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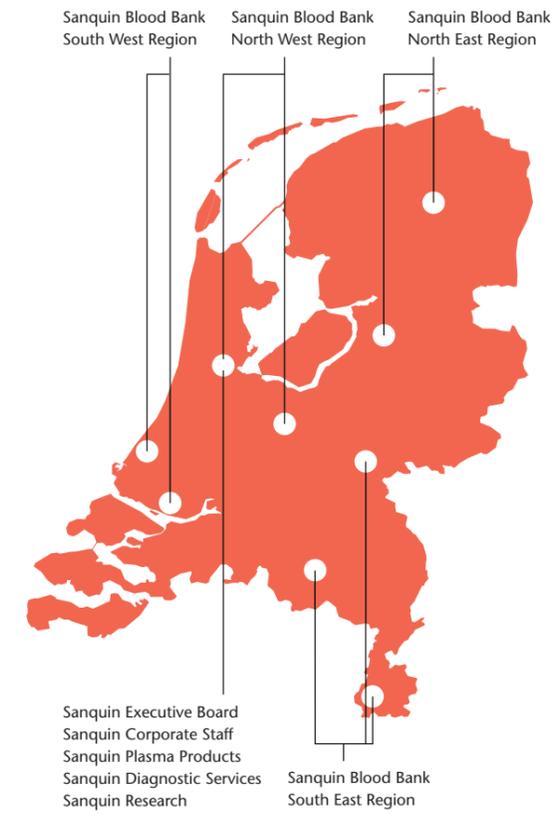


Sanquin Scientific Report 2002

Sanquin places the  
greatest demands on  
quality, safety and  
efficiency.  
And service forms an  
integral part.

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

Scientific Report 2002



## Colophon

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### Printing

Spinhex & Industrie, Amsterdam

Scientific Report 2002

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Research is the  
foundation of Sanquin.  
We are at the forefront  
of our field, and want  
to remain there.

# The number of citations of papers published by Sanquin researchers in major scientific journals is a strong measure of the quality of our research.

'Sanquin puts a lot of effort in research and development. All these research activities enable us to make better products, to offer better services and to disseminate our knowledge towards clinicians, patients and donors. Almost 250 people are intensively engaged in this – including all technical and analytical support staff. Our research is competitive, as is shown by numerous publications of our scientists in major scientific journals. Citation analysis shows that our research has a large impact: our publications are cited more than average. At least, that means that our colleagues take an interest in the things we do. We organise audits as well: we ask top notch scientists from the Netherlands and abroad to review the quality of our work. In-house, our Scientific Advisory Board and Research Programme Committee co-ordinate research efforts based on quality and relevance to Sanquin's mission statement. This has to do with research topics and focal points.

Despite all organisational and technical aspects, research is about people. We are actively looking to find the right people and work to retain them for our research. That isn't always easy. We not only want good scientists, but we are looking for those who want to share our mission and have a feeling for clinical and patient oriented research.'



Frank Miedema, Jeroen de Wit



Citation analysis

# Introduction

In 1998 the Blood Banks and the Central Laboratory of the Dutch Red Cross Blood Transfusion Service merged into the Sanquin Blood Supply Foundation. Since then, Sanquin is actively trying to further strengthen its research efforts and is focusing its research programme around its mission to ensure the Dutch blood supply and to advance transfusion medicine in such a way that the highest possible standards are met in quality, safety and efficiency. Sanquin supplies products and services, performs scientific research and offers education and training programmes.

Increasingly, Sanquin will endorse a corporate research and training programme covering a broad range of areas within the framework of blood supply, transfusion medicine and all its threats and challenges. In this 2002 Scientific Report all research activities of Sanquin are covered. Also research performed at CAF Belgium, the Belgium Foundation for Plasma Fractionation, is included in this report.

## Research portfolio

The research portfolio of Sanquin comprises research and technology projects to support the maintenance and development of Sanquin's products and services.

