transfusion studies

Transfusion-related research performed in 2008 included ongoing studies unraveling the mechanisms and consequences of transfusion induced alloimmunization and transfusion related immunosuppression. Research so far only indicated that transfusions, in particular non-leukocyte-depleted red blood cells, are associated with immune stimulation, whereas little ground has been found for a direct immunosuppressive effect of transfusions. A few recently started studies are discussed in more detail.

As described above, patients who produced antibodies can be considered as high responders at risk for broad alloimmunization.
This also applies to females with alloantibodies against paternal antigens expressed on fetal red blood cells resulting in hemolytic disease of the fetus/newborn (HDN). Mothers whose fetus received intra-uterine transfusion (IUT) for HDN are high responders towards RBC antigens. After pregnancy with IUT more than 70% possess multiple antibodies. An IUT follow-up study (LOTUS) was started in 2008 evaluating mothers and their live offspring, who have been treated with IUT, over the last 20 years. Study questions are unravelling risk factors for high respondership, antibody persistence in relation to chimerism, the role of HLA-disparity between mother and fetus and associations between RBC, leukocyte and platelet alloimmunization, besides child developmental issues related to the severity of anemia. This study is performed in close collaboration with the LUMC departments of Obstetrics, Neonatology and Immunohaematology & blood transfusion.
Within the Lotus and extended to other immunized patients we unravel HLA class II (DRB1) associations with high respondership against particular RBC antibody specificities.

Key publications

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Cord blood transplantation

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Cord blood (CB) research at the Blood Bank South West Region focuses on improving the current therapies with CB stem cells. The main objective is to increase the number of patients that can be treated with a CB transplant and to improve the outcome after CB transplantation. For this purpose, three strategies (or combinations) are pursued; *ex vivo* expansion of CB stem cells with thrombopoietin (TPO) as single growth factor, co-transplantation with non-hematopoietic cells, and multiple CB transplantation.

With regard to *ex vivo* expansion we were able to identify the *in vivo* role of the different cell subsets present after TPO expansion in the NOD/SCID mouse model. We showed that the CD34-/Lin- population is responsible for the observed acceleration of platelet recovery in the peripheral blood, and contributes together with CD34+ cells to long-term bone marrow (BM) engraftment. Cell concentration experiments showed a clear correlation between the number of CD34-/Lin- cells and both the speed of platelet recovery as well as the total number of platelets in the peripheral blood at 6 weeks after transplantation. Surprisingly, the megakaryocytes produced *in vitro* after expansion with TPO do not to play any role in platelet recovery. Because it has been shown that mesenchymal stem cells (MSC) enhance the overall engraftment when co-transplanted with CB CD34+ cells, we examined whether treatment with a combination of CB CD34+ expansion and co-transplantation of MSC act synergisticcally by shortening the time to platelet recovery as well as improve overall engraftment in the BM. Mice transplanted with a combination of CD34 expanded cells and MSC exhibited significantly accelerated platelet recovery compared to non-expanded CD34+, but this was not superior to TPO expanded CD34 cells. Moreover, the engraftment-enhancing effect of MSC was no longer observed. This suggests that combining two CB manipulations, each favourably affecting distinct aspects of CB transplantation, does not necessarily further benefit CB transplantation. The mechanisms underlying these differences are currently under study.

Compared to single CB transplantation, double CB transplantation results in a higher proportion of engraftment in adult patients. Sustained hematopoiesis is usually derived from a single donor, however, the mechanism of predomination of a particular CB donor unit has not been unravelled yet. Emerging hypotheses focus a) on difference in quality and viability between cord bloods, b) on immunological interactions between CB leading to rejection of one cord, or c) on increase of accessory cells relevant to facilitate CD34 cell homing and engraftment. In collaboration with the Erasmus MC in Rotterdam, a clinical trial was initiated to study the clinical effects of multiple CB transplantation in adult patients and to unravel the possible mechanism behind the predomination of one unit in the mouse and in patients. With discriminating HLA-allele specific monoclonal antibodies (hybridoma's provided by Arend Mulder, IHB, LUMC) we can detect donor and patient leukocyte subpopulations (and platelets) in the pancytopenic period early after double CB transplantation. Thus far, 15 patients were enrolled in the study and we could discriminate the 3 different parties in 9 of 15 patient-donor combinations (60%), while in 6 cases (40%) the lack of discriminating HLA-mismatches prevented us from distinguishing all 3 parties. A pilot study in 2 patients revealed that already from day 18 post-transplant a single CBU was predominant in all subpopulations, although differences were observed between the leukocyte subpopulations. Simultaneously, we explored in the NOD/SCID model the role of the CD34+ and CD34- CB cells in facilitating engraftment after double CB transplantation. The engraftment facilitating effect of co-transplantation of CD34+ cells only was similar compared to co-transplantation of both CD34+ and CD34- cells suggesting that enhancement of short-term engraftment in NOD/SCID mice by double cord blood transplantation is mainly mediated by the CD34+ cells in the second CB.

Key publications

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Epidemiological studies to test the hypothesis that TRALI is caused by leukocyte antibodies

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The majority of cases of transfusion-related acute lung injury (TRALI) are thought to be caused by donor leukocyte antibodies, and these antibodies are more prevalent in female than in male donors. This had led to the exclusion of plasma from female donors for transfusion in several countries. The objective of this project is to assess the relation between donor sex and the occurrence of TRALI. This project is performed in cooperation with Anske van der Bom MD PhD, Dept. Epidemiology, Leiden University Medical Centre.
A systematic review of the literature shows that, due to methodological problems, only 14 out of 82 papers on the prevalence of leukocyte antibodies in donors of TRALI patients allow for a quantitative estimate of the relative risk associated with these antibodies. Based on these papers the risk to get TRALI is 15-fold higher in recipients of antibody containing blood products than in recipients of products without antibodies. However, there seems to be a considerable publication bias favoring the role of antibodies. In an international collaborative study, patients were selected who received products either only from male donors or only from female donors. Among red cell recipients the risk of TRALI after a transfusion from a female donor was the same as the risk after transfusion from a male donor. Among recipients of plasma rich products the risk was 9-fold higher for products from female donors. Our data suggest that female donors and leukocyte antibodies are involved in some TRALI cases, especially those caused by plasma rich products.

Key publication(s):

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The research of the Unit Research & Development of the Sanquin Blood Bank South East Region runs along two related research lines: 1) Donor Recruitment and Retention, and 2) Donor characteristics and health issues regarding blood donations, including donor deferrals.

Donor Recruitment and Retention

Behavioral studies

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Psychosocial and behavioral aspects of blood donation are the key issues within this research line.

We investigated relationships between philanthropy in general, blood donation, and social capital. In our studies we do not find a strong relation between blood donation

and philanthropy. In addition, social capital does not seem to be a strong explanatory variable for blood donation. Studying and identifying donor profiles points out that in The Netherlands differences can be assessed between incidental donors versus so called multi-gallon donors. Multi-gallon donors more often have a higher socio-economic status, and live in more urbanized areas. A significant negative association exists between the duration of a donor career and the number of donations performed on the one hand, and the chance that a donor will discontinue or stop being a blood donor on the other hand.
Applying the Theory of Planned Behavior, we learned that Attitude towards donation and Self-Efficacy regarding blood donation are strong determinants for the intention to donate in so-called new donors. We could prove that the flyers we use today are not efficient in addressing the important determinants of blood donation just mentioned. Instead, they focus too much on the knowledge of blood transfusion, a seemingly unimportant determinant for becoming a blood donor.
The determinants of intended donations also are important for the remaining (future) donor career. Their development in time is the main issue in ongoing research. There we try to study whether determinants of intention to donate develop over time during a donor career and which determinants are important predictors of future donations. To this end a longitudinal study has been set up, of which the first quantitative results are expected in 2009/2010.

DOnor MAnagement IN Europe, DOMAINE

DOMAINE (Donor Management IN Europe) is a European Union co-funded project, in which blood establishments from 14 European member states and one patient-driven organization join their forces on donor management. DOMAINE aims to create a safe and sufficient blood supply, by comparing and recommending good donor management practice. It focuses on various aspects of donor management: donor recruitment strategies, donor retention strategies, deferral procedures and blood bank policy regarding patients requiring long-term transfusion. In the first phase – performed in 2008/2009 – of the project, a survey is conducted to analyze donor management practice in Europe. In total, 48 questionnaires have been sent to 37 European countries, with a response rate of 88%. The survey report will

be finalized by May 2009. The second phase (2009/2010) will concentrate on composing a manual, recommending good donor management. The final phase (2010/2011) will concentrate on instructing blood establishment professionals on the manual.

Key publications

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Donor characteristics and health issues regarding blood donations, including donor deferrals

Donor characteristics and health effects related to blood donation are the main issues within this research line. Furthermore, there is a strong focus on donor deferral.

Donor characteristics and health effects of blood donation

Donor InSight is a large study, which started in 2007. The primary aim of Donor InSight is to gain insight into donor characteristics. In total, 50,000 whole blood and plasma donors are invited to fill in an extensive questionnaire on many topics, like demography, lifestyle, health and disease, donor motivation and donor satisfaction. At the end of 2008, over 40,000 donors have received the questionnaire, thereby yielding a response rate of 63%. Furthermore, the research line focuses on donor complications as well as the effect of blood donation on disease occurrence, like cardiovascular disease and cancer.

Donor deferral

Low hemoglobin level (Hb) is an important reason for donor deferral. In The Netherlands, in total, about 10% of the donors visiting a collection centre, is deferred. Within the deferred group, from 2-3% of male donors up to 5-7% of female donors are being deferred for low Hb. Since deferral is a proven reason of donor lapse, reducing this percentage is paramount. Hb is known to be related to several factors, including: gender, physical condition, iron status, Body Mass Index, nutrition, but also environmental conditions, such as environmental temperature and donation history. To disentangle these complex relations we recently started an extensive statistical modeling study on prognostic factors of Hb. In 2008 the study population has been identified and the first modelling results are expected to be available in 2009. In a pilot study among donors, substantial iron depletion – measured through Zinc Protoporphyrin (ZPP) levels – could be observed. ZPP is an anticipated predictor of iron depleted Hb production.

Key publications

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Research performed at Sanquin Blood Bank North West Region, Research & Development

Improving materials and methods for storage of blood components

Flow cytometer method for counting of platelets in platelet concentrates

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Hematological cell counters are developed for counting of (patient) whole blood samples, but are also used for quality control of platelet contentrates (PC). However, due to the high platelet count and the absence of significant numbers of erythrocytes, these counters have a high variability in platelet counts of TC. An independent, flow cytometric method was developed for counting platelets in TC. The reproducibility of this method was investigated in an international study with 8 blood centers from 5 countries.

Five TC samples were sent around and measured in triplo at each participating laboratory. In the final protocol samples were diluted 1:100 in diluent (0.5% BSA and 10 mM EDTA in PBS) by 2 subsequent 1:10 dilutions (checked by weight). To a TruCount tube (BD Biosciences) 20 µL diluted sample was added and 20 µL (1:100 diluted) anti-CD41a-FITC (BD). After 20 minutes incubation, 400 µL diluent was added and the sample was measured on a flow cytometer. The results were analyzed with a uniformed template. All samples were also counted on the hematological counters available in the participating centers (15 counters).

In general the results for the flow cytometers are about 8% higher than those of the cell counters. The inter-centre CV is smaller for the flow cytometers (6.3%) compared to the cell counters (7.6%). A large difference was found between cell counters based on impedance technique or flow cytometry technique, with the latter showing counts much closer to the counts obtained with the newly developed method.

Counting of platelets in TC with a flow cytometer in combination with accurate dilutions, results in reproducible results with an acceptable CV. Compared to cell counters this method results in a much smaller range. The new method is potentially suitable as an independent ‘gold standard’ method for counting of platelets in TC.

DEHP in blood products for pediatric use

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Di-ethyl-hexyl-phthalate (DEHP) is a frequently-used plasticizer in PVC blood bag systems. An advantage is that DEHP protects the red cells from hemolysis, so they can be stored better. A side effect is that DEHP could leak into the blood product and can be converted into toxic mono-ethyl-hexyl-phthalate (MEHP). This is a global concern, particularly for blood products intended for pediatric use, such as pedipacks (aliquots of regular red cell concentrates: RCC) and reconstituted whole blood (WB) for exchange transfusions (RCC + plasma).

The amount of DEHP was measured in pedipacks and exchange transfusions, intended for newborns and children up to 4 years old. Routinely produced pedipacks were stored in regular bags (manufacturer A and B; because Sanquin blood banks are using two different brands) and sampled directly after preparation and at day 28, 35 and 42 after donation. Exchange transfusions, prepared in routine, were sampled directly and 24 hours after preparation. DEHP extraction was performed in all samples. Analysis of DEHP was performed by HPLC. These results were compared with historical DEHP data of routinely produced RCC.

In total, 12 pedipacks and 4 reconstitued WB for exchange transfusions were investigated. The amount of DEHP in pedipacks increased during storage, in bag A to significantly higher levels 24.9±5.5 ppm) than in bag B (20.8±2.6 ppm). These levels were comparable with adult-dose RCC from a former study, which contained 19.4±2.3 ppm on Day 35 of storage. The amount of DEHP in exchange transfusions increased with 50% during 24 hours of storage at 4°C from 8.3±1.2 to 13.3±0.5 ppm. This is faster than in pedipacks, due to better solubility of DEHP in plasma as compared to SAGM.

With a transfusion of 20 ml to a baby of 4 kg, the amount of DEHP transfused is 40-125 µg/kg/day (depending on the storage time). For an exchange transfusion with 300 ml reconstituted whole blood this amounts to 600 µg/kg/day. These amounts are similar (pedipacks) or lower (exchange transfusion) as reported in literature. The recently determined Tolerable Daily Intake (TDI) for DEHP is 48 µg/kg/day, but this is based on feeding rats during 3 generations with DEHP. The daily exposure (mainly from food) for DEHP is 2-8 µg/kg/day, thus blood transfusion is causing peak values in the exposure of DEHP.

Key publications

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SYSMEX XT 2000I Versus ADVIA 2120 cell counter

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Blood bank laboratories often analyze blood samples with exceptional blood counts. In our blood bank the cell counters Advia 2120 and Sysmex 2000i, based on resp. flowcytometer and impedance techniques are used. To check exchangeability of measurements in the upper en lower spectrum of the given linearity ranges, cell counts of blood samples with high and low cell concentrations measured on both cell counters were compared.

The erythrocyte, platelet and leukocyte counts, including subsets of neutrophils and lymphocytes, of 20 samples were determined on the Sysmex XT 2000i and the Advia 2120 as part of various experiments. Values of both cell counters were compared by linear regression. High correlation coefficients (cc) were found between the Sysmex 2000i and the Advia 2120 for all cell counts analyzed even when measured above given linearity range (cc between 0.995 and 1.000). Neutrophil measurements were spliced into counts below (n=16) and above (n=4) 53 x 10^9/L, because an obvious buckle was observed in the correlation scatter at that value. For erythrocytes, platelets and low neutrophil counts the cell counters

give similar results, but for leukocytes, lymphocytes and high neutrophil counts there were differences. Remarkably, the similarity in platelet counts is contradictory to a former comparison of these cell counters, using platelet concentrates, when counts from the Sysmex 2000i were 15-20% lower.

Improving materials and methods for blood bank processing

In vitro quality of components after slow cooling of whole blood

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Cooling and transport of whole blood (WB) at Sanquin is performed by using butane-1,4-diol plates or otherwise. In search for a hygienic, labor friendly and uniform method the use of cool boxes was investigated in a 'worst-case' scenario. The *in vitro* quality was investigated of leukocyte-reduced red cell concentrates (LR-RCCs), plasma (PL) and platelet concentrates (PCs) made from WB after overnight storage and 'slow' or 'fast' cooling.

In paired experiments (n=12), two WB were cooled either 'slow' (group A) or 'fast' (group B) and stored for 19-22h. 'Slow' cooling was performed using cool boxes and simulated WB units of +35°C. 'Fast' cooling was performed using butane-1,4-diol plates. After storage, WB was separated routinely in PL, LR-RCC and buffy coat (BC). BCs were used to prepare 1 donor unit PCs. LR-RCCs and PCs were stored up to 42 days or 8 days respectively and sampled at regular intervals for *in vitro* measurements. WBs which were cooled 'slow', were cooled down to 24-26°C after 19-22h. WBs which were cooled 'fast', reached a temperature <25°C within 2h.

At Day 1, before and after processing respectively, WBs and LR-RCCs of group A showed very low 2,3-DPG content (<1.3 µmol/ g Hb). At Day 42, LR-RCCs of group A showed also lower ATP and higher hemolysis data than LR-RCCs of group B. Hemolysis in 4/12 (33%) units of group A did not conform to the requirement <0.8%. No significant differences were seen in Factor VIII content of the PLs (group A: 0.88±0.19, group B: 0.89±019 IU/mL). PCs of group A showed significantly lower pH values at Day 1 but after storage no significant differences were seen in pH (group A: 6.78±0.25, group B: 6.84±0.31), CD62P expression and Annexin A5 binding.

It can be concluded that after slow cooling of WB *in vitro* quality of PLs and BCs/PCs is

not affected, but LR-RCCs show more hemolysis and lower ATP and 2,3-DPG values. The use of butane-1,4-plates is recommended to prevent 'worst-case' situations.

Buffy coat derived granulocyte transfusion product: a bridging product?

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Because of required donor selection, testing, and G-CSF / dexamethasone stimulation 12 hours prior to the apheresis procedure, neutropenic patients with life-threatening infections have to wait for 24-48h before apheresis derived granulocyte components derived from G-CSF and dexamethasone stimulated donors are available. This delay can be lethal, but till availability of apheresis product the patient might be helped with granulocytes isolated from pooled buffy coats (BC). To use as a bridging product, we developed a BC-derived transfusion product complying with the European and national guidelines for granulocyte transfusion products (≤500 mL, ≥$1x10^{10}$ granulocytes/unit, preferably >$0.8x10^{8}$ granulocytes/kg of patients body weight). BC-derived concentrates (n=11) were made by pooling of ten overnight stored ABO and rhesus compatible BC. The pools were centrifuged (1800 g, 5 min) in a Bottom-and-Top bag. The red blood cell and the plasma layers were removed via bottom or top outlet using a plasma clamp, resulting in a super-BC. The volume of the product was about 100 ml, with a hematocrit of about 40%, 340 x 10^{9} platelets and 23 x 10^{9} leukocytes. Leukocytes consisted for 50% of granulocytes, so the super-BC contained 1.13 x10^{10} granulocytes per unit. This super-BC was irradiated with at least 25 Gy. The composition of the products was determined on an automated impedance counter (Sysmex XT 2000i).
It can be concluded that we are able to produce BC-derived granulocyte transfusion products, complying with the European guidelines and with the national guidelines. This BC-derived product can be of value to bridge the period till availability of apheresis derived granulocyte components.

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Pathogen detection and reduction

Bacterial screening of platelet concentrates: results of 2 year active surveillance

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The BacT/ALERT system for bacterial monitoring of platelet concentrates (PCs) was introduced in the Netherlands in 2001. Samples are cultured for 7 days and as a result of the short shelf-life of PCs, they are usually released as 'negative to date'. Therefore, some of the PCs have already been transfused at the moment of a positive signal in continued cultures in the BacT/Alert. It is unclear, however, whether these PCs are associated with more transfusion reactions.
During a 2-year period clinical data were collected from all patients who received PCs released as 'negative to date' but with a positive bacterial culture after being transfused.
Data of 158 patients who received PCs with confirmed positive bacterial culture tests were analyzed. Two patients developed a transfusion reaction. In both PCs a propioni bacterium was cultured. The imputability as related to the transfusion was classified as unlikely in both patients.
Transfusions of PCs released as 'negative to date', but with a confirmed positive BacT/ALERT result after being transfused were not associated with an increased rate of transfusion reactions.

Mirasol® treatment of buffy coat platelets suspended in additive solution

Mirasol pathogen reduction technology (PRT) treatment inactivates leukocytes and a wide range of bacteria, viruses and parasites in platelet concentrates. This process involves the addition of riboflavin in combination with UV light and is currently CE-marked for plasma and platelets. The Mirasol system is validated to treat platelets suspended in 100% plasma and to add platelet additive solutions (PAS) post-illumination for storage.
To extend the range of platelet products to be treated, the *in vitro* cell quality of buffy coat platelet concentrates (BCPC) that were PRT treated in the presence of PAS was evaluated. *In vitro* cell quality of the platelets was assessed over 8 days of storage.

BCPCs were generated from 5 pooled buffy coat units in SSP+ (Macopharma) (n=15). The average yield of the units was $4.0+0.5x10^{11}$ in 360+11ml of plasma plus SSP+ ($1.1+0.1x10^{9}$/ml). Plasma carryover was on average 32+0.9%. For PRT treatment 35ml of Riboflavin (500µM) was added to test units (n=10). Platelet function was assessed in test and control units by pH, swirl, morphology, lactate production and glucose consumption rates, CD62P expression and Annexin V staining over 8 days of storage.

The pH in PRT treated units on day 8 of storage was slightly decreased (7.05±0.04) compared to untreated controls (7.37±0.03). PRT treated and untreated units maintained positive swirls throughout storage. The lactate production and glucose consumption rates on day 8 in PRT treated units was elevated (0.072±0.008 mmol/10^{12} cells/hr and 0.042±0.004 mmol/10^{12} cells/hr respectively) compared to untreated control units (0.040±0.005 mmol/10^{12} cells/hr and 0.020±0.003 mmol/10^{12} cells/hr respectively). CD62P expression and Annexin V staining was increased in PRT treated units (38±5% and 8±2% respectively) compared to untreated controls (13±1% and 5±1% respectively). No significant difference due to PRT treatment could be detected on morphology (Kunicki score, % discoid platelets).

It can be concluded that PRT treatment of BCPC in the presence of PAS generates units that can be stored for 7 days. Elevated lactate production and glucose consumption rates and CD62P expression after PRT treatment is due to cellular activation and increased metabolism of the cells. *In vitro* cell quality results obtained here on day 8 of storage are almost identical to values obtained for platelets after PRT and storage in 100% plasma on day 5 (AuBuchon et al, Transfusion 2005; 45:1335) and would predict acceptable survival and recovery *in vivo* (Goodrich et al, Vox Sang 2006; 90:279).

Key publication

De Korte D, Curvers J, de Kort W, Hoekstra T, van der Poel CL, Beckers EA, Marcelis JH. Effects of skin disinfection method, deviation bag and bacterial screening on clinical safety of platelet transfusions in the Netherlands. Transfusion 2006; 46:476-85.

Research performed at Sanquin Research, Department Blood Cell Research

Research in the Department Blood Cell Research is performed by two Principal Investigators, Dirk de Korte PhD and Arthur Verhoeven PhD. Both PI's have their own focus on Research Lines. The focus of Dirk de Korte is described here, while research lines by Arthur Verhoeven involving Dirk de Korte, as well can be found with PI Arthur Verhoeven.

New insights into RBC metabolism during storage

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Recently, we developed an improved additive solution for red cell concentrates. This chloride-free medium allowed maintenance of 2,3-DPG and ATP levels during 35 days of cold storage. This solution, based on the 'chloride-shift' proposed by Meryman in 1996, should result in an increased intracellular pH and an increased 2,3-DPG formation.

In this study, RBC units were prepared from double cytapheresis donors in either the standard storage medium SAGM (saline, adenine, glucose, mannitol; pH6.2) or the experimental medium PAGGGM (phosphate, adenine, guanosine, glucose, gluconate, mannitol; pH8.2), and the cells were metabolically characterized during 49 days of cold storage.

RBC in PAGGGM had higher 2,3-DPG and ATP levels throughout storage, but showed a similar intracellular pH. This contradicts the hypothesis in the literature that 2,3-DPG levels are primarily determined by the intracellular pH. Initial glucose-6-phosphate levels (G6P) were higher in RBC suspended in SAGM, but this difference was reversed upon storage.

We conclude that PAGGGM stimulates at least two steps in glycolysis, one of which is hexokinase. These effects are not mediated by changes in the intracellular pH.

Evaluation of the new Compoflow break-away closure on *in vitro* quality of blood products

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To open break-away closures during blood component preparation manually introduces workload and a cause for RSI. Incorrect opening can induce damage to the blood components, especially hemolysis in erythrocytes. A new, automatically to open, break-away closure has been developed by Fresenius, called Compoflow, and prototypes of this closure were tested with respect to *in vitro* quality of blood components after passing the new closure.

Blood bag systems in which the normal break-away closures were replaced by the new Compoflow break-away closure, were used to prepare blood components on the Compomat G4, including platelet concentrates from pooled buffy coats (BC-PC). The various blood components were tested for *in vitro* quality during subsequent storage.

Six units of whole blood were leukoreduced and then used for preparation of plasma and red cell concentrate (RCC), both passing the new closure. The extensive evaluation of plasma and RCC *in vitro* quality showed no significant differences to products obtained with the standard closure. Six units of whole blood were processed into plasma, buffy coats and RCC. The initial component quality showed no differences compared to units with the standard closure. However, work-up time on the Compomat was 10% shorter due to less restriction in the line after opening the closure. During storage of RCC, the maximal hemolysis was 0.28% after 42 days, with a mean of 0.22%. Parameters like morphology, ATP content and Annexin 5A binding were also indistinguishable from standard products. Finally, BC-PC which passed during preparation the new closure in the pooling set, showed the same *in vitro* quality during storage as BC-PC prepared with standard pooling sets.

No negative effects on product quality of the automatically to open Compoflow break-away closure were observed during standard component preparations. Due to the improved opening of the new closure, the work-up time for component preparation on the Compomat was about 10% shorter.

Development of an internally controlled assay for broad range detection of bacteria in platelet concentrates

At the moment, bacterial contamination of blood products is the most common microbiological risk of transfusion. The prevalence of contamination of cellular blood products is approximately 1 in 3,000 donations. The risk is greatest for platelet concentrates (PC) as they are stored at room temperature under constant agitation to preserve function and vitality. These conditions make PCs an excellent growth medium for bacteria. In the Netherlands, screening of PCs for the presence of bacteria is done by automated culturing with the BacT/Alert culturing system (BioMerieux). Although the system is sensitive, in theory it can detect 1 colony forming unit (CFU) per 5 to 10 ml PC, its use is restricted by long assay times. Slow growing bacteria or low bacterial loads are not always detected by the system. Previously a real-time PCR assay based on the 16s rRNA gene was developed as a fast alternative for culturing of PCs. However, this assay was not as sensitive as the BacT/Alert culturing system. To improve the sensitivity of the real time PCR assay, a reverse transcriptase step was added to detect RNA.

Total nucleic acids were isolated using the MagNA Pure LC automated extraction system (Roche). Hereafter, reverse transcriptase was used to make cDNA, together with random hexamer primers. The real-time PCR was performed with a previously developed 16S rRNA gene primer and probe set that detects all bacteria relevant for bacterial contamination in PCs. A RNA bacteriophage internal control (IC) was used to control RNA isolation and amplification. Two model bacteria, *Staphylococcus epidermidis* and *Escherichia coli* were used to determine the sensitivity of the assay in PCs. The total amount of RNA and DNA in growing bacteria and bacterial cultures treated with antibiotics was determined to investigate whether there are differences in sensitivity of the test dependent on the bacterial viability.

With the real time PCR based on detection of DNA, 150 CFU/ml of E. coli and 700 CFU/ml of *S. epidermidis* could be detected in PCs. By detecting RNA, sensitivity improved to 3 CFU/ml for *E. coli* and 70 CFU/ml for *S. epidermidis*. The ribosomal RNA of bacteria grown in the presence of antibiotics broke down very slowly.

Therefore, the test is suitable for detection of bacteria even if the bacteria are not actively growing.
It can be concluded that the sensitivity of 16S rRNA PCR assay improved considerably by detecting RNA instead of DNA. Because RNA of bacterial cultures treated with antibiotics breaks down very slowly, the test remains sensitive, even if the bacteria are not actively growing. The short turnaround time of the real time RT PCR assay makes it a candidate as alternative for the BacT/Alert and/or as a rapid test shortly before transfusion.

Key publications

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Development related research in SBNO

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In 2008 approval was given through the national medical ethical committee for a multi-center study to investigate the effect of fibrin glue in patients that have undergone knee or hip replacement. The principal investigator for this study is one of the orthopedists. The study will start in 2009. Besides this, R&D started a study for the extended expiring date of fibrin glue for one to two years. Preliminary data show that quality remains good for at least one year, but probably even for two years, i.e clotting time <10s and enough FXIII to prevent clot resolving within 24h. Furthermore, studies were performed for the BEST (Biomedical Excellence for Safer Transfusion) working party. We participated in two studies, one for testing red cell concentrates processed from fresh or stored whole blood in various additive solutions, and one for platelet counting using hematology analyzers and flow cytometers. Also, a pilot for a BEST study was done. In the pilot we tested *in vitro* quality of buffy coats, red cell concentrates and plasma derived from fresh and stored whole blood. The results of the pilot study show that buffy coats derived

from fresh blood had a 60% lower platelet count and an also 60% higher platelet activation compared to buffy coats from overnight stored blood. For freshly prepared buffy coats after storage the platelet count was improved, but platelet activation was not. Red cells showed a most optimal condition when prepared fresh, although red cells from stored whole blood also showed good quality. For plasma no difference in FVIII was observed between fresh and overnight stored whole blood. In the BEST study, which will be organized by R&D from SBNO, platelet concentrates derived from buffy coats from fresh or stored whole blood will be compared in a multi-national study.

Another topic of R&D is setting up a post-marketing surveillance. The name for this is TRAM Sanquin, in which TRAM stands for TRAnsfusion Monitoring. A general project scheme has been written and possibilities for a TRAM for apheresis plasma that can be stored for 7 days (since November 2008) are under investigation. The results of apheresis data from 2007, only non irradiated, non concentrated, non divided platelet concentrates in plasma with a maximum storage for 5 days, show that about 40% is discarded and that except for apheresis products for the Military Blood Bank most products are distributed at day 5 of storage. Apheresis data for platelet concentrates stored for 7 days still need to be evaluated.

As contract research one study for testing whole blood leukoreduction filters and two studies for testing trombocyte leukoreduction filters were done. Furthermore, we started preparations for a large field evaluation of the BCSI pH1000, which makes it possible to measure pH of the platelet concentrate in a sterile way without sampling at all, in clinic and blood bank.

In 2008 the department R&D of SBNO validated and implemented various methods and machines or products, such as Sysmex K4500, Microcentrifuge, CD62p and Annexin V in platelet concentrates, buffy coats and whole blood, platelet aggregation using collagen and ADP, irradiated platelet concentrates in BCSI containers, and Fresenius collection system. The implementation of these methods and machines will lead to increased user friendliness and a broader set of quality control tests both for Sanquin as well as hospitals or firms.

Three national send arounds were organized by R&D, (i) one for counting platelets in platelet concentrates, (ii) one for counting leukocytes in plasma, red cell

concentrates and platelet concentrates and (iii) one for measuring pH in platelet concentrates. These send arounds are repeated yearly. By these send arounds, participating laboratories can check whether their methods are comparable or not with that of colleague blood banks. For platelet and leukocyte counting results differed between laboratories, but pH measurements led to comparable results for all participating laboratories. Also we assisted the Processing Facility for their preparations for JACIE accreditation.

Key publications

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Chemotaxis and polarized signaling in cell motility

Rac1 binding proteins – diversity in signaling and subcellular localization

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The small GTPase Rac1 is a ubiquitous key regulator of the actin cytoskeleton and coordinates cell polarity and migration. Rac1 binds, through its hypervariable C-terminus, to proteins that are involved in cytoskeletal dynamics, vesicle transport and nuclear events.

The nuclear protein SET has been analyzed in more detail with respect to its Rac1-

dependent nuclear exit. In resting cells, SET-YFP, exits the nucleus in an apparently random fashion. Rac1 also resides in part in the nucleus and this fraction travels together with SET to the cytosol. We are currently testing potential intracellular regulators of this pathway as well as receptor agonists to further define the requirements for the Rac1-SET nuclear exit.
The adapter protein Caveolin-1 binds to Rac1 through its C-terminus. The bulk of the Caveolin-1 protein in polarized cells resides in the rear of the cell. However, Rac1 signaling is generally assumed to be required in the leading edge. We found that Rac1 activation recruits Cav1 to leading edge focal adhesions primarily in the front of migrating cells, where both proteins colocalize. Current work is aimed at defining the relevance of Caveolin-1 for Rac1 signaling, polarity and migration.
A third Rac1- binding adapter protein that we are currently interested in is CD2AP or CMS. This protein concentrates, in contrast to Caveolin-1, in the leading edge of polarized cells, and particularly in membrane ruffles, but not in focal adhesions. CD2AP is known to mediate internalization of membrane proteins and to mediate cell-cell contact. Both aspects are now under investigation in relation to Rac1 signaling.

CXCR4 signaling and turnover in migration of hematopoietic stem cells

Hematopoietic stem cell (HSC) transplantation is applied to treat various (non-)hematological diseases. The speed of hematological recovery after HSC transplantation depends on the capacity of the transplanted HSC to migrate efficiently to the bone marrow (BM) cavity. The chemokine Stromal Derived Factor-1 (SDF-1/CXCL12) and its receptor CXCR4 are critical in the homing of HSC to the BM. The initiation of directional migration of a cell in response to a chemokine is dependent on the level of surface expression of the chemokine receptor and the cell's ability to properly initiate signaling upon ligand binding. Using various modes of Rac1 inhibition, we found that surface-expression of CXCR4 on HL60 and U937 cells was significantly and specifically reduced and resulted in a blockade of CXCL12-induced chemotaxis. Furthermore, pull-down experiments showed a biotinylated CXCR4 C-terminal peptide to interact with endogenous Rac1. These data suggest that Rac1 physically interacts with CXCR4 and that this interaction may regulate the

surface expression of CXCR4. Future experiments are aimed at determining whether Rac1 controls CXCR4 traffic to or from the plasma membrane.

Chemorepulsion in the hematopoietic system

The chemorepellent Slit proteins and the Roundabout (Robo) receptors inhibit the CXCL12-induced migration of leukocytes. These data make it conceivable that a balance between positive and negative migratory cues regulate the migratory behavior of hematopoietic (stem) cells to and from tissues. Our goal is to establish the mechanism mediating the apparent inhibitory migratory signal provided by Slit and Robo. Expression analysis of the four Robo and three Slit homologues in the hematopoietic system showed that Slit2 and -3 are expressed in primary BM stroma and BM-derived endothelial and stromal cell lines, whereas Robo1 is expressed by CD34+ hematopoietic stem and progenitor cells (HSPC). Interestingly, Robo1 mRNA and surface protein expression levels were significantly higher in HSPC as compared to CD14+ monocytes. In line with this, we found that Robo1 expression was reduced during differentiation of HSPC towards CD14+ cells *in vitro*.
Furthermore, Slit3 inhibited the CXCL12-induced migration of Robo1-expressing HL60 and U937 cells, while it enhanced the directional migration of primary monocytes. HSPC migration remained unaffected by the same dose of Slit3, but lower Slit3 concentrations decreased HSPC chemotaxis. In contrast to the *in vitro* migration data, the *in vivo* homing to the BM at 24 hours after transplantation was 2-fold increased by Slit3.
In summary, Slit proteins may inhibit or promote leukocyte chemotaxis, depending on the cell type, its state of differentiation, relative expression of the Robo1 receptor and environmental, possibly tissue-specific, components.

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Leukocyte-endothelium interactions and endothelial integrity

Signaling through endothelial Ig-like Cell Adhesion Molecules

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During transendothelial migration, leukocytes use adhesion receptors, such as ICAM-1 and VCAM-1, to adhere to the endothelium. Subsequently, ICAM-1 is recruited to sites of leukocyte adhesion, and so-called 'transmigratory cups' are formed, dynamic membrane protrusions that partially surround adherent leukocytes. Uncontrolled ICAM-1 recruitment and regulation of these cups likely affect leukocyte migration across the endothelium, resulting in physiological disorders such as chronic inflammation and atherosclerosis. Thus, it is of high importance to understand the details of ICAM-1 recruitment and the subsequent formation of these cups. However, little is known about the signaling pathways that regulate these actions. Our work focuses on the understanding of the recruitment of ICAM-1 and the subsequent formation of 'transmigratory cups'. Preliminary data suggest that endothelial small GTPases, such as Rac1, RhoA, Cdc42 and RhoG are activated downstream from adhesion receptors. Moreover, we are studying how these small GTPases are activated. Recent data using biochemical approaches such as pulldown assays implicate guanine-nucleotide exchange factors, enzymes that are able to specifically activate small GTPases, in the formation of these cups. Currently, we are using siRNA techniques to reduce the expression of candidate GEFs and small GTPases and studying their effect on leukocyte transendothelial migration. Moreover, these studies will be extended in flow models to mimic physiological conditions in real-time.

In 2008, the work on the ICAM-1-binding protein filamin has been finalized. We found that clustering of ICAM-1 promotes the ICAM-1-filamin B interaction. Moreover, we found that filamin B is required for the lateral mobility of ICAM-1 in the plane of the membrane and for ICAM-1-induced transmigration of leukocytes. Reducing filamin B expression in primary human endothelial cells resulted in reduced recruitment of

ICAM-1 to endothelial docking structures, reduced firm adhesion of the leukocytes to the endothelium, and inhibition of transendothelial migration. In conclusion, this study has identified filamin B as a molecular linker that connects ICAM-1 to the cortical actin cytoskeleton and mediates ICAM-1-driven transendothelial migration.

Control of VE-cadherin-mediated cell-cell contact

The endothelial monolayer forms a barrier against plasma and leukocytes. The monolayer is formed by the connection of individual endothelial cells through specialized proteins that form the cell-cell junctions. The major cell-cell junction molecule in endothelial cells is Vascular Endothelial (VE)-cadherin; VE-cadherin is a homotypic adhesion molecule, that regulates not only the electrical resistance of the endothelial monolayer but also the passage of leukocytes. Leukocytes cross the endothelium either by migrating through cell-cell junctions, i.e. the paracellular route, or by penetrating the endothelial cell body, i.e. the transcellular route. However, the majority of the leukocytes use the paracellular route of transmigration. Currently, it is unknown what triggers the leukocyte to choose either the transcellular or the paracellular route.

Our research focuses on the mechanism of VE-cadherin function and the way by which VE-cadherin regulates the passage of leukocytes across the endothelial monolayer and regulates the integrity of the endothelium. Our data show that adhesion of leukocytes through ICAM-1 on the endothelium induces the phosphorylation of VE-cadherin and that this phosphorylation event is required for efficient passage of leukocyte across the endothelium. We have evidence that the kinases Src and Pyk2 are involved in mediating VE-cadherin phosphorylation. Currently, we are studying how endothelial cells re-establish their cell-cell contacts after damage and whether VE-cadherin (de)phosphorylation signals the recovery of the endothelial monolayer. In addition, we focus on the role of VE-cadherin phosphorylation in the formation of new vessels by use of angiogenesis experiments.

Together, our research will define new signaling pathways that are crucial for leukocyte transendothelial migration and may promote the development of new therapies to treat disorders such as chronic inflammation and atherosclerosis.

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Membrane proteins and vesicle transport

Regulation of the inflammatory response of endothelial cells by the small GTPase RhoB

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Pro-inflammatory mediators such as the cytokines TNFα, IL1β and the bacterial product lipopolysaccharide (LPS) are released during pathogen infection and chronic inflammation. These mediators activate the inflammatory response of the endothelium by inducing the expression of cytokines and leukocyte-adhesion molecules which are required for the activation, binding and extravasation of circulating leukocytes. Binding of TNF elicits TNF-receptor signaling from the plasma membrane and receptor internalization in endosomes. However, the TNF receptor continues signaling from its endosomal location. We found that stimulation of human primary endothelial cells with pro-inflammatory mediators induces the upregulation and activation of the small GTPase RhoB, a member of the Rho GTPase family, which associates to endosomes. TNF induces *de novo* RhoB synthesis through NFκB and JNK-dependent pathways. RhoB localizes to endosomes and regulates TNF-receptor intracellular traffic. RhoB expression silencing in endothelial cells prevented TNF-induced activation of the mitogen-activated kinases (MAPKs) p38 and JNK. Furthermore, RhoB downregulation with siRNA blocked TNF-induced

endothelial expression of the leukocyte adhesion molecule VCAM-1 and secretion of the cytokines IL-6 and IL-8. Thus, stimulation of endothelial cells with the pro-inflammatory cytokine TNF induces increased expression of RhoB, which in turn regulates activation of MAPKs and expression of the pro-inflammatory molecules VCAM-1, IL6 and IL8 downstream of TNF. We conclude that, through the regulation of TNF receptor traffic dynamics, RhoB contributes to TNF dependent signaling pathways that are crucial for the inflammatory response of endothelial cells.

Role of the cellular prion protein in cell migration

The prion protein (PrP) is a highly conserved glycoprotein expressed on multiple cell types, which can occur in two conformations. The cellular form, PrP^{C}, is involved in cell survival, responses to oxidative stress and T cell activation. The misfolded version of PrP^{C}, PrP^{Sc}, is pathological, being involved in a group of fatal neurodegenerative disorders known as prion diseases. PrP is anchored to the plasma membrane by a glycosyl-phosphatidyl-inositol lipid moiety and resides in cholesterol-rich membrane microdomains called lipid rafts. Ligation of PrP induces intracellular signaling, likely through the association to transmembrane proteins since PrP is fully extracellular. Potential PrP-binding molecules include several proteins involved in cell adhesion such as neural cell adhesion molecule NCAM, laminin, the laminin receptor LR/LRP and vitronectin. Current models propose that PrP signals as part of a plasma membrane-located multi-component protein complex that generates outside-in signals from the extracellular matrix to the intracellular milieu. Adhesion-induced signals have a crucial role in cell migration and survival. PrP is expressed on human endothelial cells, on peripheral blood mononuclear cells and on various human leukemic cell lines. Preliminary data suggest that PrP may be shed into the extracellular space upon endothelial cell activation. We are currently investigating the role of PrP in hematopoietic and endothelial cells by analysing multiple cell functions upon the silencing of PrP expression.