## Scientific publications

	<i>Publications total number</i>	<i>SCI publications</i>	<i>Theses</i>	<i>Average impact factor</i>
<b>2002</b>	<b>177</b>	<b>142</b>	<b>7</b>	<b>4.70</b>
2001	173	131	12	4.06
2000	169	137	12	4.64
1999	204	166	9	4.03
1998	222	167	7	4.41
1997	264	197	14	3.95
1996	281	204	11	4.07
1995	272	192	16	3.89
1994	230	185	11	na
1993	226	179	12	na

## Articles\* published in 1993 through 1997 annual reports cited\*\* in five full years after publication

Publications from	Total citations	number of SCI publications	Citations in							2002		
			1994	1995	1996	1997	1998	1999	2000		2001	
1993	3483	179	551	800	818	699	615					
1994	3599	185		596	894	871	686	552				
1995	3215	192			484	736	732	641	622			
1996	3057	204				491	736	685	615	530		
1997	2962	197					369	661	657	656	<b>619</b>	

\* Only SCI publications are included

\*\* Excluding self citations

The portfolio of strategic research is focussed at:

- haematology
- haemostasis and thrombosis
- inflammation and sepsis
- blood transmitted diseases
- alternatives for bloodproducts.

Product- en process development focuses on:

- quality and safety
- efficiency
- new cellular therapies
- (evaluation) of clinical applications
- cost-effectivity analyses
- donor studies.

Research within Sanquin includes basic, strategic, and applied studies with expertise from – among others – the fields of biochemistry, immunology, cell biology, virology, bacteriology, haematology, blood transfusion medicine, and epidemiology.

### Research budget

Besides research projects funded from within the Sanquin organisation, Sanquin obtains – in competition – funding from the Dutch Research Council (NWO), the European Commission, and several charities. Sanquin is involved in contract research as well as co-development activities.

### Research organisation

Most of the research is performed within the research division, Sanquin Research, based in Amsterdam. However, more and more, the research departments in each of the four blood bank divisions are finding their own niche, often in close collaboration with university and hospital based researchers. The focus is mainly on translational and clinically oriented research, and research closely related to day to day blood bank practice. The division of Plasma Products has its own department of product- and process development.

Much of the research in the fields of immunology, immuno-haematology, and infectious diseases is being performed within the setting of the collaborative Landsteiner Laboratory of Sanquin and the Academic Medical Centre of the University of Amsterdam. Long standing collaboration exists with the Leiden University Medical Centre, and several other university based clinical and research departments.

### Research management

In 2002 research policy has been further strengthened. Sanquin's research policy is based on both intrinsic scientific developments and problems and questions arising from the primary processes of all Sanquin divisions. The Executive Board of Sanquin is ultimately responsible for research policy, the director of Sanquin Research has a delegated responsibility in enforcing Sanquin's research programme and structure.

### Scientific advisory board

Prof. EJ Ruitenber PhD (chairman, Utrecht University & Free University, Amsterdam)

Prof. DKF Meijer PhD (University of Groningen)

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

Prof. HC Hemker MD PhD (Maastricht University)

A de Jonge PhD (Semaia Pharmaceuticals)

Prof. A Brand MD PhD (Sanquin Bloodbank South West Region & Leiden University)

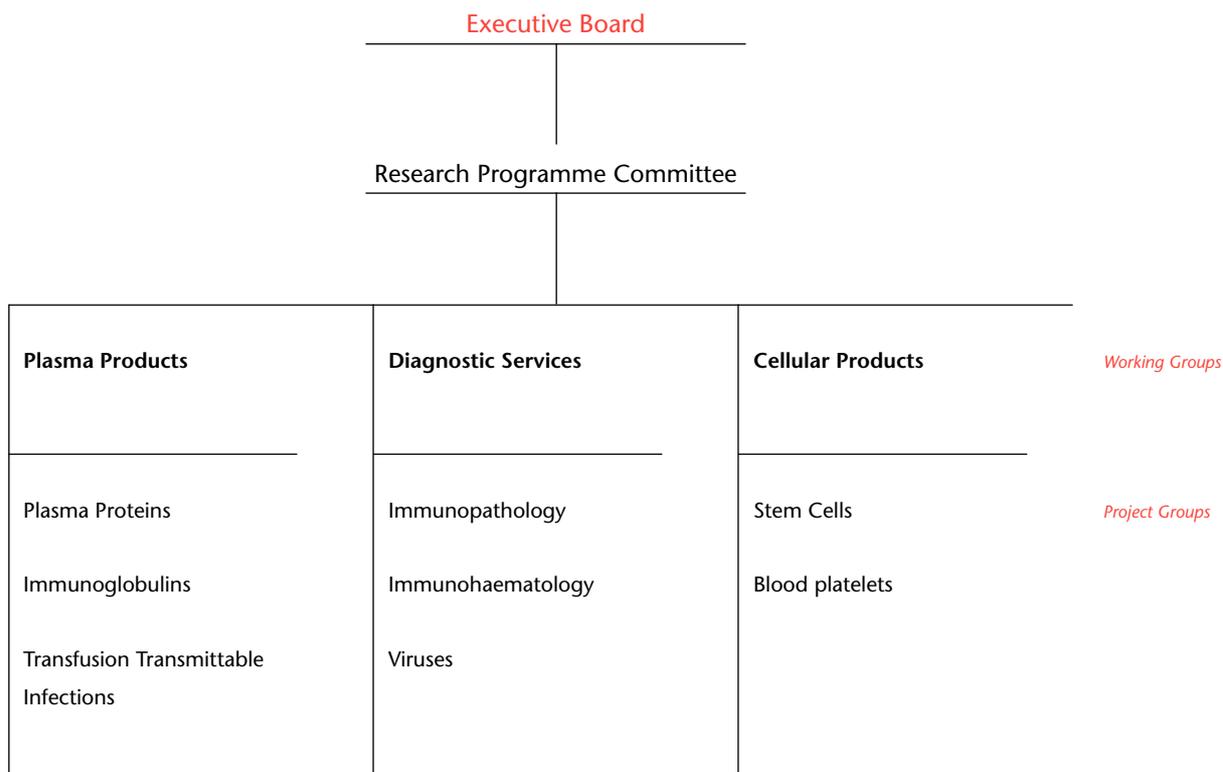
Prof. MM Levi MD PhD (University of Amsterdam)

Prof. F Miedema PhD (Sanquin Research & AMC, University of Amsterdam)

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

JW Smeenk MSc (executive secretary)

Sanquin Executive Board receives recommendations from the Scientific Advisory Board through an annually updated five year planning document – indicating areas of interest, as well as various independently drawn policy documents on various subjects relating to content and organisation of research.



The Research Programming Committee consisting of four division directors covering the research portfolios plasma products, diagnostic services, and cellular products is chaired by the director of Sanquin Research. Based on the planning document mentioned above, this committee advises the executive board on start and (dis)continuation of projects funded from Sanquin’s own resources.

On each of the stakes, working groups of senior investigators, decide on what projects proposals to write – in close collaboration between all divisions involved. Projects are submitted in competition to strengthen quality and relevance of the projects involved. Active researchers discuss progress of research projects and new ideas in project groups in order to generate new project proposals for funding from both internal and external sources.

### Quality Assessment

Some years ago a system of recurrent site visits by peer review committees was introduced. In 2002 the department of Transfusion Technology was reviewed, as was Sanquin’s research on Immunohaematology, where research from both Sanquin Research and Sanquin Blood Bank North East Region was reviewed. The core of the peer review committees are members of the Research Assessment Board, consisting of Dutch, as well as international members. The Peer Review Committee on Transfusion Technology consisted of professors MM Levi (AMC, University of Amsterdam) and JR Hess (Baltimore, USA). Members of the Peer Review Committee on Immunohaematological Research were professors CG Figdor (University of Nijmegen), VWM van Hinsbergh (TNO-Institute for Prevention and Health), D Anstee (Bristol, UK), P Lansdorp (Vancouver, Canada), and SC Silverstein (New York, USA).

## Research Assessment Board

### National members

Prof. RM Bertina MD PhD (Leiden University)  
 Prof. FC Breedveld MD PhD (Leiden University)  
 Prof. WE Fibbe PhD (Leiden University)  
 Prof. F Grosveld PhD (Erasmus University Rotterdam)  
 Prof. ADME Osterhaus PhD (Erasmus University Rotterdam)  
 Prof. JJ Sixma MD PhD (Utrecht University)

### International members

Prof. D Anstee MD, PhD (University of Bristol, United Kingdom)  
 Prof. R Carell PhD (University of Cambridge, Cambridge, United Kingdom)  
 Prof. RA Flavell PhD (Yale University, New Haven, USA)  
 Prof. LW Hoyer MD (American Red Cross, Rockville, MD, USA)  
 Prof. MD Kazatchkine MD PhD (INSERM, Hospital Broussais, Paris, France)  
 Prof. RA Koup MD PhD (University of Texas, Southwestern Medical Center, Dallas, USA)  
 Prof. D Lane MD PhD (Imperial College School of Medicine, London, United Kingdom)

## Academic affiliations:

### Education & training in Blood Transfusion Medicine, Immunology and laboratory techniques

Sanquin research departments attract many students who participate in scientific projects.