Publications

Rondaij MG, Bierings R, van Agtmaal EL, Gijzen KA, Sellink E, Kragt A, Ferguson SS, Mertens K, Hannah MJ, van Mourik JA, Fernandez-Borja M, Voorberg J. Guanine exchange factor RalGDS mediates exocytosis of Weibel-Palade bodies from endothelial cells. Blood 2008; 112:56-63.

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Research in the Department of Plasma Proteins is performed by two Principal Investigators, prof Koen Mertens PhD and Jan Voorberg PhD. Both PI's are mutually involved within the overall focus Hemostasis and Thrombosis on the different research lines. The research lines of Koen Mertens are described here, while research lines by Jan Voorberg involving Koen Mertens as well can be found with PI Jan Voorberg.

Structure and function of hemostatic proteins

The coagulation cascade comprises several serine proteases that act in combination with a non-enzymatic cofactor on phospholipid-containing membranes. During the past decade we have been focusing on the mechanism by which activated factor IX assembles with its co-factor factor VIII. While the biochemistry of hemophilia continues to be the focus of this research line, we are also addressing other, related proteins that have potential impact on the hemostatic system. One example of these proteins is the Factor Seven Activating Protease (FSAP). This is a 'novel' serine protease that has been reported to activate single-chain urokinase-type plasminogen activator (scuPA) and Factor VII (FVII), suggesting a key role in hemostasis. Since its discovery in 1996, numerous additional functions have been proposed, including inhibition of smooth muscle cell proliferation and migration. With its decreasing specificity, the physiological role of FSAP has become increasingly unclear. Rigorous studies have been limited by the difficulty of obtaining intact FSAP from blood or recombinant sources. Therefore, the aim of our study was to produce recombinant (r) FSAP suitable for functional studies, and to re-assess its role of FSAP as a trigger of coagulation and fibrinolysis. To overcome the problem of autoactivation and subsequent degradation, we constructed an FSAP variant in which the natural activation site (R313-I314) was replaced by a cleavage site for the bacterial protease thermolysin. While expression of wild-type FSAP invariably resulted in the accumulation of degraded FSAP, 293 cells produced FSAP-R313Q in intact form. This was purified by immunoaffinity chromatography, activated by thermolysin, and analyzed for its enzymatic activity. Activated rFSAP displayed the same affinity for chromogenic peptide substrates as plasma derived FSAP (pdFSAP). rFSAP retained its capability to activate scuPA (Km 62 nM). Vmax was increased through interaction with negatively charged surfaces like polyphosphate and heparin (3- and 6-fold, respectively). Surprisingly, rFSAP proved incapable of activating purified FVII, even in the presence of Ca2+ ions and lipid vesicles of varying composition, including up to 40 mol% negative phospholipids such as phosphatidylserine and cardiolipin (CL). On membranes of 100% CL FVII cleavage did occur, but this resulted in transient activation and rapid degradation. We conclude that FVII is surprisingly resistant

to activation by rFSAP. Intracellular membranes that are rich in CL, when exposed on apopototic cells, might function as cofactor supporting local FSAP activity. We found no evidence, however, in favor of a more general role of FSAP in FVII activation. We are currently addressing FSAP variants with a mutation in the serine protease domain. These are of particular interest because similar mutations are also occurring in factor IX, and are associated with hemophilia B. Studying dysfunctional FSAP variants might contribute to understanding the physiological role of this controversial serine protease.

Key publication

Fribourg C, Meijer A, Mertens K. The interface between the EGF2 domain and the protease domain in blood coagulation factor IX contributes to factor VIII binding and factor X activation. Biochemistry 2006; 45:10777-85.

Cellular receptors involved in coagulation factor clearance

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It has been well established that von Willebrand Factor (VWF) protects factor VIII (FVIII) from rapid clearance from the circulations. The underlying mechanism, however, remains poorly understood. In 1999, we and others demonstrated that FVIII interacts with the low-density lipoprotein receptor-related protein (LRP). Subsequently we found that also low-density lipoprotein receptor (LDLR) binds FVIII. *In vitro*, VWF blocks the interaction between FVIII and LDLR or LRP. Using a mouse model we found that, despite the presence of endogenous VWF, both LRP and LDLR contribute to the catabolism of FVIII *in vivo*. This suggests that dissociation of the FVIII-VWF complex in plasma or at the cell surface drives the LRP/LDLR dependent clearance of FVIII. We are therefore focusing on catabolism of FVIII in cellular models. We first assessed the effect of VWF on the binding of FVIII to the cell surface employing confocal microscopy. To this end, a functional FVIII derivative containing yellow fluorescent protein (FVIII-YFP) was incubated with LRP-expressing or LDLR-expressing CHO cells at 4ºC in the presence and absence of VWF. The results showed that there was a distinct yellow fluorescent staining of the cell surface irrespective of the presence of VWF. Similar experiments performed at 37ºC, however, revealed

yellow fluorescent focal spots rapidly appearing inside the cells, but only in the absence of VWF. Co-localization studies demonstrated that these spots originated from FVIII-YFP present in early endosomes. These findings suggest that VWF blocks the transfer of FVIII to its endocytic receptors but not the binding of FVIII to the cell surface. Intriguingly, in the presence of ristocetin, FVIII-YFP was localized inside the cells not only in the absence of VWF but also in its presence. In agreement with this finding, flow cytometric analysis confirmed that VWF was no longer able to prevent endocytosis of FVIII-YFP in the presence of ristocetin. These results suggest that in the complex with FVIII-YFP complex, switching VWF into its active conformation triggers the endocytic uptake of FVIII. The restored endocytosis of FVIII was not the consequence of a loss of affinity for upon VWF activation. This could be concluded from solid phase binding studies that showed that FVIII-VWF complex formation is indistinguishable in the presence and absence of ristocetin. We propose that switching VWF within the VWF-FVIII complex into its active conformation initiates a sequence of molecular events at the cell surface, which ultimately lead to the endocytic uptake of FVIII by cells expressing LRP or LDLR. Apart from these established endocytic receptors, however, also other, so far unidentified membrane constituents may contribute to FVIII uptake. We are currently addressing this issue employing a proteomics approach.

Key publication

Meijer AB, Rohlena J, van der Zwaan C, van Zonneveld AJ, Boertjes R, Lenting PJ, Mertens K. Functional duplication of ligand-binding domains within low-density lipoprotein receptor-related protein for interaction with receptor associated protein, alpha2-macroglobulin, factor IXa and factor VIII. Biochim Biophys Acta 2007; 1774:714-22.

Novel strategies for factor VIII delivery

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Gene therapy provides an attractive alternative for protein replacement therapy in Hemophilia A patients. However, human clinical trials have shown disappointing results so far. Recent insights have suggested to direct factor VIII (FVIII) gene delivery to cells that also express its natural carrier protein von Willebrand factor

(VWF) e.g. platelets and endothelial cells. VWF synthesized in endothelial cells is stored in storage organelles called Weibel-Palade bodies (WPBs) that release their content upon agonist-induced stimulation. Co-storage of the VWF/FVIII complex in WPBs and subsequent release of this complex upon agonist-induced stimulation has the potential of secreting large amounts of FVIII at sites of vascular injury as well as directly increasing FVIII half-life by protecting FVIII from proteolytic attack and premature clearance. In this study, we explored the feasibility of Blood Outgrowth Endothelial Cells (BOECs) as a cellular FVIII delivery device with particular reference to long-term production levels, intracellular storage in Weibel-Palade bodies and agonist-induced regulated secretion. Human BOECs were isolated from citrated venous blood collected from healthy donors, transduced at passage 5 with a single exposure to a lentiviral vector encoding human B-domain deleted FVIII-GFP. Transduced BOECs were characterized by FACS and immunohistochemical analysis. Intracellular distribution analysis was performed by subcellular fractionation followed by a density gradient. Secretion pathways of VWF and FVIII were determined by brefeldin A (BFA) treatment, which blocks constitutive release of newly synthesized proteins. Transduction with the lentiviral vector encoding FVIII-GFP resulted in 80% positive cells. The FVIII-GFP transduced BOECs expressed 1.6±1.0 pmol/1x10^6 cells/24 h FVIII antigen and 0.45±0.23 pmol/1x10^6 cells/24 h endogenous VWF. Immunohistochemical analysis demonstrated that FVIII-GFP was stored in WPBs together with VWF and P-selectin. Although agonist-induced (PMA) secretion resulted in an 8-fold increase of VWF, FVIII levels were only slightly increased compared to the non-stimulated control. Subcellular fractionation revealed that the ratio of FVIII:VWF in the WPBs was on average 1:15. Treatment of transduced BOECs with BFA demonstrated that basal secretion is responsible for the majority of the VWF secreted without stimulation, whereas FVIII is mainly released via the constitutive pathway. We conclude that lentiviral transduction of BOECs results in long-term FVIII-GFP expression and secretion of high FVIII levels. FVIII co-localizes with VWF and P-selectin in the WPBs, however, regulated secretion of FVIII from the WPBs seems to be restricted. The limited FVIII increase after stimulation seems to result from low sorting efficiency of FVIII to the WPBs. Therefore, the potential of regulated FVIII secretion from endothelial cells remains to be established.

Key publication

Van den Biggelaar M, Bierings R, Storm G, Voorberg J, Mertens K. Requirements for cellular co-trafficking of factor VIII and von Willebrand factor to Weibel-Palade bodies. J Thromb Haemost 2007; 4:2235-42.

Role of Beta2-glycoprotein-I in hemostasis

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We have recently shown that patients suffering from the antiphospholipid syndrome (APS) have antibodies with reactivity against several domains of beta2-glycoprotein I (beta2GPI). Domain I of beta2GPI proved to contain a major binding site for thrombosis-related antiphospholipid antibodies. In this study we investigated the heterogeneity of the antibody response against beta2GPI by isolating a panel of human monoclonal antibodies from two APS patients. Two patients were selected based on a history of recurrent thrombosis and the presence of anti-beta2GPI IgG antibodies in plasma. B-cells were isolated from these patients and cDNA coding for the variable heavy chain repertoire was cloned into pHEN1-VLrep. Single chain Fv fragments displayed on phages were selected for reactivity towards beta2-glycoprotein I and domain I. Subsequently, several clones were selected for full length IgG production and tested for reactivity towards different domains of beta2GPI and for LAC activity in an APTT-based assay. After several panning-rounds we selected 4 phages from the two patients for single chain Fv (scFv) production. The four clones were assigned to two groups based on their homology to variable heavy (VH) germline segments. Two clones bound domain I of beta2GPI (both used VH4-4) and two clones bound beta2GPI outside domain I (both VH3-23). Subsequently, we constructed scFv's from those 4 clones. Interestingly, for all four clones the transformation from the phages (multiple binding sites) to scFv's (one binding site) resulted in an almost complete loss of reactivity. To test whether this reduction in reactivity was based on the need for bivalency of beta2GPI-binding sites, we constructed full length IgG's. Indeed, all four clones regained their reactivity towards beta2GPI when produced as full-length IgG1. These unique patient-derived monoclonal antibodies are now being

characterized for LAC activity and other beta2GPI-dependent molecular interactions. Our current data raise the possibility that pathogenic anti-domain I antibodies contribute to the thrombotic complications observed in patients with APS.

Key publication

De Laat B, Mertens K, de Groot, PG. Mechanisms of disease: antiphospholipid antibodies – from clinical association to pathologic mechanism. Nat Clin Pract Rheumatol 2008; 4:192-9.

Prof Hanneke (J) Schuitemaker PhD

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As from mid December 2007, the research group of Professor Schuitemaker moved to the Academic Medical Center of the University of Amsterdam. The group is still part of the joint Sanquin - AMC Landsteiner Laboratory. Professor Schuitemaker also acts as advisor to Sanquin with regards to virological issues.

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Host genetic factors that influence the clinical course of HIV-1 infection

The effect of Trim5 polymorphisms on the clinical course of HIV-1 infection

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The antiviral factor tripartite interaction motif 5alpha (Trim5alpha) restricts a broad range of retroviruses in a species-specific manner. Although human Trim5alpha is unable to block HIV-1 infection in human cells, a modest inhibition of HIV-1 replication has been reported. Recently two polymorphisms in the Trim5 gene (H43Y and R136Q) were shown to affect the antiviral activity of Trim5alpha *in vitro*. In this study, participants of the Amsterdam Cohort studies were screened for polymorphisms at amino acid residue 43 and 136 of the Trim5 gene, and the potential effects of these polymorphisms on the clinical course of HIV-1 infection were analyzed. In agreement with the reported decreased antiviral activity of Trim5alpha that contains a Y at amino acid residue 43 *in vitro*, an accelerated disease progression was observed for individuals who were homozygous for the 43Y genotype as compared to individuals who were heterozygous or homozygous for the 43H genotype. A protective effect of the 136Q genotype was observed but only after the emergence of CXCR4-using (X4) HIV-1 variants and when a viral load of 10(4.5) copies per ml plasma was used as an endpoint in survival analysis. Interestingly, naive CD4 T cells, which are selectively targeted by X4 HIV-1, revealed a significantly higher expression of Trim5alpha than memory CD4 T cells. In addition, we observed that the 136Q allele in combination with the -2GG genotype in the 5'UTR was associated with an accelerated disease progression. Thus, polymorphisms in the Trim5 gene may influence the clinical course of HIV-1 infection also underscoring the antiviral effect of Trim5alpha on HIV-1 *in vivo*.

Association of HLA-C and HCP5 gene regions with the clinical course of HIV-1 infection

Recently, a genome-wide association analysis revealed single-nucleotide polymorphisms (SNPs) in the gene regions of HLA-C and HCP5 to be associated with viral load at set point and SNPs in the RNF39/ZNRD1 gene region to be associated with HIV-1 disease course. We studied whether the association of these SNPs with viral load

at set point could be replicated and whether these SNPs also associated with other clinical outcomes of HIV-1 infection in 335 HIV-1-infected homosexual participants from the Amsterdam Cohort Studies on HIV infection and AIDS (ACS).
Significant associations between the minor allele variants of SNPs HLA-C rs9264942 and HCP5 rs2395029 and a lower viral load at set point could be replicated in the ACS. Moreover, these SNPs were significantly associated with delayed progression to AIDS, AIDS-related death, and a CD4 T-cell count below 400 cells/mul. Both minor allele variants were independent predictors of disease progression, also when a CCR5 Delta32 heterozygous genotype was included in the analysis. However, predictive value was not independent from viral load and CD4 T-cell count at set point. The SNPs in the RNF39/ZNRD1 gene region were associated with set point CD4 T-cell count but not with disease course in the ACS.
The minor allele variants of SNPs in the HLA-C and HCP5 gene regions are also in the ACS associated with a lower viral load at set point and additionally with delayed HIV-1 disease progression. The association of these SNPs with the relatively early course of infection may help unravel their mode of action.

Key publications

Van Manen D, Kootstra NA, Boeser-Nunnink B, Handulle MA, van 't Wout AB, Schuitemaker H. Association of HLA-C and HCP5 gene regions with the clinical course of HIV-1 infection. AIDS 2009; 23(1):19-28.

Van Manen D, Rits MA, Beugeling C, van Dort K, Schuitemaker H, Kootstra NA. The effect of Trim5 polymorphisms on the clinical course of HIV-1 infection. PLoS Pathog 2008; 4(2):e18.

Characterization and optimization of HIV-1 specific humoral immunity

Autologous neutralizing humoral immunity and evolution of the viral envelope in the course of subtype B human immunodeficiency virus type 1 infection

Most human immunodeficiency virus type 1 (HIV-1)-infected individuals develop an HIV-specific neutralizing antibody (NAb) response that selects for escape variants of the virus. Here, we studied autologous NAb responses in five typical CCR5-using progressors in relation to viral NAb escape and molecular changes in the viral envelope (Env) in the period from seroconversion until after AIDS diagnosis. In sera from three patients, high-titer neutralizing activity was observed against the earliest autologous virus variants, followed by declining humoral immune responses against subsequent viral escape variants. Autologous neutralizing activity was undetectable in sera from two patients. Patients with high-titer neutralizing activity in serum showed the strongest positive selection pressure on Env early in infection. In the initial phase of infection, gp160 length and the number of potential N-linked glycosylation sites (PNGS) increased in viruses from all patients. Over the course of infection, positive selection pressure declined as the NAb response subsided, coinciding with reversions of changes in gp160 length and the number of PNGS. A number of identical amino acid changes were observed over the course of infection in the viral quasispecies of different patients. Our results indicate that although neutralizing autologous humoral immunity may have a limited effect on the disease course, it is an important selection pressure in virus evolution early in infection, while declining HIV-specific humoral immunity in later stages may coincide with reversion of NAb-driven changes in Env.

Key publications

Bunnik EM, Pisas L, van Nuenen AC, Schuitemaker H. Autologous neutralizing humoral immunity and evolution of the viral envelope in the course of subtype B human immunodeficiency virus type 1 infection. J Virol 2008; 82(16):7932-41.

Quakkelaar ED, van Alphen FP, Boeser-Nunnink BD, van Nuenen AC, Pantophlet R, Schuitemaker H. Susceptibility of recently transmitted subtype B human immunodeficiency virus type 1 variants to broadly neutralizing antibodies. J Virol 2007; 81(16):8533-42.

Host CTL pressure driving HIV-1 evolution

Molecular evolution of human immunodeficiency virus type 1 upon transmission between human leukocyte antigen disparate donor-recipient pairs

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To address evolution of HIV-1 after transmission, we studied sequence dynamics in and outside predicted epitopes of cytotoxic T lymphocytes (CTL) in subtype B HIV-1 variants that were isolated from 5 therapy-naive horizontal HLA-disparate donor-recipient pairs from the Amsterdam Cohort Studies on HIV-1 infection and AIDS. This was performed in collaboration Debbie van Baarle PhD, José Borghans PhD and prof Frank Miedema PhD from the Department of Immunology of Utrecht University Medical Center.

In the first weeks after transmission, the majority of donor-derived mutations inside and outside donor-HLA-restricted epitopes in Gag, Env, and Nef, were preserved in the recipient. Reversion to the HIV-1 subtype B consensus sequence of mutations inside and outside donor-HLA-restricted CTL epitopes, and new mutations away from the consensus B sequence mostly within recipient-HLA-restricted epitopes, contributed equally to the early sequence changes. In the subsequent period (1-2 years) after transmission, still only a low number of both reverting and forward mutations had occurred. During subsequent long-term follow-up, sequence dynamics were dominated by forward mutations, mostly (50-85%) in recipient-HLA-restricted CTL epitopes. At the end of long-term follow-up, on average 43% of the transmitted CTL escape mutations in donor-HLA-restricted epitopes had reverted to the subtype B consensus sequence.

The relatively high proportion of long-term preserved mutations after transmission points to a lack of back selection even in the absence of CTL pressure, which may lead to an accumulating loss of critical CTL epitopes. Our data are supportive for a continuous adaptation of HIV-1 to host immune pressures, which may have implications for vaccine design.

A nonprogressive clinical course in HIV-infected individuals expressing human leukocyte antigen B57/5801 is associated with preserved CD8+ T lymphocyte responsiveness to the HW9 epitope in Nef

The human leukocyte antigen (HLA) B57 allele and the closely related HLA-B5801 allele are overrepresented among human immunodeficiency virus type 1 (HIV-1)-infected individuals with a long-term nonprogressive clinical course of disease (known as 'long-term nonprogressors', LTNPs). These alleles are, however, also present among individuals with normal disease progression (known as 'progressors'). In a comparison of HLA-B57/5801-expressing progressors and LTNPs, we observed a similar prevalence of escape mutations in 4 Nef epitopes and a similar reactivity of CD8+ T cells against 3 of 4 of these epitopes and their autologous escape variants. However, LTNPs tended to have frequent and preserved CD8+ T cell interferon-gamma responses against the wild-type HW9 Nef epitope, whereas progressors did not maintain a specific CD8+ T cell response. This finding is in line with the findings of a more exhausted phenotype of CD8+ T cells in progressors, as is demonstrated by their enhanced level of expression of inhibitory receptor 'programmed death 1' (PD-1). The results of the present study suggest that preservation of HW9-specific T cell responses is associated with a more benign clinical course of infection.

Key publications

Navis M, Matas DE, Rachinger A, Koning FA, van Swieten P, Kootstra NA, Schuitemaker H. Molecular evolution of human immunodeficiency virus type 1 upon transmission between human leukocyte antigen disparate donor-recipient pairs. PLoS ONE 2008; 3(6):e2422.

Navis M, Schellens IM, van Swieten P, Borghans JA, Miedema F, Kootstra NA, van Baarle D, Schuitemaker H. A nonprogressive clinical course in HIV-infected individuals expressing human leukocyte antigen B57/5801 is associated with preserved CD8+ T lymphocyte responsiveness to the HW9 epitope in Nef. J Infect Dis 2008; 197(6):871-9.

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Phagocyte activation

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Neutrophils and macrophages recognize pathogens by means of a variety of surface receptors. These include non-opsonic pattern recognition receptors (PRRs), such as scavenger receptors, Toll-like receptors (TLR) or NOD-like receptors (NLR), that interact with more or less conserved microbial structures, and opsonic receptors, such as Fc- or complement receptors, that recognize antibodies or complement fragments covering the microbes. In addition, we are studying the role of several families of immunoreceptors in the regulation of innate immune cell activation.

CD163

CD163 is a member of the scavenger receptor cysteine-rich group B (SRCR-B) family selectively expressed by the majority of mature tissue macrophages. It mediates recognition and clearance of hemoglobin-haptoglobin complexes and this appears particularly relevant during excessive hemolysis. Furthermore, our previous work has demonstrated a role for CD163 in erythroblast adhesion and this interaction promotes the contact between macrophages and erythroblasts in erythroblastic islets, which is important for proper erythropoiesis. Since some related members of the scavenger receptor group B cysteine-rich scavenger receptor family to which CD163 belongs play a role in microbial recognition we studied a potential role for CD163 as a bacterial receptor. Indeed, our findings have shown that CD163 mediates recognition of intact gram-positive of and gram-negative bacteria. Moreover, bacterial binding to CD163 triggers the production of a variety of proinflammatory cytokines. This suggests that CD163 on mature tissue macrophages may act as a sensor for bacterial infection in many tissues.

Key publication

Fabriek BO, van Bruggen R, Deng DM, Ligtenberg AJ, Nazmi K, Schornagel K, Vloet RP, Dijkstra CD, van den Berg TK. The macrophage scavenger receptor CD163 functions as an innate immune sensor for bacteria. Blood 2009; 113(4):887-92.

TLR and NLR

Members of the TLR and NLR families act as receptors for conserved microbial components, such as lipopolysaccharide (LPS) and others, and their role in macrophage activation is relatively well documented. We are focussing on the role of TLR and NLR in neutrophil function. Studies with neutrophils from an individual suffering from deficiency of IRAK-4, a protein that interacts with MyD88 and is pivotal for the MyD88-dependent pathway of TLR signaling, indicate that IRAK-4 forms an absolute and common requirement for TLR signaling in neutrophils.
We are also studying the role of NLR protein family members in phagocytes. NLR proteins act as putative sensors for microbial and host components that together with caspase-1 and adaptor proteins form complexes, termed inflammasomes. Upon activation, these inflammasomes mediate the cleavage of pro-IL1β into active IL1β. Sustained activation of inflammasomes, either by excess of ligand (e.g. uric acid crystals in gout) or by activating mutations, leads to autoinflammatory syndromes. Work in collaboration with the group of prof Jurg Tschopp (Lausanne, Switzerland) has shown that phagocytosis, even of inert particles such as asbestos or silica, constitutes a stimulus for inflammasome activation in macrophages and this process may require the formation of reactive oxygen species that are probably not formed by a NADPH oxidase. Our results also provide evidence for the existence of functional inflammasomes in neutrophils.

Key publications

Dostert C, Pétrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. Science 2008; 320(5876):674-7.

Koller B, Kappler M, Latzin P, Gaggar A, Schreiner M, Takyar S, Kormann M, Kabesch M, Roos D, Griese M, Hartl D. TLR expression on neutrophils at the pulmonary site of infection: TLR1/TLR2-mediated up-regulation of TLR5 expression in cystic fibrosis lung disease. J Immunol 2008; 181(4):2753-63.

Fc- and complement-receptors

Opsonization by complement of antibodies is often required for an effective uptake and removal of microbes by phagocytes. This involves receptors for complement, such as CR3 or Fc- receptors (FcRs) that recognize the Fc region of antibodies. In our evaluation of FcR gene variation in the human population we have identified a rare gain-of-function FcγRIIa variant with an additional exon that extends the cytoplasmic tail of the receptor. Individuals carrying this FcγRIIa allele often suffer from acute or chronic inflammatory reactions, and some have actually experienced severe anaphylactic reactions upon IVIG treatment. Neutrophils from individuals with this mutation show a more potent degranulation response. Furthermore, expression of the variant FcγRIIa resulted in a higher CD32-mediated Ca2+ response than the common variant.

Key publications

Brouwer N, Dolman KM, van Houdt M, Sta M, Roos D, Kuijpers TW. Mannose-binding lectin (MBL) facilitates opsonophagocytosis of yeasts but not of bacteria despite MBL binding. J Immunol 2008; 180(6):4124-32.

Breunis WB, van Mirre E, Bruin M, Geissler J, de Boer M, Peters M, Roos D, de Haas M, Koene HR, Kuijpers TW. Copy number variation of the activating FCGR2C gene predisposes to idiopathic thrombocytopenic purpura. Blood 2008; 111(3):1029-38.

Hartl D, Krauss-Etschmann S, Koller B, Hordijk PL, Kuijpers TW, Hoffmann F, Hector A, Eber E, Marcos V, Bittmann I, Eickelberg O, Griese M, Roos D. Infiltrated neutrophils acquire novel chemokine receptor expression and chemokine responsiveness in chronic inflammatory lung diseases. J Immunol 2008; 81(11):8053-67.

Immunoreceptors

While much attention is focussed on the receptors and pathways that trigger the activation of phagocytes and other innate immune cells, such as NK cells, little is known about the mechanisms by which the activity of innate immune cells is controlled. We are investigating the role of several families of immunoreceptors in this context. Apart

from Fc-receptors, already mentioned above, these include signal regulatory proteins (SIRP), primarily found on myeloid cells, and killer immunoreceptors (KIR), expressed on NK cells. SIRPα is a typical inhibitory receptor that is selectively expressed on myeloid and neuronal cells. It acts as a receptor for the broadly expressed surface molecule CD47, and the ligation of SIRPα by CD47 results in the recruitment and activation of tyrosine phosphatases, such as SHP-1 and SHP-2, to immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the cytoplasmic tail of SIRPα. We propose that, in analogy to MHC class I molecules that restrict NK cell function via killer inhibitory receptors, CD47 acts as a 'self' molecule to control phagocyte functions. Indeed, there is solid evidence now that CD47-SIRPα interactions negatively regulate the clearance of erythrocytes and platelets by macrophages *in vivo*. We are investigating whether also other phagocyte activities are controlled via SIRPα. We have demonstrated, for instance, that SIRPα is a negative regulator of the phagocyte oxidative burst. Over-expression of full-length SIRPα in PLB985 cells strongly suppresses the oxidative burst, and this appears due to a down-regulation of $gp91^{phox}$. Furthermore, macrophages and granulocytes from SIRPα-mutant mice display an enhanced oxidative burst and an enhanced expression of $gp91^{phox}$. This may suggest that SIRPα limits activity of the phagocyte NADPH oxidase in order to prevent excessive 'collateral' oxidative damage to the host during infection and this has futher been substantiated in bacterial infection models. In collaboration with the group of prof V Everts (Dept Oral Cell Biology, ACTA, Amsterdam) we have demonstrated that osteoclasts from SIRPα-mutant mice that carry a defect in SIRPα signaling have an increased bone resorption capacity, both *ex vivo* as well as *in vivo*. Osteoclasts with a defect in SIRPα signaling also display an increased actin ring formation that form an integral component of the so called sealing zone, that allows osteoclasts to strongly adhere to the bone surface and create a specialized extracellular compartment for bone resorption. Finally, we have obtained evidence that interactions between CD47 on tumor cells and SIRPα on macrophages form a barrier for antibody-dependent cellular cytotoxicity of phagocytes towards tumor cells. How individual differences with respect to gene composition, polymorphisms and copy number variation in immunoreceptors relate to the susceptibility and progression of disease, including infection, (auto)immunity and cancer is being studied by Multiplex Ligation-dependent Probe Amplification (MLPA).

Key publication

Van den Berg TK, van der Schoot CE. Innate immune 'self' recognition: a role for CD47-SIRPalpha interactions in hematopoietic stem cell transplantation. Trends Immunol 2008; 29(5):203-6.

Immunodeficiencies

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A variety of genetic defects in phagocyte function give rise to an increased susceptibility to bacterial and fungal infection. We are investigating in (pediatric) patients the genetic basis of such immune defects and the resulting consequences for phagocyte development and function.

CGD

Chronic granulomatous disease (CGD) is caused by mutations in genes encoding the subunits of the phagocyte NADPH oxidase that plays a pivotal role in the oxidative killing of micro-organisms in the phagosome. As the European Registry for Genetic Database on CGD Apart from a number of previously undescribed mutations in NADPH oxidase genes that we have identified. We have also identified a number of patients with a novel subtype of CGD, termed partial CGD, characterized by strongly diminished oxidative burst in response to bacterial peptide f-Met-Leu-Phe (fMLP), but relatively normal responses to phorbol ester and serum-treated zymosan (STZ). We are currently characterizing the defect(s) in partial CGD in more detail. Activation of the NADPH oxidase is controlled by assembly of the complex at the phagosomal membrane, but the dynamics and requirements of this process have not been properly documented. We have generated a panel of cell lines expressing fluorescently tagged NADPH oxidase components and are using fluorescence resonance energy transfer (FRET) and other advanced fluorescent imaging techniques to study this at the single-cell level. This is done in collaboration with the group of dr C Otto (University of Twente).

Key publications

Wolach B, Gavrieli R, de Boer M, Gottesman G, Ben-Ari J, Rottem M, Schlesinger Y, Grisaru-Soen G, Etzioni A, Roos D. Chronic granulomatous disease in Israel: clinical, functional and molecular studies of 38 patients. Clin Immunol 2008; 129(1):103-14.

Van Manen HJ, Verkuijlen P, Wittendorp P, Subramaniam V, van den Berg TK, Roos D, Otto C. Refractive index sensing of green fluorescent proteins in living cells using fluorescence lifetime imaging microscopy. Biophys J 2008; 94(8):L67-9.

Shwachman-Diamond Syndrome

Our current efforts are also directed to characterize other types of neutrophil defects in greater detail. For instance, Shwachman-Diamond Syndrome (SDS) is an autosomal, recessively inherited disorder characterized by bone marrow failure with significant risk of developing pediatric acute myeloid leukemia (AML) and myelodysplasia (MDS), which is often refractory to treatment. Neutropenia and defective neutrophil chemotaxis are the most frequently observed hematological abnormalities in SDS patients. In this respect, the role of SBDS (the protein defective in SDS) is studied in normal myeloid differentiation and function by transfection and knock-down strategies. We have found that SBDS co-localizes with the microtubule organizing center as well as with the mitotic spindle, suggesting a potential role for SBDS in regulating chromosome segregation during mitosis. We have conducted life-cell-imaging intracellular trafficking studies with SBDS mutant proteins, which provides evidence for post-translational modifications and interactions of SBDS with nuclear and cytoplasmic binding partners, including microtubules. We anticipate that the defective SBDS function in SDS results in cell migration and division defects, potentially contributing to the increased risk of developing leukemia.

LAD/variant syndrome

We have also performed studies to identify the genetic defect in the leukocyte adhesion deficiency variant syndrome, that was originally described in our laboratory in 1997. LAD/v is characterized by recurrent non-pussing infections and a Glanzman-type bleeding disorder, caused by impaired leukocyte extravasation and platelet

aggregation, respectively, which in turn are the result of a defect in integrin inside-out signaling. Although it had been suggested that mutation of the Rap1 exchange factor CALDAG-GEF was responsible for LAD/v, our previous studies already demonstrated convincingly that this was not the case. Using homozygosity mapping we localized the defect to a 13 MB region on chromosome 11, containing the FERMT3 gene. All patients, and none of the controls, were shown to have mutations in FERMT3. These mutations lead to (different) premature stopcodons in the kindlin-3 protein, encoded by FERMT3, a protein that associates with integrins in focal adhesions in hematopoietic cells. Thus, mutations in the FERMT3/kindling-3 gene are responsible for LAD/v syndrome. Studies to understand the role of kindlin-3 in phagocytes and its participation in integrin activation are ongoing.

Key publication

Kuijpers TW, van de Vijver E, Weterman MA, de Boer M, Tool AT, van den Berg TK, Moser M, Jakobs ME, Seeger K, Sanal O, Unal S, Cetin M, Roos D, Verhoeven AJ, Baas F. LAD-1/variant syndrome is caused by mutations in FERMT3. Blood 2009; 113(19):4740-6.

Apoptosis

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Neutrophils have a short life-span and die by apoptosis. In fact, apoptosis of neutrophils constitutes an important mechanism for limiting the inflammatory response. We have shown that in neutrophils mitochondria are not important for ATP synthesis, but that their primary role may rather be in the regulation apoptosis in these cells. An important factor in the regulation of neutrophil survival is G-CSF. G-CSF promotes the development of immature granulocytes and the mobilization of neutrophils from the bone marrow, but also prolongs survival of mature neutrophils by delaying apoptosis. The mechanisms underlying this effect have remained elusive. Our findings show now that G-CSF acts at a level downstream of caspase-8 and mitochondria, and upstream of the caspases-9 and -3. In particular, G-CSF controls the increased calcium (Ca2+) influx during apoptosis, thereby preventing activation of Ca2+ dependent calpain. Calpain inhibition resulted in the stabilization of the X-linked inhibitor of apoptosis (XIAP) and hence inhibited caspase-9 and -3 in

human neutrophils. Counter intuitively, we have now also shown that mitochondria in neutrophils produce reactive oxygen species and that these can mediate the pro-survival effect of TNFα on these cells.
Apoptosis is also believed to play an important role during myeloid development. We have thus far focused on the myeloid inhibitory receptor SIRPα that seems to provide pro-apoptotic signals in myeloid cells. This may be important during acute myeloid leukemia (AML). SIRPα is expressed on mouse and human hematopoietic stem cells and throughout myeloid development, but expression on AML is significantly reduced as observed for both SIRPα mRNA and protein. This appeared due, at least in part, to an indirect mechanism of epigenetic silencing of SIRPα gene expression as shown by studies with inhibitors of DNA methylation and histone deacetylation and by methylation analysis of the SIRPα promoter region. Importantly, reconstitution of expression in t(8;21) and t(15;17) AML cell lines and triggering of SIRPα by agonistic antibodies induces apoptosis. This suggests that the growth of myeloid leukemic cells may be controlled by SIRPα, and that a low SIRPα expression on AML may contribute to their uncontrolled proliferation and survival.

Key publications

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Granulocyte transfusion

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Granulocyte transfusion provides support for neutropenic patients suffering from life-threatening infections. The large numbers of granulocytes required can be mobilized from the bone marrow of donors by treatment with G-CSF and dexamethasone. We have shown that this treatment does not significantly affect

the major effector functions of neutrophils. Moreover, G-CSF and dexamethasone prolong the potential storage time *in vitro*. In order to identify components or signaling pathways involved in extending survival we have performed microarray analysis of neutrophils before and after G-CSF plus dexamethasone treatment. Among a large number (~1000) of regulated genes we observed a strong upregulation of calpastatin, the endogenous inhibitor of calpains, a family of calcium-dependent cysteine proteases known to be involved in neutrophil apoptosis. The mRNA expression of calpastatin was induced by G-CSF/dexamethasone treatment, both *in vivo* and *in vitro*, while the protein expression of calpastatin was stabilized during culture. Clearly, this upregulation of calpastatin upon treatment with G-CSF and dexamethasone may contribute to the increased survival of neutrophils. Apart from calpastatin we have also observed regulation of components of the TLR signaling pathway(s) and studies to reveal the functional consequences of this are ongoing.

Key publications

Drewniak A, Boelens JJ, Vrielink H, Tool AT, Bruin MC, van den Heuvel-Eibrink M, Ball L, van de Wetering MD, Roos D, Kuijpers TW. Granulocyte concentrates: prolonged functional capacity during storage in the presence of phenotypic changes. Haematologica 2008; 93(7):1058-67.

Drewniak A, van Raam BJ, Geissler J, Tool ATJ, Mook O, van den Berg TK, Baas F, Kuijpers TW. Changes in gene expression of granulocytes during in vivo G-CSF/dexamethasone mobilization for transfusion purposes. Blood 2009 Apr 6.

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Notwithstanding that blood products for transfusion are very safe, with new technologies there are always means to improve safety even further. As blood is allogeneous human material of humans who are exposed to a changing environment with new threats, complete blood safety can never be obtained. In addition, emerging infectious diseases may require new safety interventions. Dutch governmental policy is to balance such developments aiming at 'optimal blood safety' *versus* what is considered as 'maximum blood safety': complete risk aversion. Optimal blood safety however is still not well defined, but cost-utility or cost-effectiveness evaluations of safety measures are included in the decision process. Risk assessments underlying these analyses draw attention not only to the decision models but also to communication of present risk – or safety – level to regulatory bodies and the public. With increasing pressure on cost containment in health care,

cost-effectiveness analyses of blood safety interventions are internationally becoming more relevant. For these analyses nationally representative data on clinical blood use and blood recipient profiles, including recipient survival, are needed in addition to risk analyses of adverse outcomes of blood transfusion.
Given a new emerging infectious disease, the risk of disease or negative health outcome for recipients of different blood products needs to be assessed. Such assessments require modeling of the transfusion chain as well as costs and effects of given interventions: properties of the (new) infectious agent, donor epidemiology and donation behavior, test characteristics, processing and inactivation steps and distribution of the end products.

In collaboration with the Department of Medical Technology Assessment of the Julius Center for Health Sciences and Primary Care at the Utrecht University, a 'Transfusion Technology Assessment' group has been formed, with the explicit mission to perform risk assessments and cost-effectiveness analyses on blood safety and to establish a nationally representative database of clinical blood use and blood recipient profiles. This means measurement and modeling of costs and effects associated with emerging threats given the national blood transfusion data and the evaluation of proposed blood safety interventions.

Ten years of blood transfusions: use, disease and survival (PROTON-study)

In the Netherlands about 954,500 blood component transfusions are given annually. As Sanquin and hospitals are separate organizations, and hospitals have diverse information systems, little quantitative information on transfusion recipient profiles is known. The distribution of various patient groups, underlying diseases, the amount and type(s) of components transfused, and the survival of the recipients are parameters required for evaluating the (cost-) effectiveness of blood safety interventions. Information on patients receiving blood components between January 1995 and December 2006 has been collected from 21 Dutch hospitals. The dataset is in 2008 now complete and contains information on more than 2 million transfusions (component type, number of transfusions) and the transfused patients (age, gender). These data were linked to mortality and diagnoses databases (LMR) at

Statistics Netherlands (CBS). Distributions of blood components, age and diagnoses are compared for various hospitals. The data were extrapolated to estimate the distributions of blood recipient characteristics in the Netherlands as a whole. The dataset now contains information on 244,273 patients who received 2,123,144 blood product transfusions. Distributions of transfusion patient characteristics show similar patterns for hospitals of the same category. The age distribution of academic hospitals shows a large peak for newborns. In both academic and general hospitals most of the blood goes to elderly patients. Most of the red blood cells (RBCs) given in academic hospitals are transfused to patients with neoplasms and circulatory diseases. Even numbers of RBCs are given more often than odd numbers, so RBCs are mostly given in pairs. The post-transfusion survival rate is higher in general hospitals than in academic hospitals. Survival of women who are transfused around child delivery does not differ significantly from survival of the general population. Recipient survival in terms of blood use and underlying disease was analyzed. Censuring the survival rates proved to be complex and a model for proper calculation of the survival was performed in collaboration with the Department of Applied Mathematics of the Technical University Delft. The PROTON study provides quantitative information on various characteristics of blood transfusion recipients. The similarities in distribution patterns of patient characteristics in hospitals of the same category suggest that the (randomly sampled) hospitals included in the study can be used to extrapolate these distributions to national level. In 2008 the model was further successfully validated for representation of the total Dutch transfusion chain. Identifying differences between hospitals on their use of blood can be used for the optimization and of blood usage. The PROTON data are essential for cost-utility analyses on new safety interventions in the blood supply.

Identifying relevant parameters for prediction of national blood demand

It was concluded in a recent report on the 2nd evaluation of the Dutch Blood Act, encompassing the obligation of Sanquin to provide sufficient supply *(Tweede evaluatie Wet inzake bloedvoorziening, ZONMW)*, that changes in the characteristics of the national population will affect changes in the populations of blood recipients and donors. It is possible that measures need to be taken for the continuation of sufficient national supply and to meet the national demand. The Executive Board of

Sanquin has asked the TTA group to identify key parameters in the PROTON study relevant for the prediction of possible changes in the distribution and demand of blood components. In 2008 the PREDICT study was started based on the PROTON database and demographic models of Statistics Netherlands (CBS). It appears that other factors in addition to demographic variation determine the demand and use of blood. This study will be finalized in 2009.

Cost-Utility of Blood In Transfused patients (CUBiT-study)

The incremental cost-effectiveness ratio (ICER) of new blood safety measures may show poor cost-effectiveness, as the effects of recent measures are modest in comparison to the already achieved blood safety level. Reporting such unfavorable ICER's of new safety measures such as NAT in the medical literature, may generate biased opinions as to the value of blood transfusion in itself. The Executive Board of Sanquin has asked the TTA group to start a study, based on the PROTON data, to establish the cost-utility of blood as a therapy in different transfused patient categories. Given the succesful validation of the PROTON data base, the top-ten indications and recipient profiles are selected. Formal expert elicitation for estimating survival of untransfused patients will be started.