Historically there is a strong collaboration with the Academic Medical Centre (AMC) of the University of Amsterdam. Sanquin staff members participate in research programs and curricula of the AMC Research Institute for Immunology (JJ van Loghem Institute) and the Research Institute for Infectious Diseases. The collaborative AMC – Sanquin Landsteiner Laboratory is housed within Sanquin premises. At many Dutch universities, staff from various Sanquin divisions are involved in training programmes for undergraduate and graduate students in (medical) biology, pharmacy, medicine, and health sciences as well as laboratory technicians. Of course, Sanquin is also involved in training of specialist in blood transfusion medicine, other medical specialties, and training of nurses.

## Professorships of Sanquin staff members

Prof. RC Aalberse PhD (Biological immunology, Faculty of Biology, University of Amsterdam)  
 Prof. LA Aarden PhD (Molecular immunology, Academic Medical Centre, University of Amsterdam)  
 Prof. A Brand MD PhD (Blood transfusion medicine, Faculty of Medicine, Leiden University)  
 Prof. CE Hack MD PhD (Immuno-pathophysiology of non specific immunity, Free University, Amsterdam)  
 Prof. K Mertens PhD (Pharmaceutical plasma proteins, Faculty of Pharmacy, Utrecht University)  
 Prof. F Miedema PhD (Immunology of AIDS, Academic Medical Centre, University of Amsterdam)  
 Prof. DJ van Rhenen MD PhD (Blood transfusion medicine, Faculty of Medicine and Health Sciences, Erasmus University Rotterdam)  
 Prof. D Roos PhD (Non-specific immunology, Academic Medical Centre, University of Amsterdam)

## Awards/Grants

In 2002, Sanquin obtained three prestigious fellowships from the Dutch National Research Council. Two so-called VENI grants, to give a researcher the opportunity to develop their own ideas for a three year period (Neeltje Kootstra and José Borghans), Ellen van der Schoot received a so-called ASPASIA grant.

During the 4<sup>th</sup> annual research conference, organised by the Dutch Blood Transfusion Society and Sanquin, the annual Van Loghem Research Prize for the best oral presentation was awarded to Koert Dolman (Sanquin Research). The Dutch Blood Transfusion Society award for the best poster was granted to Judith van Drunen, and co-workers of Sanquin Blood Bank South West Region. The annual PhD award for the best published paper was won by Jaap van Buul (Sanquin Research). For the 27<sup>th</sup> time the international Groningen symposium of the Blood Bank North East Region was organised; this year entitled 'Cellular engineering and cellular therapies'.

Prof. F Miedema PhD  
 Prof. DJ van Rhenen MD PhD

# Department of Clinical Viro-Immunology

## Academic staff

*J Schuitemaker PhD (head)*  
*D van Baarle PhD*  
*FAC van Engelenburg PhD*  
*Prof F Miedema PhD*  
*JWAM van Oers PhD*  
*MThL Roos PhD*  
*NA Tesselaar PhD*

## Postdoctoral fellows

*J Borghans PhD*  
*I van den Nieuwenhof PhD*  
*A van Leeuwen-Gorter*

## PhD-students

*T Beaumont*  
*C Bronke (AMC)*  
*F Danisman (GG&GD)*  
*MD Hazenberg MD*  
*CA Jansen*  
*N Kloosterboer*  
*FA Koning*  
*S Kostense*  
*IS Kwa*  
*E Quakkelaar*  
*E Piriou*  
*R van Rij*  
*E Stalmeijer*  
*N Vrisekoop*