Modeling emerging Infections in the Transfusion Chain (MITCH-study)

Recent collaboration initiatives of the Julius Centre and the TTA group with scientists and prof RA Coutinho of the Centre for Infectious Disease Control (Cib) of the National Institute for Public Health and Environment (RIVM), strengthens the knowledge base for infectious disease modeling in the transfusion chain. Factors such as global climate change, increased traffic, and intensive agricultural methods generated increasing concern about recent outbreaks of emerging infections. Rational decisions for blood safety based on quantitative risk assessments are needed. The Executive Board of Sanquin has asked the TTA group to develop risk models for emerging Transfusion Transmitted Infections (TTI) in the transfusion chain. As emerging infections can be unpredictable by nature, a generic TTI model in the transfusion chain will be developed encompassing all relevant model parameters for biological and epidemiological characteristics of example TTI's, representative of different

possibly emerging TTI's. This model will allow ad hoc introduction of actual emerging infectious disease parameters into the model, thereby allowing timely assessment of the quantitative risk to the blood supply. The risk analysis and decision processes include structured expert opinion elicitation and decision frameworks. Such strategies are common in other fields like industrial risk management, water management and environment, however they are not yet practiced in the blood transfusion chain. In 2008, in collaboration with the National Centre of Infectious Disease Control (Cib / RIVM) the risk modeling of Q-fever transmission after the 2008 outbreak in The Netherlands was started. The implications of this study will have European (ECDC) dimensions.

Viral risks of plasma-derived medicinal products

New European legislation (EMEA guideline CPMP/BWP/5180/03) requires a viral risk assessment for HBV, HCV, HIV, Parvo B19 and HAV for all new market applications of plasma products. A risk model was developed for Sanquin Plasma Products on the basis of viral and test characteristics, donor epidemiology and Sanquin Virus Safety Studies (VSS) inactivation data. The model has been discussed at confidential meetings of the International Plasma Fractionation Association (IPFA) with risk assessors of the Biotechnology Products Laboratory (BPL) of the United Kingdom. The results of model sensitivity analyses show that the residual risk is mainly determined by the viral incidence rate, screening test sensitivity, viral reduction capacity and the product yield. The production pool size and type of donation (apheresis or whole blood donation) have low impact on the residual risk. Increasing the inventory hold period has a modest impact on the residual risk, only 0.5 logs for 1 year increase in hold period. The results show that there is large dispersion in the residual risk estimates (2 to 6 logs) depending on type of virus. Monte-Carlo (probabilistic) simulations are essential when estimating residual risks of blood products. This approach in contrast to traditional risk estimation allows incorporation of complex process specific decision strategies into the risk model. It also allows modeling of uncertain model parameters, like incubation time, duration of the window phase or viral load of an infected donation. Counter-intuitive findings were that production pool size and type of donation e.g. apheresis or

whole blood donation only have a limited impact on the residual risk. The detailed results of the study remain proprietary of Sanquin, and are to be submitted to the European Medicines Evaluation Agency (EMEA), however the methodology of the risk assessment, which is first in this field, has been presented at several conferences and has recently been published (Janssen et al, Transfusion 2008). In 2008 the probabilistic risk analysis methods for plasma products were further implemented in the field, including the organization of a dedicated session at the American Association of Blood Banks annual conference. In 2009 it is expected that this approach will be further welcomed by EMEA and the EU Commission.

Experimental design and analysis of Virus Validation Studies

A study was performed to evaluate the effectiveness of the design and analysis of the robustness of virus validation studies. The aim is to improve on the output of these expensive experiments through evaluation of the current experimental design process and by applying advanced statistical techniques for analyzing the results. This work is performed in collaboration with the Department of Applied Mathematics of the Technical University Delft. It was found that indeed more information on relevant process parameters can be obtained by applying more advanced modeling techniques (N van Geloven, *Statistical evaluation and design of virus validation robustness studies,* Masters Thesis TU Delft, May 2007).

Determining the frequency of positive test results of additionally tested manufacturing pools

A study was performed to predict the frequency of positively NAT tested manufacturing pools for a plasma product. In this case the pool size of minipool NAT testing approaches the order of magnitude of the pool size of the manufacturing pools. It was found that given the donor epidemiology, additional NAT testing of the manufacturing pool can improve the safety of these plasma products, however at considerable loss of manufacturing pools. An alternative strategy may be to reduce the pool size of minipool NAT testing. The results of the study remain proprietary of Sanquin.

vCJD risk of plasma-derived medicinal products

A risk model was developed for Sanquin on the basis of expert opinion on variant Creutzfeldt-Jakob Disease (vCJD) and estimates on donor epidemiology and production process inactivation data. Monte-Carlo simulations were used for estimating the contamination risk of blood components and plasma products. Model outcomes have been discussed at confidential meetings with the Medical Advisory Board of Sanquin. The methodology has been discussed at the international risk assessment meetings of the International Expert Advisory Group of Health Canada workshops on Iterative Risk Assessment Processes for Policy Development Under Conditions of Uncertainty and Emerging Infectious Diseases. The results of the study remain proprietary of Sanquin.

Monitoring of viral infection incidence rates among blood donors in The Netherlands

Presently EMEA requires manufacturers of plasma products to report on the prevalence and incidence rates of HIV, HBV and HCV in donor populations. A proprietary report is written for Sanquin. An important measure of residual blood safety is the incidence rate. The goal of the project is to develop a monitoring tool, enabling the detection of significant deviations in incidence rates in repeat tested donors. First a developed monitoring tool is used to check changes in the incidence rates on the national level. Second, incidence rates variability is explored and regional differences within The Netherlands are examined. Furthermore, statistical tests are used to evaluate and thus enable controlling the infection rates in the repeat tested donor population. For this purpose, two statistical tests were developed. As there is a dependency between donation frequency of the infected donors and the estimated HBV incidence rate, an improved estimation process is proposed. To this end the correction of the observed inter-donation interval is made. Advice has been provided on adjusting the present reporting on confirmed positive donors (Van der Bij et al, Transfusion 2006). The report has been submitted to EMEA and will be published in 2009.

Cost-effectiveness of (HBV-) NAT testing in The Netherlands

Sanquin is to replace its HIV / HCV NAT testing program in 2008, as NAT laboratories will be further centralized and technology needs replacement. In addition NAT for Parvovirus B19 is performed for plasma products and NAT for HBV and HAV is considered. Based on the data of the infectious disease epidemiology among donors of Sanquin, the different NAT test characteristics available for donor screening (especially for HBV) and the recipient survival characteristics of the TTA transfusion chain model, the (incremental) cost-effectiveness ratio's (CER) of the different NAT options will be estimated with priority on the decisions regarding addition of HBV NAT to the test algorithms. For the modeling the HBV infection, collaboration with the National Institute for Public Health and Environment (RIVM) has been established. The results are in press.

Council of Europe Reporting on the collection, testing and use of blood and blood components in Europe

The annual reporting on the collection, testing and use of blood and blood components in the Council of Europe Member States is an assignment of the Council of Europe to the TTA group since 2001. As the 2004 and 2005 data have been communicated, the 2007 data are to be included in a Trend Analysis on Blood Transfusions in Europe since 2001. The robustness of the data on most countries became apparent by consistency over time. A draft was submitted for review. The reports are published by the Council of Europe, EDQM Department of Biological Standardization, OMCL Network and Healthcare, Strassbourg.

Key publications

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Immune response to blood group antigens

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Blood group antigens

In previous years we have developed a high throughput genotyping array for blood group antigens (BloodChip). In 2008 a validation study on 3000 samples has been completed. The diagnostic accuracy observed was high, and all discrepancies between genotyping and serology except for 2 (a new O-allele of the ABO-system and bigC expression from an r's gene) were solved in favor of the BloodChip.

The existence of cell-free fetal DNA in the maternal circulation during pregnancy has opened new possibilities for non-invasive prenatal diagnosis (NIPD), especially for blood group antigens. The diagnostic applications are however hampered by lack of a generic control marker for circulating fetal DNA. The promoter of *RASSF1A* gene is hyper-methylated in the placenta and hypo-methylated in maternal blood cells, which allows the use of methylation-sensitive restriction enzymes. Initial results with this potential fetal DNA identifier however showed weak positive results in virtually all male plasmas. We developed a modified protocol based on the application of a double digestion with Bstu1 and Hha1. Our present results indicate that *RASSF1a* can be used as universal fetal identifier for non-invasive fetal genotyping assays.

Antibodies against the platelet alloantigen HPA1a are present in 1:450 pregnant women. In several countries it is therefore considered to screen pregnant women for the presence of anti-HPA1a antibodies. So far no robust NIPD assays for HPA1a have been described, with all assays hampering from aspecific amplification of the maternal HPA1b allele. Since the C>T mutation in the HPA1b allele results in a restriction site for Msp1, pre-digestion of plasma DNA will cut the maternal HPA1b DNA sequences while leaving the fetal HPA1a allele intact. Indeed, with this approach we have been able to develop a specific and sensitive NIPD assay for HPA1a.

Antibodies against blood group antigens

Despite the numerous theoretical blood group mismatches only a minority of patients form alloantibodies upon transfusion and pregnancy. The induction of IEA seems not to be a random effect that is only related to the number of transfusions. Based on mathematic modeling it has been suggested that only 13% of individuals are high

responders to alloimmunization against red blood cell antigens. In the coming years a genome-wide association study on genetic risk factors will be performed. In a pilot study we identified HLA-DRB1*15 and a CTLA4 polymorphism (rs231775) as a risk factor for high respondership to RhD antigen. CTLA4 is a key negative regulator of T-cell proliferation.

Various biological activities of immunoglobulin G including antibody-dependent cellular cytotoxicity (ADCC) are modulated by the structural feature of the N-glycan in the Fc part that interferes with its binding to IgG-Fc Receptors. By analyzing the Fc-glycosylation of the pathogenic, affinity-purified IgG1 alloantibodies formed during pregnancy against antigens of the fetus (HPA-1 or RhD) at the glycopeptide level using mass spectrometry, we found markedly decreased levels of core-fucosylation as well as increased levels of galactosylation and sialylation as compared to glycosylation patterns of total serum IgG1 of the same patients. Because IgG1 Fc-core-fucosylation is known to influence ADCC activity, modulation of core-fucosylation may have a profound effect on disease severity and prognosis. Studies in large patient cohorts will have to be performed to establish such correlations.

FcRn

Human IgG3 displays the strongest effector functions of all human IgG subclasses but has a short half-life, suggesting FcRn-mediated IgG salvage to be defective for IgG3. We previously observed that human IgG1 inhibited FcRn-mediated transcytosis and recycling of IgG3. The observed inhibition was due to a single amino acid difference at position 435, where IgG3 has an arginine instead of the histidine found in all other known IgG species, as H435-engrafted IgG3 inhibited FcRn-mediated transport and rescue of R435-IgG1. In 2008 these differences in binding have been confirmed by Biacore analysis. The studies have been extended by *in vivo* analysis. In humans the half-lives of H435-containing IgG3 allotypes were shown to be comparable to IgG1. In an animal model H435-IgG3 also proved better suited for protection against pneumococcal challenge, demonstrating that H435-IgG3 is a formidable candidate for monoclonal antibody therapies in patients.

Key publications

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Grootkerk-Tax MG, Ait Soussan A, de Haas M, Maaskant-van Wijk PA, van der Schoot CE. Evaluation of prenatal RHD typing strategies on cell-free fetal DNA from maternal plasma. Transfusion 2006; 46(12):2142-8.

Hematopoiesis and cellular therapies

Bone marrow microenvironment

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Mesenchymal stromal cells are widely used for cellular therapy. However, high cell numbers are needed for therapy since the homing ability of these cells to the tissues of interest is low. More insight in the trafficking of these cells is therefore of utmost importance. Cell cycle is a process that has been demonstrated to be involved in homing of HSC. We now observed that the migratory MSC fraction contained significantly less cells in S- and G2/M-phase as compared to the non-migrating MSC. By microarray analysis genes that are differentially expressed between migrating and non-migrating MSC were identified, including NR4A1, NR4A2, CYR61, SMAD7, AXIN1, ID3 and HIST1H2AK. The largest group of upregulated genes in the migrating MSC subpopulation are involved in (or related to) the G-protein-coupled receptor protein signaling pathway. SDF exposure induced large differences compared to cultured MSC, and the data were enriched for genes involved in the (regulation of) cell cycle, response to wound healing and regulation of cell differentiation. These results indicate that besides promoting MSC migration, SDF may also induce other (paracrine) functions, that MSC may have in the injured niche.

Carlijn Voermans has demonstrated during her stay at the laboratory of dr. Reya in the USA that the ECM protein βig-h3 is strongly upregulated in regenerating mouse HSC. Ectopic expression of βig-h3 causes an accelerated differentiation of HSCs and rapid exhaustion of murine, primitive progenitors *in vivo* as well as *in vitro*. We now demonstrate that human CD34+ cells adhere to βig-h3 and that overexpression of βig-H3 accelerates differentiation of these cells towards megakaryocytes.

Megakaryocytopoiesis

We have shown that MEIS1 is uniquely restricted to MKs and platelets. In primary hematopoietic progenitor (CD34+) cells two splice variants of MEIS were identified, which were differentially expressed in the different stem cell sources. During megakaryocytic differentiation both MEIS1 transcripts were upregulated. Downregulation of MEIS1 using lentiviral short-hairpin RNAs (shRNA) in DAMI and primary cells resulted in reduced proliferation. This effect was caused both by cell cycle arrest and by increased apoptosis. Comparative transciptional profiling showed a total of 255 and 273 up- and down-regulated genes, respectively upon MEIS1-knock down. Consistent with the role of MEIS1 in MK differentiation, down-regulated genes were significantly enriched with functional categories such as response to wounding, hemostasis, and coagulation. Among these genes are several regulators of platelet volume, recently identified in genome wide association studies.

Cellular neovascularization therapy

We have previously shown that Endothelial colony-forming cells (CFU-EC) are monocytic cells. CD4+ T-cells facilitated the monocytic colony formation, by the secretion of (a) soluble factor(s) upon CD3-MHC-class II interaction. Last year we showed that monocytes activated by this T cell factor(s) showed an increased revascularizing potential in the ischemic hind limb model in nude mice.
Also blood outgrowth endothelial cells (BOEC) are candidate for vascular cell therapy. It had been suggested that cord blood derived BOECs are superior. However, we showed that the phenotype and functional characteristics of BOECs isolated from cord blood and peripheral blood were almost equal. A slightly more angiogenic phenotype favors CB-BOEC, but addition of VEGF to PB-BOEC induces equal proliferation and tube formation.

Key publications

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In 2008, we have integrated the topics of the research line 'Immune regulation' into the other research lines of the department Immunopathology. Our work on the major inhibitor of classical complement pathway, C1-inhibitor (C1-Inh), has been integrated in the complement and inflammation research line of Lucien Aarden. Our work on the function of the granzymes and intracellular serpins has been integrated in the research line 'Immune regulation by T cells'.

Immune regulation

Immune regulation by B cells

The research line 'Immune Regulation by B cells' was previously a designated project within the research line 'Antigen presentation'. This research line addresses the question how the humoral and cellular immune responses are regulated by MHC-mediated antigen presentation in B cells.

In human B cells, effective class II-Ag presentation depends on MHC class II, but also on HLA-DM and HLA-DO, the chaperones that regulate the composition of the antigenic peptide repertoire. B cell chronic lymphocytic leukemia (B-CLL) is characterized by a chronic immune dysfunction of a.o. the T cell compartment. This year, we continued our collaboration with dr Arjan van de Loosdrecht and drs Martine Chamuleau (Dept Hematology, VUmc) on aberrant class II antigen presentation in B cell chronic lymphocytic leukemia (B-CLL) and showed that this correlates with the known shift in the patients' T cell population towards the effector phenotype. This points to an antigen driven process of immune activation. In addition, we showed that transcriptional deregulation of HLA-DR, HLA-DM and HLA-DO results from hyperactivation of CIITA, the transcriptional master-regulator of the class II genes. Inclusion of extra parameters showed that especially mRNA levels of DOA, the alpha chain of the HLA-DO complex, seem to be predictive for survival of the patients.

Previously, we showed that B cells behave as professional phagocytes of bacteria and particles when recognition is triggered via the specific B cell receptor (BCR). Phagocytosis of *Salmonella*, our model pathogen, leads to survival of the bacteria in a latent state in the B cells. This is subsequently followed by extracytosis of the bacteria and reinfection in other tissues followed by local multiplication. This year,

we investigated in animal models whether B cells in mice indeed contribute to dissemination of *Salmonella in vivo*, in collaboration with Maria Rescigno (Milan, Italy). Our first experiments show that indeed this seems to be the case. Thus, B cells can serve as a niche for survival of *Salmonella* from the innate immune system and as a transport vehicle for systemic dissemination. In addition, phagocytosis of *Salmonella* does lead to efficient antigen presentation of bacterial antigens to CD4+ T helper cells. This process aids in the formation of specific antibodies, and therefore an efficient humoral immune response is mounted against *Salmonella*. Next to activation of CD4+ T cells, we also investigated whether B cells that had taken up *Salmonella* are able to activate CD8+ cells. We showed that efficient cross presentation of *Salmonella* antigens by B cells leads to activation of CD8+ T cells that are cytotoxic and able to kill *Salmonella*-infected target cells. This process requires CD4+ help. Thus, B cells are able to activate *Salmonella*-specific cytotoxic T cells that are needed to eliminate cells that have been infected with this facultative intracellular pathogen.

Immune Regulation by Dendritic cells

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The research line 'Immune Regulation by Dendritic cells' was previously a designated project within the research line 'Antigen presentation'. In our dendritic cell (DC) research program we aim to develop clinically approved, validated and cost-efficient monocyte-derived DC products. For the development of immuno-activatory DCs we extended our research on our newly developed DC maturation cocktail, MPLA-plus-IFNγ. We demonstrated that DCs matured with this cocktail are both able to migrate and to produce IL-12. Migration and Il-12 production are both prerequisites for anti-tumour immuno-activatory DC therapy. We have shown that these DCs induce a strong Th1 response, which is a prerequisite for the induction of a good CTL response against the tumor. *In vitro* we have now also demonstrated that our MPLA-plus-IFNγ-matured DCs can induce superior CTL responses. Next to the induction of high percentages of specific CTLs, we also demonstrated specific killing of tumor cell lines by these CTLs. In collaboration with dr Sheila Krishnadath and dr Francesca Milano (Dept Gastroenterology, AMC) and prof Martien Kapsenberg (Dept Histology and Cell Biology, AMC) we are setting up a phase I/II trial to study the toxicity and

use of MPLA-plus-IFNγ-matured DCs in the treatment of patients suffering from esophageal cancer. We showed that autologous DC can induce an immune response against esophageal cancer cells *in vitro*.
We have continued our collaboration with dr Carlijn Voermans and prof Ellen van der Schoot (Dept of Experimental Immunohematology) and dr Hans Vrielink (Blood Bank North West Region) to isolate monocytes via a closed system through a specialized leukocytapheresis method (Haemonetics®) combined with the ElutraTM system. Currently we are optimising DC culture conditions in closed bags and validating freeze/thaw processes of the product. In an additional collaboration with the group of prof Ellen van der Schoot and dr Jaap Jan Zwaginga the contribution of activated CD4+ T cells to colony formation of endothelial cells was demonstrated.
Next to the development of a DC product for the treatment of cancer, we are also studying the potential of tolerizing DC therapy in autoimmune disease and transplantation. We have set up a suppression assay in which we can compare the regulatory T-cell-inducing capacity of different methods to generate tolerance-inducing DCs. At the moment we are evaluating the different methods to create stable tolerance-inducing DCs.

Key publications

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Immune Regulation by T cells

The research line 'Immune Regulation by T cells' was previously a designated project within the research line 'Antigen presentation'. Within this research line, we work on two projects: T-cell epitopes and granzymes.

T-cell epitopes

Patients that suffer from RA are successfully treated with antibodies against TNFα. In a large number of these patients however, therapy fails because patients mount an antibody response against the therapeutic antibody. We are investigating whether we can identify the T cell epitopes in the therapeutic antibody adalimumab that may play a role in antibody formation. This research is performed in collaboration with Genmab (Paul Parren PhD and Janine Schuurman PhD) and Algonomics (Ignace Lasters PhD and Jurgen Pletinckx PhD). In 2008, we indeed identified epitopes in adalimumab that generated CD4+ T cell reactivity. Reactivity was observed both in patients that mounted an antibody response against adalimumab and in patients that did not show formation of inhibitory antibody levels. We are currently extending our patient analyses to define more clearly which T cell epitopes in adalimumab generate T cell expansion and whether these epitopes exist only in the variable portion of the therapeutic antibody. For this project we are also generating tetrameric MHC class II molecules as tools to monitor antigen-specific T-cells in relation to antibody formation against the therapeutic antibody. We have expressed monomeric class II complexes associated to CLIP (Class II Associated Invariant Chain Peptide) in insect cells. Class II/CLIP complexes are a natural intermediate in the MHC class II antigen presentation pathway in which the CLIP renders a stabily expressed MHC class II complex. In antigen-presenting cells, CLIP is exchanged for the antigenic peptides that form the final repertoire of the class II molecules. In our recombinant system, CLIP allows stable secretion of the recombinant MHC class II complex. We are now in the process of exchanging CLIP for the antigenic peptides of interest. In this way, we aim to generate large production batches of MHCII/CLIP that may be used for the versatile generation of arrays of different MHC II tetramers that each carries the desired antigenic peptide of interest.