## Technical staff

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*F van Alphen*  
*C Beugeling*  
*AE van den Blink*  
*BDM Boeser-Nunnink*  
*A. Boots*  
*N Brinkhuis*  
*SM Broersen*  
*D Dam*  
*SJ Dekker*  
*K van Dort*  
*L Dekker*  
*E Gijsen*  
*S de Haan*  
*A Harskamp-Holwerda*  
*B Hooibrink*  
*J Kant-van Eenbergen*  
*HGH Korsten*  
*M Mangas-Ruiz*  
*A Meinesz*  
*S van Miltenburg*  
*CW Mollenaar*  
*NM Nanlohy*  
*AC van Nuenen*  
*J Ottenhoff-Mollenaar*  
*SA Otto*  
*N Palmer*  
*V Purwaha*

*R Ran*

*GMW van Schijndel*  
*H Smit-Rietkerk*  
*P van Swieten*  
*FG Terpstra*  
*KE Vandenberghe till 4/02*  
*J Vingerhoed till 8/02*  
*JA Velstra-Visser*  
*TJK van der Vorst*  
*N Willemse*  
*CA Witte*

## Guest

*A Tsegaye (Ethiopia)*

## Undergraduate students

*I Bergval*  
*B Everts*  
*M Navis*  
*I Rood*

## Secretary

*GSM Damhuis*

## Persistent hyperactivation of the immune system may lead to erosion of the naive T-cell pool and CD4<sup>+</sup> T-cell depletion.

The major research themes in the Department of Clinical Viro-Immunology concern the immunopathology and the viro-pathogenesis of HIV infection. Immunity against other viruses, such as EBV and CMV, is studied in relation to the pathogenesis of AIDS and other clinical diseases. Another line of research focuses on prion-related disease (TSE).

### Immunopathology of HIV infection

#### Chronic immune activation is the major driving force for CD4<sup>+</sup> depletion

A long standing line of research is directed towards the pathogenic mechanism by which HIV-1 infection causes depletion of CD4<sup>+</sup> T-cells, immune deficiency and ultimately AIDS. In the past year, we have shown that increased peripheral cell division rates that characterise HIV-1 infection are caused by chronic immune activation through the persistently active virus, rather than by a homeostatic response to T-cell depletion. HIV-1 infection is characterised by chronic generalised CD8 and CD4 T-cell hyperactivation, the biological impact of which is not completely understood. We have proposed recently that chronic immune activation may in fact be the driving force for depletion of in particular the naive CD4 T-cell pools, which are the most difficult to replenish and will hence lead to CD4<sup>+</sup> T-cell depletion (Hazenberg et al, Nat Immunol 2000). We, therefore, studied the relation between chronic immune activation and CD4 T-cell depletion in HIV-1 infection in a prospective cohort study among participants of the Amsterdam Cohort Studies on HIV-1 infection and AIDS with a known seroconversion date (n=102). Pre- and post-seroconversion (1 and 5 years after seroconversion) CD4<sup>+</sup> and CD8<sup>+</sup> T-cell activation marker expression, T-cell proliferation and T-cell numbers were analysed in Cox proportional hazard analyses for predictive value of these parameters for progression to AIDS. Pre-seroconversion low CD4<sup>+</sup> T-cell numbers or elevated levels of CD4<sup>+</sup> T-cell activation were associated with increased risk for develop-

ment of AIDS after HIV-1 seroconversion. Progression to AIDS was associated with loss of both CD4<sup>+</sup> and CD8<sup>+</sup> naive T-cells. We confirmed the predictive value of CD8 T-cell activation and showed in addition that in the course of infection, low CD4<sup>+</sup> T-cell counts, increasing proportions of dividing CD4<sup>+</sup> and CD8<sup>+</sup> T-cells or elevated CD4<sup>+</sup> T-cell activation marker expression became independent predictors of progression to AIDS. These data support the hypothesis that persistent hyperactivation of the immune system may lead to erosion of the naive T-cell pool and CD4<sup>+</sup> T-cell depletion.