Granzymes

We are studying the role of granzymes and granzyme-inhibiting serpins. Cytotoxic T cells and natural killer cells produce Granzyme A (GrA) and Granzyme B (GrB). In conjunction with perforin, GrA and GrB induce target cell apoptosis. The activity of Granzyme B is regulated by the human intracellular serpin SerpinB9, and many

cells (like tumor cells and dendritic cells) are protected against the action of GrB via expression of SerpinB9. Expression of the granzymes, perforin or SerpinB9 may be indicative for the severity of inflammatory disease in which CTL and NK cells are the major effector cell types. In collaboration with drs Reinout Bem and Job van Woesel PhD (Pediatric Intensive Care Unit, Emma Children's Hospital, AMC), we demonstrated that both Granzyme A and Granzyme B are activated in children with severe respiratory syncytial virus infection. CTL and NK also play an important role in rejection processes in organ transplantation. This year, we extended our collaboration with Ajda Rowshani MD PhD and prof Ineke ten Berge MD PhD (Dept Internal Medicine, AMC) and investigated in a large cohort of patients who had undergone renal transplantation whether the presence of Granzyme A and B, perforin and SerpinB in the urine of patients who have undergone renal transplantation correlates with transplant rejection. We have now shown that especially GrA mRNA levels may be a predictive non-invasive biomarker of acute and chronic renal rejection. In addition, we collaborated with dr Pawel Kaliniski (Pittsburgh, USA) to demonstrate novel interactions between CD8+ T cells and dendritic cells involving differential granzyme production and the expression of intracellular granzyme inhibitors. Finally, we generated active recombinant GrA in the yeast *Pichia pastoris* and continued our research into uptake of GrA by target cells. In addition, we are investigating how GrA exerts its function and how this is related to the structure of the protein.

Key publications

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C1 Inhibitor

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We investigated possible new fields of clinical application for C1-Inh in close collaboration with Sanquin Plasma Products. In addition, in collaboration with Roel Bennink MD PhD (Dept Nuclear Medicine, AMC) we demonstrated that the clearance of radiolabeled recombinant C1-Inh (produced in yeast) from the circulation is regulated by the liver and is much faster than that of plasma-purified C1-Inh. Thus, plasma-derived C1-Inh is the product of choice for treatment with C1-Inh.

Key publications

Ramaglia V, Wolterman R, de Kok M, Vigar MA, Wagenaar-Bos I, King RH, Morgan BP, Baas F. Soluble complement receptor 1 protects the peripheral nerve from early axon loss after injury. Am J Pathol 2008; 172(4):1043-52.

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Research in the Department Blood Cell Research is performed under the supervision of two Principal Investigators, Dirk de Korte PhD and Arthur Verhoeven PhD. Both PI's have their own focus on Research Lines. The focus of Arthur Verhoeven is described here, while research lines by Dirk de Korte involving Arthur Verhoeven as well can be found with PI Dirk de Korte.

Red blood cell function

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Previous studies in our group, in collaboration with the group of prof C Ince (Dept of Physiology, AMC) have shown that long-term storage of human red blood cells (RBC) negatively affects tissue oxygenation in a rat model. Since RBC have been shown to produce the vasodilator nitric oxide (NO) from nitrite, a reaction catalyzed by (deoxygenated) hemoglobin, and this NO release is probably important for oxygen delivery to hypoxic tissues, we have investigated the effect of storage on this pathway. Mouse RBC have recently been shown to express endothelial cell type NO synthase (eNOS), enabling them to produce nitric oxide (NO) with arginine as substrate. This pathway was therefore also considered in this study.
Leukodepleted RBC concentrates were prepared according to standard blood bank procedures and used either within 3 days ('fresh') or after 5-6 weeks of storage in SAGM at 4°C ('stored'). Release of NO (in the gas phase) was measured in RBC suspensions at 37°C with a Sievers 280i analyzer under a continuous stream of helium. Gas flow through the suspension itself was avoided to minimize hemolysis. After 6 min of deoxygenation, nitrite or arginine was added and NO released from the cells was measured over the next 10 min.
To investigate the presence of eNOS, different antibodies raised against eNOS were used for immunoblotting of RBC lysates, with human umbilical cord vein endothelial cells (HUVEC) as positive control. A sandwich ELISA was used to quantify the amount of eNOS present in RBC and HUVEC, with recombinant eNOS as positive control.
Reproducible measurements of nitrite-induced NO release required static conditions of the RBC suspensions, after first being mixed to induce about 50% deoxygenation of the hemoglobin (Hb). Spiking experiments with NO donors confirmed the notion that most NO is scavenged by the RBC themselves, but that some NO is able to escape from the surface of the cell suspensions. The amount of NO released from the surface was proportional to the amount of cells in the incubation up to an hematocrit of 5%. Nitrite-induced NO release showed an optimum at about 50% Hb saturation, confirming published data obtained with purified Hb.
Nitrite-induced NO release from stored RBC was 25% higher than from fresh RBC units. Met-Hb, the other product of nitrite reduction by deoxy-Hb, was 50% higher in the

stored units. The rate of Met-Hb reduction (after removal of nitrite) was the same in fresh and stored RBC.

Release of NO from RBC could thus be demonstrated with nitrite as substrate, but arginine, the substrate of eNOS, did not elicit a detectable response. Immunoblotting with a monoclonal eNOS antibody showed the presence of an 80-kDa band in lysates and membranes from human RBC, but not the 140-kDa band of intact eNOS as present in HUVEC. A polyclonal eNOS antibody also showed only intact eNOS in HUVEC, but not in RBC lysates or membranes. Results of the sandwich ELISA indicated that, on a cell basis, HUVEC contain 200,000 times more intact eNOS protein than human RBC.

We conclude from these data that

1) storage of human RBC does not result in a decrease in the ability to release NO in response to nitrite under in vitro conditions;
2) human RBC are unlikely to contain sufficient eNOS to allow NO release from arginine;
3) NO release by human RBC, although probably of great importance for RBC function, appears not to be related to storage-induced defects in oxygen delivery as observed in our rat model.

Key publications

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

Raat NJ, Hilarius PM, Johannes T, de Korte D, Ince C, Verhoeven AJ:. Rejuvenation of stored human red cells reverses the renal microvascular oxygenation deficit in an isovolemic transfusion model in rats. Transfusion 2009; 49:427-34.

Red blood cell aging

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Storage under blood bank conditions induces changes in erythrocytes that lead to the clearance of a significant fraction of the transfused erythrocytes (up to 20%) within 24 h after transfusion. The exposure of phosphatidylserine (PS), an important 'eat-me' signal for macrophages, may be one of the mechanisms involved. Our group studies the maintenance of phospholipid (PL) asymmetry in the plasma membrane of RBC, which is pivotal to prevent the exposure of phosphatidylserine (PS), an important 'eat-me' signal. PL asymmetry is maintained by strict control of scrambling activity, responsible for outward translocation of PS, and by the activity of an ATP-dependent flippase, which transports PS back to the inner leaflet of the membrane.

Spur cell anemia is an acquired red cell defect associated with elevated free cholesterol levels in the plasma and, as a consequence, a high ratio of cholesterol to phospholipid in the RBC membrane. In one spur cell patient, we had the opportunity to investigate the effect of elevated cholesterol on the ATP-dependent flippase activity and the ATP-independent scrambling activity. The erythrocytes of this patient were also analyzed for morphology, membrane proteins, osmotic resistance, ATP levels, cholesterol and PL content.

Flippase activity was measured by means of NBD-PS translocation and scrambling activity by measurement of PS exposure and NBD-PC translocation. In artificially cholesterol-loaded and depleted erythrocytes, the same analyses were performed. An elevated chol/PL ratio as observed in spur cells, increases osmotic resistance, viscosity and impairs deformability by loss of the biconcave shape. These changes are expected to complicate the passage of these cells in the spleen. However, we also observed that excess cholesterol profoundly inhibits scrambling activity, activated either by elevation of cytosolic free Ca2+ or by exposure to singlet oxygen. Conversely, depletion of the RBC membrane enhanced scrambling activity. Flippase activity was also affected, but to a lesser extent by cholesterol loading or depletion.

Taken together, the effect of excess cholesterol on PL translocation prevents exposure of PS as a phagocytosis-inducing signal for macrophages. The sequestration of spur cells may therefore be less effective than expected based on their aberrant shape.

As noted above, storage under blood bank conditions induces changes in RBC erythrocytes that lead to the clearance of a significant fraction of the transfused cells within 24 h. This constitutes a heavy burden on the macrophages with unknown consequences for e.g. cytokine secretion by these cells. Normally, *in vivo* aged erythrocytes are phagocytosed by macrophages residing in the red pulp of the spleen, but also in this case the mechanisms involved are largely unknown. We recently have begun to study the interactions between macrophages, isolated from human spleen, and erythrocytes, aged either *in vivo* or *in vitro*.
Spleen macrophages were isolated from human spleen samples by Percoll fractionation. Expression of cell surface markers was assessed by flow cytometry and/or confocal microscropy, and cytokine secretion during erythrophagocytosis was determined by a membrane cytokine array.
The spleen macrophages expressed various cell surface markers that could play a role in phagocytosis of aged erythrocytes, including Fc receptors, SIRPα and Stabilin-2, a recently identified phosphatidylserine (PS) receptor. Also, erythrocytes isolated from human spleen proved to have a significant higher exposure of PS than erythrocytes from peripheral blood. Expression of CD44 (a hyaluronic acid receptor) was also significantly higher. Furthermore, we found that spleen macrophages secrete, amongst others, IL-10, MIP-1alpha, MIP-1beta and IP-10 during phagocytosis of these *in vivo* aged erythrocytes. Experiments with erythrocytes aged *in vitro* or genetically altered erythrocytes (derived from manipulated stem cell cultures) will be carried out in the near future.

Key publication

Verhoeven AJ, Hilarius PM, Dekkers DW, Lagerberg JW, de Korte D. Prolonged storage of red blood cells affects aminophospholipid translocase activity. Vox Sang 2006; 91:244-51.

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Biology of von Willebrand factor

Von Willebrand Factor (VWF) and factor VIII (FVIII) circulate in plasma in a non-covalent complex. It is generally assumed that VWF acts as a molecular chaperone that protects factor VIII from proteolytic degradation in the circulation. This is illustrated by the decreased factor VIII levels that are found in plasma of patients with von Willebrand disease variants that fail to interact with factor VIII. In these patients, factor VIII levels are reduced to approximately 20% of that observed in normal plasma, resulting in a mild bleeding tendency. Conversely, elevated levels of VWF have also been linked to increased levels of blood coagulation

factor VIII. Elevated levels of factor VIII have been shown to increase the risk for venous thrombosis. This observation underscores the important role for VWF in the regulation of circulating levels of factor VIII. As yet, limited information is available with respect to regulation of plasma levels of VWF. Biosynthesis of VWF occurs in vascular endothelial cells where it is stored in rod-shaped endothelial cell-specific storage organelles, the Weibel-Palade bodies. Besides VWF, these Weibel-Palade bodies contain a number of other proteins, including P-selectin, angiopoietin-2, osteoprotegerin and a number of other components. Upon stimulation of endothelial cells by agonist such as thrombin or epinephrine, Weibel-Palade bodies undergo exocytosis, resulting in release of surface expression of their contents. The small GTP-binding protein Ral has been implicated in regulated exocytosis via its interaction with the mammalian exocyst complex. We have previously demonstrated that Ral is involved in exocytosis of Weibel-Palade bodies. Little is known about intracellular signaling pathways that promote activation of Ral in response to ligand binding of G protein-coupled receptors. Recently, we have shown that RNAi-mediated knockdown of RalGDS, an exchange factor for Ral, results in inhibition of thrombin- and epinephrine-induced exocytosis of Weibel-Palade bodies while overexpression of RalGDS promotes exocytosis of Weibel-Palade bodies. A RalGDS variant lacking its exchange domain behaves in a dominant negative manner by blocking release of Weibel-Palade bodies. We also provide evidence that RalGDS binds calmodulin (CaM) via an amino-terminal CaM-binding domain. RalGDS association to CaM is required for Ral activation because a cell-permeable peptide comprising this RalGDS CaM-binding domain inhibits Ral activation and Weibel-Palade body exocytosis. Together our findings suggest that RalGDS plays a vital role in the regulation of Ral-dependent exocytosis after stimulation with Ca(2+)- or cAMP-raising agonists.

Following release of VWF from endothelial cells ultra large VWF multimers remain anchored to the surface of endothelial cells. These so-called ultra large (UL) VWF strings provide multiple binding sites for platelets. This provides an initial step in maintaining vascular integrity following injury. Under physiological conditions UL-VWF strings are rapidly processed by the VWF cleaving protease ADAMTS13. Cleavage of UL-VWF by ADAMTS13 provides a means to rapidly downregulate adhesion of blood platelets to UL-VWF strings following release of Weibel-Palade bodies from endothelial cells.

Key publication

Rondaij MG, Bierings R, van Agtmaal EL, Gijzen KA, Sellink E, Kragt A, Ferguson SS, Mertens K, Hannah MJ, van Mourik JA, Fernandez-Borja M, Voorberg J. Guanine exchange factor RalGDS mediates exocytosis of Weibel-Palade bodies from endothelial cells. Blood 2008; 112:56-63.

Immune response to hemostatic proteins

Hemophilia

Hemophilia is an X-linked bleeding disorder that is caused by a deficiency of factor VIII (hemophilia A) or factor IX (hemophilia B). 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% of the patients with hemophilia B. Inhibitor development renders patients unresponsive to coagulation factor replacement therapy. The eradication of inhibitory antibodies in patients with hemophilia A can be accomplished by frequent administration of high or intermediate doses of factor VIII (FVIII), so-called immune tolerance induction (ITI). This study monitored the distribution of IgG subclasses of anti-FVIII antibodies during ITI. FVIII-specific antibodies of subclass IgG1 were detected in all inhibitor patients tested, anti-FVIII IgG4 in 16, IgG2 in 10 and IgG3 in one of 20 patients analyzed. Levels of anti-FVIII IgG1 and IgG4 correlated well with inhibitor titres as measured by Bethesda assay. In low-titre inhibitor patients, anti-FVIII antibodies consisted primarily of subclass IgG1 whereas, anti-FVIII antibodies of subclass IgG4 were more prominent in patients with high titre inhibitors who needed prolonged treatment or who failed ITI. Longitudinal analysis of 14 patients undergoing ITI revealed that the relative contribution of IgG subclasses was constant for most of the patients analyzed. In two patients, the relative contribution of IgG4 increased during ITI. Overall, our findings document the distribution and dynamics of anti-FVIII IgG subclasses during ITI. Future studies will need to address whether monitoring the relative contribution of anti-FVIII subclasses IgG1 and IgG4 may be useful for the identification of patients who are at risk of failing ITI.

Thrombotic thrombocytopenic purpura

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 ADAMTS13. In the absence of ADAMTS13, ultra large VWF (UL-VWF) polymers, originating from endothelial cell specific organelles, designated Weibel-Palade bodies, accumulate in the circulation. These UL-VWF polymers mediate the formation of platelet-rich thrombi in the microcirculation that give rise to hemolytic anemia and thrombocytopenia. In plasma of the majority of patients with acquired TTP, antibodies directed towards ADAMTS13 are present. We have isolated a panel of human monoclonal antibodies from the immunoglobulin repertoire of a patient with acquired TTP. Inspection of the primary sequence of the variable heavy chain of these antibodies revealed that these were encoded by a single variable heavy chain gene segment designated VH1-69. Both monoclonal antibodies inhibited the VWF processing activity of ADAMTS13 under different experimental conditions. Epitope mapping revealed that this class of antibodies interacts with the spacer domain of ADAMTS13. A similar epitope-specificity was observed for polyclonal antibodies present in patients with acquired TTP. These findings suggest that the isolated human monoclonal antibodies are representative for the pathogenic antibodies in plasma of patients with acquired TTP. Recently, we have shown using an anti-idiotypic antibody specifically recognizing the VH1-69 variable heavy chain gene segment that anti-ADAMTS13 antibodies present in plasma of patients with acquired TTP frequently incorporate this gene segment. Based on these findings we propose that the frequent usage of the VH1-69 gene segments underlies the restricted epitope specificity of anti-ADAMTS13 antibodies that develop in patients with acquired TTP.

Key publication

Van Helden PM, van den Berg HM, Gouw SC, Kaijen PH, Zuurveld MG, Mauser-Bunschoten EP, Aalberse RC, Vidarsson G, Voorberg J. IgG subclasses of anti-FVIII antibodies during immune tolerance induction in patients with hemophilia A. Br J Haematol 2008; 142:644-52.

Hans L Zaaijer MD PhD

Blood-borne Infections

On November 1st 2008, the Sanquin department for the study of blood-borne infections (BOI) came into existence.

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The coming years BOI will focus on the following topics:

Occult hepatitis B virus (HBV) infection

HBV infection is the most common viral infection detected in Dutch blood donations. Recently the testing for HBV DNA was added to the screening of Dutch blood donations. As a consequence 'occult' (i.e. HBsAg negative) cases of HBV infection are discovered. A preliminary analysis already indicates that various mechanisms cause occult HBV infection among Dutch blood donors. To determine the relevance of occult HBV infection for the safety of blood and blood products, the coming years all HBsAg-negative, HBV DNA-positive cases will be studied in detail, including HBV genomic analysis. HBV sequencing results will be compared to occult HBV sequences obtained from immunodeficient and immunosuppressed patients.

Q-fever

In 2008 the Netherlands experienced the largest outbreak of Q-fever among humans ever recorded. Fortunately only one case of probable transmission of *Coxiella burnetii* via blood transfusion is known (1977). To corroborate the conclusion that *C. burnetii* does not pose a relevant threat to the blood supply, a study on *C. burnetii* bacteremia is foreseen. If an outbreak of Q-fever occurs in 2009, serum samples from blood donors in affected areas will be tested for *C. burnetii* DNA and for antibodies against *C. burnetii*, in co-operation with the Center for Infectious Disease Control, Cib, Bilthoven, and the Laboratory for Medical Microbiology – Bosch Medicentrum (Den Bosch).

Parvovirus B19

In the Netherlands 'parvovirus-safe' blood is available upon request. This blood is collected from blood donors who test positive for IgG antibodies to Parvovirus B19, during at least six months. It becomes clear that in healthy, asymptomatic persons, such as blood donors, parvovirus viremia may last for months or even years. Probably the presence of neutralising antibodies prevents blood-borne transmission of parvovirus from viremic blood donors. To better understand the dynamics of parvovirus infection, it is planned to study in detail the appearance of various anti-parvovirus antibodies and the course of viremia in blood donors with recent parvovirus infection.

Fast and standardized decision-making in case of emerging infections

To ensure the safety of blood, for each emerging infection Sanquin rapidly must assess whether measures must be taken to prevent blood-borne transmission of the agent involved. In 2008 the Sanquin Working group for emerging blood-borne infections (WOBI) evaluated 256 signals on emerging infections, with possible implications for the safety of the blood supply. Applying an *ad hoc* approach, in

20 meetings WOBI considered the relevance of outbreaks of dengue, Q-fever, chikungunya, hepatitis A, malaria, West Nile fever, Rift Valley fever, tick-borne encephalitis, etc. To ensure optimal decision-making, it is desirable to define and apply a more standardized procedure for the evaluation of emerging infections. BOI aims to develop a standardized decision making tool, specifically designed to decide rapidly whether and which preventive measures must be instituted in case of an emerging infection.

HBV X protein

Hepatitis B virus features only few viral proteins. The function of the HBV X protein remains unknown. As of 1 April 2009, in co-operation with Experimental Virology, Clinical Virology and the Laboratory for Viral Immuno Pathology (AMC) in the Landsteiner Laboratory, a new hypothesis regarding the function of the HBV X protein is being explored.

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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 leaders: H ter Hart (h.terhart@sanquin.nl); I Prins (i.prins@sanquin.nl)

Development work on a manufacturing process for holotransferrin was started in close collaboration with L von Bonsdorff, Sanquin Oy, Finland. The development of this new product and the manufacturing installation was finalized in 2008. Holotransferrin will be used for a new drug product, Oxaliplatin-encapsulated transferrin-conjugated PEG-liposomes for targeting Oxaliplatin (I-OHP) to colon cancer cells via cancer manifesting transferrin receptors. Clinical studies using this new product are planned for 2009.

As spin-off of the development of holotransferrin, a project was started to develop an apotransferrin product. Apotransferrin was manufactured using the manufacturing installation for holotransferrin as well. The resulting product was characterized and virus validation studies were performed with excellent results. A clinical study with this product is planned for 2009.

Project leaders: M Kleijn; A Koenderman (a.koenderman@sanquin.nl)

To enhance the virus safety of Cetor®, a high-purity C1-inhibitor product, a 15 nm Planova-filtration step was developed and implemented in the manufacturing process for a next generation C1-inhibitor product. For the USA Cinryze™ was developed using USA plasma as starting material. This product was registered in the USA in October 2008.

In close collaboration with Sanquin Research, a project was started to characterize C1-inhibitor products. With Sanquin Virus Safety Services prion removal studies were performed for the new 15 nm Planova filtration step with good results.

To develop new indications for Cetor® collaborations were started with research groups of the University Medical Center Utrecht and the Radboud University Nijmegen Medical Centre.

Project leader: GJ Derksen (g.derksen@sanquin.nl)

A project was started to develop a second intermediate pure FVIII product besides Aafact in close collaboration with dr R Laub from CAF-DCF (Brussels, Belgium).
To guarantee the virus safety, studies on the feasibility of the use of two new virus reducing techniques (20 nm filtration and UVC technology) are started.

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

In close collaboration with ProThera Biologics, East Providence, USA a project was started to develop a manufacturing process for an Inter-Alpha Inhibitor Protein ('IAIP') product.

Manager: A Koenderman (a.koenderman@sanquin.nl)

In collaboration with the Dept of Immunopathology, studies on IgG products are ongoing to study dimer formation and polymerization of IgG and its significance in the occurrence of adverse events in patients and the role of sialylation of IgG.

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The CAF-DCF Product Development Division (former R&D) is located in Brussels. For its staff, research and development means ensuring both the efficacy of plasma-derived medicinal products and their biological safety regarding pathogens and environmental pollutants.

The paradigm of plasma derivative safety is approached by a combination of different approaches: NAT screening, the evaluation of critical virus epidemiological data, neutralization by specific antibodies, virus infectivity testing in a cell model, virus inactivation/virus elimination validation studies, and pathogen reduction techniques (including UVC irradiation developed in our Division). The CAF portfolio of UVC patents granted in Europe, Israel, and the US has been sold to the company Sartorius. Focusing on therapeutic proteins (IVIG, albumin, AGP, FVIII) and their excipients in plasma or concentrates, the division develops both immunological methods and biochemical-biophysical techniques and exploits them in industrial applications or as tools for monitoring IVIG infusion.
In 2008, we developed computational (*in silico*) tools to visualize albumin and FVIII interactions with drugs, stabilizers, and immunoglobulin.

Effects of stabilizers on albumin (HSA) antioxidant capacity and brown color

Action of caprilic acid, mandalate, and acetyl tryptophan on albumin antioxidant capacity

Albumin (HSA) is mostly used clinically for its oncotic and colloid properties, but it is recognized for its high ligand-binding capacity, antioxidant functions, and esterase activity. This could be of importance in acute liver failure. HSA is made from pooled human plasma and pasteurized (60°C-10h) in the final vial in the presence of caprylate (Capr), with or without N-acetyl-tryptophan (AcTrp) or mandalate.
The binding capacity for six ligands recognizing different HSA domains was studied by intrinsic and total quenching fluorescence in six pharmaceutical-grade HSAs stabilized with Capr ± AcTrp or ± mandalate. Experimental R&D HSA preparations (control, after addition of stabilizer(s), after pasteurization) were included.

All the studied pharmaceutical-grade albumins were highly purified proteins (>95%). Binding studies showed that the specific affinities (K_a) of dansylsarcosine ($2.75\text{-}0.30 \times 10^5$ M^{-1}), warfarin ($5.00\text{-}3.00 \times 10^5$ M^{-1}), bilirubin ($7.15\text{-}2.90 \times 10^5$ M^{-1}), cis-parinaric acid ($9.10\text{-}0.02 \times 10^5$ M^{-1}), copper ($3.60\text{-}0.15 \times 10^6$ M^{-1}) and hemin ($3.30\text{-}0.002 \times 10^9$ M^{-1}) depended on the HSA preparation. The number of binding sites ranged from 0.5 to 2 per HSA molecule.
After addition of stabilizer(s) to the purified HSA preparation, a weak reduction (<1 log) of bilirubin and dansylsarcosine binding affinity and esterase activity was observed. In contrast to Capr addition, AcTrp addition caused a significant reduction (>1 log) of copper binding and even a greater reduction of cis-parinaric acid binding. AcTrp drastically reduced hemin binding in both pharmaceutical and experimental HSA preparations. Both SH chemical titrations and mass spectra showed that the majority (61 to 77%) of HSA molecules contained free SH functions. In the presence of AcTrp, this amount was reduced by up to 30%, with the appearance of a new major albumin compound. Pasteurization in the presence of stabilizer(s) did not induce any additional effect, as shown by the products' stability towards urea. An *in silico* model was established, showing that AcTrp binds to the IIIA domain and the IA/IIA domain, shared with Capr. AcTrp drastically modifies by allostery the shape of HSA, as shown by binding experiments with hemin, ci-parinaric acid, and copper, this having as consequence a reduction of the HSA antioxidant capacity. Furthermore, a number of diseases are associated with low plasma antioxidants, such as schizophrenia, Alzheimer's disease, and cancer.

Acetyltryptophan and the brown color of albumin (HSA)

The European Pharmacopeia allows albumin to range from colorless to yellow, amber, or green. Processing albumin to its final vial and pasteurization can lead to a dark brown color. This study was conducted to identify if albumin was involved in the color of the therapeutic protein solution stabilized by caprylate and AcTrp. The presence of a small amount of transferrin could not be the cause of the appearance of the brown color. The analysis by absorbance spectrophotometry revealed, in addition to the major peak at 280 nm (protein peak), a compound with the expected maximum absorbance between 300 and 400 nm. Synchronous

fluorescence studies targeting tryptophan residues (a powerful tool for protein analysis) revealed no significant difference between the various fractions V, intermediate fractions of the Cohn process, and 20% albumin products. Size-exclusion chromatography demonstrated the presence of a reduced AcTrp content in the total volume of the brown preparations. An additional small compound was found with a MW of 2.3-kDa, suggesting that this 2.3-kDa compound could be formed at the expense of AcTrp. This small compound shows specific fluorescence properties and is probably a pigment, produced by degradation followed by polymerization of AcTrp derivatives. It is well known that AcTrp is a fragile amino acid liable to react chemically in the presence of dissolved oxygen and able to form higher-molecular-weight compounds.

These results were confirmed by a mass spectrophotometry study showing that starting intermediate fractions V and the albumin present in the brown preparations contained only albumin with, as major isoform, mercaptanalbumin, a powerful antioxidant, although the HSA mercaptan was strongly reduced in the brown preparation. A third HSA isoform was detected, probably a nitrosylated HSA molecule. An additional small 3.85-kDa compound was found by ESI-Q-TOF, most probably equivalent to the former identified 2.3-kDa in the brown HSA preparations. The molecular weight discrepancy with the 2.35-kDa SEC compound was probably attributable to the fact that only an apparent molecular weight could be measured by SEC broad-spectrum absorbtion at 600 nm.

FVIII epitopes and ligand binding sites and new FVIII concentrates

Computational prediction of FVIII epitopes recognized by natural autoantibodies in IVIG commercial concentrates, patient inhibitors, and ligands

'*In silico*' methods have been widely applied to pharmacology hypothesis development and testing. Inhibitory antibodies directed against blood coagulation factor VIII (FVIII) impair FVIII replacement therapy, constituting a serious complication in hemophilic and autoimmune patients. Identifying B-cell FVIII epitopes and mapping them on the molecule remain important challenges.

By means of a combination of different algorithms, more than 30 hypothetical linear epitopes were predicted on the FVIII molecule surface. We selected several major predicted sequences, spanning all FVIII domains, for specific antibody induction in rabbits. All peptides tested successfully induced production of specific anti-FVIII rabbit antibodies, supporting the relevance of our approach. Using IVIGs as starting material, substantial amounts of Ig, specific for each FVIII peptide, were purified on peptide-Sepharose columns. Our results confirm the diversity of FVIII epitopes recognized by natural human anti-FVIII autoantibodies. All IgG subclasses were found in the affinity-isolated anti-peptide material, with overrepresentation of IgG2 and IgG4. Evidence was also found for new FVIII epitopes. Five human anti-peptide preparations displayed FVIII-neutralizing activity, ranging from 1.3 to 5.3 BU/mg. The inhibitors of two hemophiliac brothers were investigated, and the results showed that their inhibitor specificities were different. Further docking studies showed that small ligand molecules successfully recognized the FVIII epitopes.

A new FVIII concentrate treated by SD and UVC

Concentrates of FVIII SD-UVC have been produced previously by CAF-DCF from 10 kg cryoprecipitate (3 consistency batches). All the studied parameters (FVIII, vWF activities, thrombin activation, FIX activation, lipid binding), neoantigen detection in a rabbit model, and virus validation studies indicate that the UVC irradiation step enhances the safety of FVIII concentrates. This project continued in 2008 thanks to the collaboration of G-J Derksen, S Caan I Yin and A Koenderman. This year, a new improved design for a UVC irradiator was developed. Two UVC irradiators were constructed with a GMP static mixer and fully validated. One was delivered to Sanquin for further FVIII- SD-UVC pilot production. The new FVIII process was improved and could be used starting from cryoprecipitates of different compositions and origins. The FVIII molecule will be analyzed using a battery of tests developed in our laboratory.

Protein contents in plasma pools made with donations from unpaid European donors versus paid US donors

National and international bodies, including the WHO, recommend that blood and its products be obtained from unpaid donors. Yet, because of the difficulty of obtaining sufficient quantities of plasma though voluntary donations, most of the world's present supply of polyvalent intravenous immunoglobulins (IVIG), albumin, and clotting factor concentrates are made with donations from paid donors. We analyzed individual cryoprecipitate-depleted plasma pools made with about 800 to 2,700 liters collected from unpaid donors in 4 European countries and from paid donors in 6 different US blood transfusion centers. Eleven protein markers were selected, with different half-lives. The US plasma pools showed reduced amounts of total protein, albumin, and even more strongly reduced amounts of total immunoglobulin and specific anti-pneumococcus antibodies. In contrast, all plasma pools showed similar levels of IgA, fibrinogen, and alpha-1 glycoprotein. These results confirm and extend previous studies showing that with intensive plasmapheresis (high volume collection and reduced time intervals between two donations) as practiced with US paid donors, it is not possible to maintain high physiological levels of plasma proteins in paid donors.

B19 infectivity in hepatocarcinoma cells and neutralization by IVIG

Human Parvovirus B19 virus is the virus most commonly responsible for mild diseases, including erythema infectiosum in childhood and arthropathy in young adults. B19 virus can also cause more severe disease, such as transient aplastic crisis in patients suffering from chronic hemolytic disorders and fetal infection during pregnancy, which can lead to spontaneous abortion, fetal hydrops, or fetal death. B19 is a frequent contaminant of blood and plasma-derived medicinal products and transmission of this virus has been shown to occur through the administration of contaminated products. Inactivation of the virus is difficult and as a consequence, manufacturers of blood products have to implement screening measures to reduce the load of Parvovirus B19

in manufacturing plasma pools through the use of quantitative NAT (EMEA and FDA regulations).

In collaboration with P Caillet and A Op den Beek, we have developed different assays to measure Parvovirus B19 infectivity and neutralization activity, based on erythroid cells and on a hepatoblastoma and a hepatocarcinoma cell line (HepG2 and HuH7). The model is relevant, since several successive rounds of progeny are successfully obtained. Briefly, subconfluent cells are infected mandatorily at a low multiplicity of infection or m.o.i (0.1), and 48 hours post-infection the viral progeny in the cellular supernatant is measured by quantification of the viral DNA with commercial quantitative B19 PCR tests (Lightcycler, Roche). B19 amplification is efficiently prevented by preincubation of the virus with antibodies raised against the VP capsid proteins or with IVIG (Multigam, CAF-DCF, Gammaquin & Nanogam, Sanquin, Octagam, Octapharma and Sandoglobulin, CSL). Under proper conditions, these systems allow up to 10^7-fold amplification of the viral population, as measured by quantitative PCR. Our cell culture assay is very sensitive, since an input of 0.1 IU gives a viral production (demonstrating that 1 IU is more than 1 infectious virus), while the most sensitive PCR commercial quantitative B19 PCR test, the LightCycler-Parvovirus B19 quantification kit, requires at least 10 IU/ml to detect a specific signal. Different genotype-1 B19 strains (WHO strain and blood-donor units) efficiently multiply in hepatoblastoma and hepatocarcinoma cell lines for up to 5 successive rounds, leading to efficient production. The viral progeny of the fifth successive round was sequenced and found identical to the inoculum of the first round, demonstrating the genomic stability of the B19 produced in this infectivity test.

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The Medical Department is responsible for the design and conduct of clinical trials with recently developed or improved plasma products in order to obtain and maintain marketing authorization. The second objective of clinical trials is to get more insight in the optimal treatment with these products or to obtain evidence for new indications. The Medical Department closely cooperates with clinical investigators in the Netherlands e.g. the Dutch Inter-University Working Party on the Study of Immune Deficiencies and the Dutch Society of Haemophilia Physicians, and with clinical investigators abroad.

To ensure safety of the products the Medical Department has implemented an appropriate system of pharmacovigilance to collect, collate and evaluate information about suspected adverse or unexpected reactions of medicinal products. Pharmacovigilance is performed both passively based on received reports on adverse events and actively by performing post authorization safety studies (PASS) in randomly selected patient groups. Periodic Safety Update Reports (PSURs) provide the authorities pharmacovigilance data. PSURs have been prepared for GPO / Albumine 5% / Cealb (albumin 20%) and Aafact (factor VIII concentrate), both for a three year review period. Moreover, the 6th and 7th PSUR and the summary bridging report for Nanogam were compiled.

Medical information and advice is provided to medical specialists, physicians, nurses and pharmacists on the optimal use of plasma products. In 2008, the selection of specific units of erythrocytes for immunization in order to obtain specific source plasma for the fractionation of RheDQuin (anti-rhesus (D) immunoglobulin) has been transferred to Blood Bank North West Region.

Clinical trials performed in 2008

Nonafact®

The clinical efficacy, immunogenicity, and safety of Nonafact® in regular patient treatment has been assessed in a multi-centre clinical trial, entitled '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)', in five Hemophilia Treatment Centres in the Netherlands. In total, thirteen patients participated in the study for 2 years. The clinical study report has been finalized in September 2008.
The post-marketing study confirmed the results of the pre-authorization studies. The clinical response of Nonafact was excellent in almost all cases in which hemophilia B patients were treated for major bleedings or underwent surgery. Nonafact was shown to be a safe product. In the two year follow-up study no adverse events related to Nonafact occurred. Furthermore, no signs of thrombo-embolic events, no abnormal lab values and no induction of inhibitors to factor IX were found.

Nanogam®

A multi-centre, controlled, cross-over clinical study, 'Treatment in patients with recurrent infections and IgG Subclass Deficiency, and/or Deficient Anti-polysaccharide Antibody Response' is performed in cooperation with the Dutch Inter-University Working Party on the Study of Immune Deficiencies. The aim of the study is to compare the efficacy of Nanogam® with that of antibiotics in the treatment of recurrent (upper respiratory tract) infections in patients with IgG-subclass deficiency or a deficient anti-polysaccharide antibody response. The study was divided into two parts: treatment of adult patient and treatment of children (≥ 5 years). The study in adult patients has been started in seven centres in 2007. The children part will be initiated in 2009. The results of this study will be used to compile a protocol for optimal treatment of patients with these disorders.

The investigator-initiated DRIP study, 'Desensitization of highly pre-sensitized dialysis patients waiting for kidney transplantation by Rituximab, IVIG-L and rescue Plasmapheresis', has started in order to assess the efficacy of treatment with rituximab in combination with Nanogam® to reduce allo-antibody levels in patients with high anti-HLA antibody titres, awaiting renal transplantation. The primary objective of the study is to achieve a negative cross-match and transplantability. Furthermore, patient and graft survival and graft function will be assessed. The study has been initiated in two Dutch renal transplant centres.

An investigator-initiated TIKI study, 'Treatment with or without IVIG in Kids with acute ITP' will be started in the beginning of 2009. In this randomized controlled study the influence of treatment with intravenous gammaglobulin on the course of the disease will be assessed. The hypothesis that early IVIG treatment in children with newly diagnosed acute ITP reduces the risk of development of chronic disease will be tested.

Preparations were started for the 'Immunoglobulin therapy for patients with idiopathic cardiomyopathy and endomyocardial Parvovirus B19 persistence - a prospective, double-blind, randomized, placebo-controlled clinical trial' (MD2009.01). Fifty patients with idiopathic cardiomyopathy and Parvovirus B19 persistence in the heart will be included, and the effect of IVIg on virus presence and cardiac functional capacity before and 6 months after IVIg therapy will be evaluated. The patients will be randomized to either receive IVIg or placebo (GPO) on top of their standard heart failure regimen. This study will start in 2009.

Nanofiltered Cetor®

To optimize viral safety, a double 15 nm filtration was introduced in the production process of Cetor®, a highly purified C1-esterase inhibitor concentrate. This improvement in the manufacturing has been submitted as a variation for marketing authorization. Therefore, a multi-centre study 'Pharmacokinetics, clinical efficacy and safety of C1 inhibitor concentrate (C1-esteraseremmer-N) for the treatment of hereditary (and acquired) angioedema' was set up in collaboration with five Medical Centres in the Netherlands. The study comprised three parts, pharmacokinetics (part A, phase II), treatment of attacks of angioedema (part B, phase III) and prophylactic use of C1 inhibitor (part C, phase III). The clinical study reports of part A and B of the study have been finalized in 2007. The results of part A showed that the pharmacokinetic properties of C1-esterase inhibitor and Cetor® are similar. In part B of the study the results clearly demonstrate that C1-esterase inhibitor-N is highly effective and safe in the treatment of acute angioedema attacks.
The prophylactic study, part C, has been completed in 2008 and the clinical study report will be finalized in 2009. The study was performed in the Academic Medical Centre Amsterdam. It provided data on both safety and efficacy to prevent

angioedema attacks in five hereditary and one acquired angioedema patient. The study demonstrated that the addition of the nanofiltration step in the production process of Cetor did not affect the efficacy in prophylactic treatment of angioedema patients. C1-esterase inhibitor-N was shown to be effective and safe.

Key publications

Frakking FN, Brouwer N, van de Wetering MD, Kleine Budde I, Strengers PF, Huitema AD, Laursen I, Houen G, Caron HN, Dolman KM, Kuijpers TW. Safety and pharmacokinetics of plasma-derived mannose-binding lectin (MBL) substitution in children with chemotherapy-induced neutropaenia. Eur J Cancer 2009; 45(4):505-12.

Strengers PFW, van Twuyver, E. Blood, blood components, plasma, and plasma products. Aronson, J (Ed): Side Effects of Drugs, Annual 30, Elsevier, Amsterdam, 2008:381-93.

Sanquin Reagents

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Sanquin Reagents has developed a broad range of blood grouping and immunology reagents for laboratories, including several innovative products for diagnostic use and for fundamental and clinical research. Sanquin reagents are available worldwide through a network of distributors, and bulk reagents for manufacturing are supplied directly form Amsterdam. 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 within Sanquin and/or with other companies and institutions.

R&D projects

The project portfolio in 2008 consisted of ongoing projects in the fields of blood grouping and immunology reagents.

The following development projects were continued or started in 2008

1) Assays to detect free, human immunoglobulin light chains (kappa, lambda) in blood.
2) Latex-based assays to quantify human IgG subclass species in blood for the Immage instrument.
3) A blood donor bloodgrouping chip based on DNA genotyping of red cell antigens.
4) QualiCard, a calibration tool for Magister/Cellbind.
5) Papain red cell panels for gelcard column techniques.

Products and patents

New products & processes

The following new products were commercially introduced in 2008:

1) PeliChange/-Screen: a kit to exchange peptides in MHC multimers (class I) using UV-cleavable peptides, and an ELISA to check for the efficacy of the peptide exchange. These tools can be used to identify new T-cell epitopes,

and can be used in research applications in cancer, transplantation and infection.

2) FlexMer products: for MHC-based cell isolations and stainings; these reagents consist of MHC monomers (Flex-Monomers), fluorochromes (Flex-PE / Flex-APC), magnetic beads (Flex-Beads) and a detach set (Flex detach set).
3) Various white label reagents.

In addition, a new manufacturing line to automate the labelling of Cellbind cards has been implemented.

Patents

Ongoing patent applications: 1) cleavable peptides in MHC class I molecules and 2) multiplex PCR & specific DNA sequences that are used in the bloodgrouping chip, and 3) Detection of antigen-specific T-cell responses.

Quality system

Two ISO certificates were renewed in 2008 (ISO 9001 & ISO 13485).

Publications

Zeerleder S, Zwart B, te Velthuis H, Stephan F, Manoe R, Rensink I, Aarden LA. Nucleosome-releasing factor: a new role for factor VII-activating protease (FSAP). FASEB J 2008; 22(12):4077-84.

Bakker AH, Hoppes R, Linnemann C, Toebes M, Rodenko B, Berkers CR, Hadrup SR, van Esch WJ, Heemskerk MH, Ovaa H, Schumacher TN. Conditional MHC class I ligands and peptide exchange technology for the human MHC gene products HLA-A1, -A3,-A11, and -B7. Proc Natl Acad Sci U S A 2008; 105(10):3825-30.

Sanquin Pharmaceutical Services

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proceed on next page

Sanquin Pharmaceutical Services (SPS) is a business unit specialized in a broad array of pharmaceutical services aiming at the development and quality testing 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, including viral reduction studies.

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 Clinical Laboratory Practice) accredited. Other QC services such as protein characterization, stability test programs, formulation studies and as well as the validation of client based assays are part of their dedicated activities.

Virus and prion validation studies

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Virus Safety Services (VSS) is a separate unit within SPS, dedicated to conducting virus and prion validation studies of plasma-derived products and other biologicals. VSS has extensive experience in validation of various process steps, including the more delicate ones, like column and nanofiltration steps. VSS uses for their activities state-of-the art BSL3 facilities, including strict separation between virus negative and virus positive areas. An Endorsement of Compliance with the OECD principles of GLP based on assessments performed according to the Netherlands GLP Compliance Monitoring Program and according to Directive 2004/9/EC was granted for these activities in 2005. VSS provides tailor-made solutions for virus validation problems using a broad range of virus systems, which meet with the latest requirements of national and international regulatory bodies. The following virus systems are available:

- 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;
- BPV (Bovine parvovirus), a specific model virus for Parvovirus B19;
- 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;
- MuLV (Murine Leukaemia Virus), a relevant virus for many biotech derived products;
- 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.

Sanquin Diagnostic Services

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Sanquin Diagnostic Services excels in routine and top-reference 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 to all Health Care Institutions and commercial companies in the Netherlands and abroad. 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 continuous innovation reflected by introduction of 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 2008

A number of new tests and services has been introduced in 2008:

- Level tests for omalizumab (anti-IgE) and natalizumab (anti-VLA-4)
- Factor H antigen and activity assays (to determine deficiencies)
- Assay to detect anti-ADAMTS-13 antibodies with a higher specificity than the existing assays currently available
- Assay for the detection of antibodies directed against the first domain of beta2-glycoprotein I
- Assay to determine the ADAMTS-13 antigen levels in plasma of patients
- A new fully automated platform for Nucleic Acid Amplification testing (NAT) of donations covering HBV, HCV, HIV-1, and HIV-2 detection in a multiplex format was validated and introduced
- New quantitative PCR assays for HBV, HCV, HIV-1 for application in clinical diagnostics and blood donor confirmation
- An EIA test for detection of anti-HTLV I/II was validated and implemented

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 2008 in the following fields:

- Development of a centralized ELISA facility;
- Use of array technologies for diagnostic purposes;
- Development of a single nucleotide polymorphism (SNP) platform.

In the following paragraphs, the above-mentioned developments will be described in more detail.

Immunocytology

We have previously validated PHOX2B as the first neuroblastoma specific target for the detection of minimal residual disease in neuroblastoma. The clinical relevance of the PHOX2B-PCR has been tested retrospectively. Patients with low tumor load at diagnosis (n=11) tended to have a better outcome than patients with high tumorload (n=37) (5-y-OS 63.6±14.5% versus 24.8±7.5%; p=0.06). The molecular response was measured at two time points. Fifteen patients had an early molecular response of the bone marrow (between 2 to 4 months) and this was associated with good outcome (5-y-OS 66.0±12.4% versus 16.7±7.6%; p=0.001). After induction chemotherapy BM of 13 patients were still MRD positive. Presence of BM MRD after induction chemotherapy (5-y-OS 7.7%±7.4 versus 51.9±11.9; p<0.001) was associated with poor outcome. For both time points, the prognostic value of molecular response was independent of INSS response, MYCN amplification and age at diagnosis, (p=0.02, p=0.01 respectively). Based on these retrospective data the value of MRD detection in neuroblastoma will be evaluated by our group in a prospective clinical trial in the Netherlands and Germany.

Key publications

Stutterheim J, Gerritsen A, Zappeij-Kannegieter L, Kleijn I, Dee R, Hooft L, van Noesel MM, Bierings M, Berthold F, Versteeg R, Caron HN, van der Schoot CE, Tytgat GA. PHOX2B is a novel and specific marker for minimal residual disease testing in neuroblastoma. J Clin Oncol 2008; 26:5443-9.

De Haas V, Dee R, Cheroutre G, van den Berg H, van der Schoot CE. Gene expression profile of slowly responding subclones might represent different profiles already at diagnosis and might be used for prediction of outcome. Leukemia 2009; 23(4):816-9.

Immunohematology

In 2008 we focussed on the development of a universal fetal marker to be used in fetal non-invasive typing assays. Furthermore, the potential use of fast-PCR protocols was evaluated, which will further improve the one-day blood group antigen molecular typing service and improve the logistics for the non-invasive fetal typing assays.
The potential use of antigen-coated bead-based antibody in platelet-leukocyte serology was evaluated in terms of sensitivity and specificity. Furthermore, the use of functional assays to predict the clinical significance of platelet autoantibodies or heparine-induced antibodies was further explored.

Key publication

Koelewijn JM, Vrijkotte TG, van der Schoot CE, Bonsel GJ, de Haas M. Effect of screening for red cell antibodies, other than anti-D, to detect hemolytic disease of the fetus and newborn: a population study in the Netherlands. Transfusion 2008; 48(5):941-52.

Immunopathology

The technology for development of specific tests for levels of and antibodies to biologicals was adopted from the reasearch department of Immunopathology and is now broadly applicable. As a result, level tests for omalizumab (anti-IgE) and natalizumab (anti-VLA-4) are now available.

The determination of antibodies to aquaporine-4, as a new diagnostic test for Neuromyelitis optica, has become available for routine patient samples. In close collaboration with R Hintzen PhD (Erasmus MC, Rotterdam) a clinical validation of the assay is ongoing.

Next to the measurement of antibodies against cyclic citrullinated peptide, which is now used as a standard assay for rheumatoid arthritis patients, we have studied the fine specificity and the kinetics of anti-citrullinated protein antibodies (ACPA) in rheumatoid arthritis patients treated with anti-TNF biologicals.
Factor H is the most important non-cell-bound inhibitor of the alternative pathway of complement activation. A shortage (or deficiency) in functional factor H leads to uncontrolled activation of the alternative complement activation pathway, resulting in usage of C3 and terminal complement pathway factors. Factor H-deficiency is associated with development of MPGNII and is accompanied by hypocomplementemia. Functional factor H-mutations occur in a subgroup of atypical hemolytic uremic syndrome (aHUS). These mutations almost never lead to hypocomplementemia of decreased factor H concentrations. To determine factor H-deficiency, we perform both antigen and activity assays.

Key publications

Bos WH, Bartelds GM, Vis M, van der Horst AR, Wolbink GJ, van de Stadt R, van Schaardenburg D, Dijkmans BA, Lems WF, Nurmohamed MT, Aarden L, Hamann D. Preferential decrease of IgG4 anti-citrullinated protein antibodies during treatment with TNF blocking agents in patients with rheumatoid arthritis. J Rheumatol 2008; 35(3):425-8.

Stapel SO, Asero R, Ballmer-Weber BK, Knol EF, Strobel S, Vieths S, Kleine-Tebbe J, EAACI Task Force. Testing for IgG4 against foods is not recommended as a diagnostic tool: EAACI Task Force Report. Allergy 2008; 63(7):793-6.

Red Blood Cell diagnostic services

In 2008, efforts have been focussed to standardize and validate our new tests for red cell membrane defects. These include the expression of Band 3 (EMA test), enhanced osmotic resistance by a modification of our current AGLT-test and quantitative membrane protein analysis by SDS/PAGE followed by densitometry. In some special cases, we also evaluate the extent of phospholipid asymmetry in the red cell membrane to understand possible changes in red cell clearance.

Key publication

Van der Schoot CE, de Haas M, Engelfriet CP, Reesink HW, Panzer S, Jungbauer C, Schwartz DM, Mayr WR, Castilho L, St-Louis M, Long A, Denomme G, Semple E,Fernandes B, Flegel WA, Wagner F, Doescher A, Poli F, Villa MA, Paccapelo C,Karpasitou K, Veldhuisen B, Nogués N, Muñiz-Diaz E, Daniels G, Martin P, Finning K, Reid ME. Genotyping for red blood cell polymorphisms. Vox Sang 2009; 96(2):167-79.

Blood Coagulation

The antiphospholipid syndrome is characterized by vascular thrombosis and/or specific pregnancy morbidity. Antiphospholipid antibodies are a heterogeneous population of antibodies recognizing different phospholipids-binding proteins. It is now generally accepted that antibodies with affinity for beta2-glycoprotein I are best correlated with clinical symptoms related to the antiphospholipid syndrome. It has been shown in a single center study that antibodies directed against the first domain of beta2-glycoprotein I are better correlated with thrombosis than antibodies directed against the other domains of beta2-glycoprotein I (De Laat et al. Blood 2005; 105:1540-5). We have conducted an international multi-center study to verify these results (submitted for publication). Indeed, we were able to show that an anti-domain I assay correlated up to 4 times better with clinical symptoms than a regular anti-beta2-glycoprotein I assay. The anti-domain I assay will be included into our thrombotic arsenal of testing this year.
Thrombotic thrombocytopenic purpura, better known as TTP, is characterized by a pentad of clinical symptoms; microangiopathic hemolytic anemia, thrombocytopenia, neurologic symptoms, renal involvement and fever. These symptoms are caused by a deficiency of ADAMTS-13 (von Willebrand factor cleaving protease). TTP can be either congenital or acquired due to the formation of antibodies directed against ADAMTS-13. The assays currently available lack specificity and sensitivity, which makes it difficult to diagnose a patient with TTP. Therefore we have developed several different assays for the laboratory diagnosis of TTP. We have developed an assay to detect anti-ADAMTS-13 antibodies with a higher specificity than the existing assays currently available. In addition, an assay was developed to determine the ADAMTS-13 antigen

levels in plasma of patients. This ADAMTS-13 antigen assay is able to quantify antigen levels more accurately in the lower range than existing assays. Due to the higher specificity and sensitivity of our assays we are able to better diagnose patients with TTP and better monitor ADAMTS-13 levels during treatment.

Key publication

De Laat B, Eckmann CM, van Schagen M, Meijer AB, Mertens K, van Mourik JA. Correlation between the potency of a beta2-glycoprotein I-dependent lupus anticoagulant and the level of resistance to activated protein C. Blood Coagul Fibrinolysis 2008; 19(8):757-64.

Infectious Diseases (blood donor screening)/Viral diagnostic services

Donor and plasma screening

A new fully automated platform for Nucleic Acid Amplification testing (NAT) of donations covering HBV, HCV, HIV-1, and HIV-2 detection in a multiplex format was validated and introduced during 2008. Special attention was paid to the validation of the sensitivity of the assay for the three markers. In addition, the assay was validated for application of in process testing of manufacturing pools. Additional assays were validated/developed to specify the viral marker in case of a reactive multiplex positive sample. The in house assay to detect HIV-2 RNA was characterized in a study organized by the National Institute for Biological Standards and Control, (NIBSC) for the WHO Biological Standardization program.
Since November 2008, all blood donations from Sanquin are tested with this new platform. Detection of these viruses is performed in small test pools enabling sensitive detection, especially important for HBV DNA to detect pre-seroconversion (HBsAg)- and 'occult' phase samples. Several 'HBV DNA only' donations were identified most of them representing donations in the occult phase of infection. 'Look back' is planned to study whether these donors transmitted HBV in the past.

Recent studies have shown that the Parvovirus B19 genome is more variable than previously published; new genotypes were discovered and even conserved regions

revealed more point mutations. Sanquin Plasma Products screened > 4.5 million donations with two B19 DNA NAT assays. One of these assays only detects genotype 1. About 280 donations were excluded for fractionation due to a high Parvovirus B19 load. In 8 cases discrepant results between the two assays were found; 3 as result of another genotype (gt2) and 5 due to mismatches in the primer or probe binding site. None of the assays detected all donations to be excluded. Based on these results both assays amplifying two different regions of the virus will be continued to obtain maximum viral safety.

Viral Diagnostic Services

In 2008, new quantitative PCR assays for HBV, HCV, HIV-1 for application in clinical diagnostics and blood donor confirmation have been introduced. The new generation assays combine ultimate sensitivity with quantification of the viral target over a large dynamic range. This test format makes it possible to use these tests for confirmation of infection and clinical follow up of treatment. This makes separate qualitative and quantitative tests for these viruses redundant. With respect to HIV-1 the new assay is improved (LTR and gag amplification) to detect also isolates with rare mismatches in the previous assays using only one region for amplification (gag). An EIA test for detection of anti-HTLV I/II was validated and implemented on an automated platform for serological tests. These tests complete the package of assays for 'Cito' screening of blood, tissue and organ donations. This makes it possible to complete testing in < 1 hour. This service will improve the viral safety of urgently needed blood components and tissue/organs.

Key publications

Corcoran A, Kerr S, Elliott G, Koppelman MH, Doyle S. Improved detection of acute Parvovirus B19 infection by immunoglobulin M EIA in combination with a novel antigen EIA (2007). Vox Sang 2008; 93:216-22.

Koppelman MH, Rood IG, Fryer JF, Baylis SA, Cuypers HT. Parvovirus B19 genotypes 1 and 2 detection with real-time PCR assays. Vox Sang 2007; 93:208-15.

HLA diagnostic services and Paternity testing

Allogeneic hematopoietic stem cell transplantation (HSCT) is a well-established therapy for several malignant disorders. The success of this therapy is based on human leukocyte antigen (HLA) matching between patient and donor. However, even in the HLA identical transplantation setting complications like graft-versus host disease or a relapse of the original malignant disease can occur. If an additional diagnostic tool would exist, than the selection of a stem cell donor could be further optimized, reducing the risk of complications after the transplantation. Recently, it has become evident that other immune-related genes may influence the outcome of HSCT. The polymorphic killer-cell immunoglobulin-like (KIR) genes have an impact on the clinical outcome after transplantation. The impact of KIRs seems to be very much dependent on transplant protocols, with the application of donor T-cell depletion possibly being the most important variable. Yet, the exact role of KIR genes in stem cell transplantation is still unknown.

In the HLA identical transplantation setting, KIR genotypes can differ between patient and donor, because the KIR complex and HLA region are located on different chromosomes, chromosome 19 and 6 respectively, and therefore segregate independently. Currently, the KIR family consists of 15 KIR genes. The KIR genes code for both inhibitory (KIR2DL and KIR3DL) and activating (KIR2DS and KIR3DS) receptors. These receptors have been shown to be highly polymorphic both at the allelic as well as haplotypic level. The number and type of KIR genes present on haplotypes can vary between individuals.

In this retrospective study, we evaluated the impact of KIR genes on the overall patient survival in related non-T-cell depleted HLA identical stem cell transplantation. The study group consisted of 83 patients who received a non-T-cell depleted HLA identical sibling stem cell transplantation. Patients were diagnosed with acute myeloid leukemia, chronic myeloid leukemia, chronic lympocytic leukemia, multiple myeloma, non-Hodgkin's lymphoma and myelodysplastic syndrome. KIR genotyping was performed by means of PCR-sequence specific primer method in which the presence or absence of 15 KIR genes was detected. Three hematologists of the VUmc and the AMC gathered the clinical endpoints; overall patient survival,

transplant-related mortality and disease progression at one and two years after transplantation. 78 patients received stem cells from an HLA identical donor in which KIR2DS4 was present. The patient survival of these patients was significantly better than of patients who received stem cells from donors in which KIR2DS4 was absent (p=0.001). We found the same correlation between KIR3DL1 and overall survival. The presence of KIR3DL1 in the stem cell donor (n=78) was correlated with a better overall patient survival (p=0.001).

These data show that in related non T-cell depleted HLA identical stem cell transplantation, the presence of KIR2DS4 and KIR3DL1 in the donor has a positive impact on the overall patient survival.

In conclusion, KIR2DS4 and KIR3DL1 genotyping could be used as an additional diagnostic tool for optimum donor selection. Currently, stem cell donor selection is primarily based on HLA matching. If a patient has more than one HLA identical sibling, selection is based on additional clinical criteria. One of the most important of these is the cytomegalovirus serologic status of the sibling compared to the status of the patient. Subsequently, several other selection criteria are used, such as Epstein Barr virus serology, blood group, medical history, gender and age of the potential donor. In future, KIR genotyping might be an additional selection criterion to reduce complications after stem cell transplantation. Our findings suggest that one should select for stem cell donation the HLA-identical sibling in which KIR2DS4 and KIR3DL1 are present.

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The mission of Sanquin Consultancy Services (SCS) is to provide guidance and advice services to restricted economy countries.

Objectives are

1) support restricted economy countries in developing safe, efficacious and sustainable blood supply systems based on current quality principles,
2) provide modular training programmes on transfusion medicine for restricted economy countries focused on the managerial and quality aspects of the transfusion chain, and
3) extend and strengthen the training and consultative potential within the Sanquin organization.

In close collaboration with the Academic Institute for International Development of Transfusion Medicine (IDTM) educational programmes and applied research in health sciences related to the field of transfusion medicine have been developed. Existing educational and scientific collaborations of the Academic Institute IDTM and SCS at the University of Groningen contribute to the development of safe, efficacious and sustainable blood supply systems based on current quality principles.

Educational programmes supported by the Academic Institute IDTM, University of Groningen:

Noordelijke Hogeschool Leeuwarden (NHL)

Two BBA students from China worked on 1) the evaluation mechanisms of new marketing material (website and brochure of SCS and IDTM) and 2) how to initiate the IDTM MMTM China project – strategy and road map for implementation to accommodate fellows from the SCS projects.

Collaborative scientific projects of the Academic Institute IDTM, University of Groningen and Sanquin Consulting Services:

1) University of Amsterdam (UvA), Faculty of Economy and Econometry, Dept of Operations Research.
   PhD University of Amsterdam – Solving large structured Markovian decision problems for perishable-inventory management and traffic control.
2) Groningen University (RUG), Dept of Social Pharmacy, Pharmaco-Epidemiology and Pharmacotherapy, Faculty of Pharmacy.
   PhD University of Groningen - Health Economics of blood transfusion safety in developing countries.
3) Groningen University (RUG), Faculty of Medical Sciences and Makerere University, Faculty of Medicine, Kampala, Uganda.
   PhD fellow from Kampala, Uganda working on 'Appropriate use of the limited supplies of homologous blood in Uganda.

Valorization, patent portfolio and licensing

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Besides results of research projects published in scientific and professional publications, Sanquin also disseminates knowledge in the form of patents and other forms of know-how. In 2008 Sanquin focused its IP efforts on the maintenance of its patent portfolio in the area of blood coagulation, and building and expanding its portfolio regarding the monitoring of clinical efficacy of therapeutic monoclonal antibodies and enhancement thereof. Seven patent applications were filed in 2008. Most often in-licensing parties both seek the opportunity of patent protection for future product pipelines and the expertise of our inventors. Therefore a license contract in general incorporates a joint research agreement enabling Sanquin to generate funding for its research and enabling third parties to evaluate the patent proposition.

Below you will find an overview of the valorization status of Sanquin Research patents and hybridoma's of the last five years is shown. Most patents/hybridomas listed have a primary therapeutic application.

Patent	Status in 2008
MHC Multimers	Open for licensing
Dendritic Cells Maturation	Open for licensing
Diagnostic methods involving determining gene copy numbers	Out licensing discussions ongoing
Anti Antibody	Out licensing discussions ongoing
Anti-FVIII	Open for licensing
FVIII mutants	Open for licensing
Mabs for intact hemostatic proteins	Open for licensing
C1-est inhibitor in AMI	3rd party out licensed
Trombose PCR/FV Leiden	3rd party out licensed
CD 97	3rd party assigned

Sanquin hybridoma's 2002-2007	Status in 2008
CD 19	Open for licensing
Anti c-1q / Anti c3-2 / 2C8	Open for licensing
4-7B	3rd party licensed
CD70	3rd party licensed
IL 6	partly out-licensed/ discussions ongoing

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 AMCSanquin Landsteiner Laboratory for Blood Transfusion Research, housed in Sanquin's premises in Amsterdam.

2nd source of funding
Dutch Medical Research Council (ZON/MW)
European Commission
Netherlands Genomics Initiative (NROG)
Netherlands Organization for Scientific Research (NWO)
SENTER/Novem

3rd source of funding (Charities, private funding organizations, non-Dutch Research councils)
Dutch AIDS Fund (SAF)
Dutch Cancer Fund /KWF
Dutch Heart Foundation
Dutch Thrombosis Foundation
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
National AIDS Therapy Evaluation Center
Tekke Huizinga Foundation

4th source of funding: Contract and co-development partners*
Ablynx
Academic Hospital, University of Maastricht
Academic Medical Center, University of Amsterdam
Algonomics
American Red Cross
Amcell Corporation
Amgen
ASAC
A-Viral ASA
Bayer
Baxter BioScience
Baxter Health Care
Baxter Oncology
BCSI
Berna Biotech
Biogen
BioMérieux
BioSafe
Biotest Pharma GmbH
Caridian BCT
CARIM, Cardiovascular Research Institute Maastricht
Cerus Corporation
Crucell
Direvo

Diaclone
DSM Biologics
Fenwal
Finnish Red Cross
Fresenius Kabi
Genmab
GlaxoSmithKline
Guava Technologies
Haemonetics
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Miltenyi Biotec
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Morphosis AG
Natal Bioproducts Institute
Navigant Bonville
Netherlands Vaccin Institute
NIZO laboratories
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Organon/Schering Plough
Ortho-Clinical Diagnostics
Pharming
Philips
ProLacta
Région de Bruxelles-Capitale
Roche Diagnostics
Roche Pharmaceuticals
Schering Corporation
Seattle Genetics
Slotervaart Hospital
Staten Serum Institute
Synaps BV
Trubion
University Medical Center Utrecht
Vitaleech Bioscience
Vrije Universiteit Medical Center, Amsterdam
Wageningen University and Research Center
Wyeth
Zentech s.a.
Zentral Laborator Bern

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Publicaties in peer reviewed journals

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PhD theses

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Rachel van Beem

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