We have extended our work on the interpretation of T-cell receptor excision circles (TRECs) with regards to estimating thymic out put by studies in HIV infected children and in children with various congenital immunodeficiencies and in collaboration with the LUMC, in severe combined immunodeficiency disease (SCID) patients that received a BMT up to 20 years ago. These data show that TRECs may be used to estimate thymic out put when one controls carefully for parameters that greatly and rapidly impact TREC content such as T-cell proliferation and turnover. In the past year a prestigious VENI post doc fellow funded by the Dutch National Research Council has started who investigates, using immunoscope TCR repertoire probing, the mechanism of CD4 depletion. It will be studied whether this happens randomly or through clonal deletion mainly. Finally to study the role of immune activation in CD4<sup>+</sup> T-cell depletion, studies in a CD70 transgenic mouse model developed in our institute by Van Lier and co-workers have started to do in depth T-cell turnover studies and to study the contribution of the thymus, in particular.

#### Quantitative and qualitative aspects of virus-specific cellular immunity

Despite readily detectable virus specific CD8<sup>+</sup> T-cells in most HIV-infected patients, immune surveillance is eventually lost, leading to progression to AIDS. In addition, loss of control over Epstein-Barr virus (EBV), a widespread human  $\gamma$  herpesvirus, may cause AIDS-related Non Hodgkin's Lymphomas (AIDS-NHL). Furthermore, Cytomegalovirus (CMV) infection

can cause serious clinical complications in HIV-infected subjects. This line of research aims to unravel the role of cellular immunity against different viruses in HIV-infection.

### Phenotypic and functional analysis of virus-specific T-cells in the course of HIV-infection

Considering the variable outcome of infection with different viruses, it could be possible that virus-specific T-cells differ in cytotoxic capacity. This difference may become more pronounced in persistent virus infection. Therefore, we investigated possible differences in virus-specific cytotoxic potential in relation to phenotype, the perforin and granzyme B content of HIV, CMV and EBV specific CD8<sup>+</sup> cells in the course of HIV-infection in HLA-A2<sup>+</sup> individuals. In all individuals, CMV- and to a lesser extent EBV-specific T-cells expressed less CD27 and contained more perforin positive T-cells compared to HIV-specific CD8<sup>+</sup> T-cells. In the course of HIV infection, in LTNP CD27 expression of CMV-specific T-cells decreased, while CD27 expression by HIV- and EBV-specific CD8<sup>+</sup> T-cells remained constant. Interestingly, perforin expression of all virus-specific T-cells in LTNP increased over time, which was not found in individuals who progressed to AIDS. In LTNP but not in progressors, EBV- and CMV-specific T-cells showed a trend towards more co-expression of perforin and granzyme B compared to HIV-specific cells in the course of HIV-infection. These data suggest that the differentiation status of virus-specific CD8<sup>+</sup> T-cells is mainly associated with disease progression. In addition, CMV-specific CD8<sup>+</sup> T-cell immunity was analysed in HIV-infected individuals developing CMV-disease by determining IFN $\gamma$  production of CMV-specific CD8<sup>+</sup> after stimulation with the immunodominant epitope of the pp65 protein (NLVPMVATV) in combination with direct staining using HLA-A2 NLV tetrameric complexes. Preliminary analysis of the group with CMV disease (n=10) showed that CMV-specific CD8<sup>+</sup> T-cells can be detected (varying from 0.07 to 8.52% of CD8<sup>+</sup> T-cells), but only a small proportion of these cells seemed to produce IFN $\gamma$  (0 to 1.64% of CD8<sup>+</sup> T-cells). In long term nonprogressors (n=5) CMV-specific CD8<sup>+</sup> T-cells could be detected as well (0.41 to 5.66%),

which produced IFN $\gamma$  to a higher extent (0-1.99%). EBV load is high in all HIV-infected individuals, does not predict the appearance of lymphomas and does not correlate with lower CD4<sup>+</sup> T-cell counts, suggesting that immunodeficiency is not the only factor involved in development of lymphomas. Therefore, we investigated EBV load in 28 homosexual men before and after HIV seroconversion. Furthermore, in 7 individuals we measured both total numbers and function of EBV-specific CD8<sup>+</sup> T-cells using HLA-peptide tetramers and ELISPOT assays for IFN $\gamma$ . EBV load increased significantly after HIV seroconversion (p<0.001). Interestingly, no further increase was observed between 1 and 5 years after HIV-seroconversion. There was a trend towards an increase in EBV-specific CD8<sup>+</sup> T-cells, whereby the % IFN $\gamma$ -production after *ex vivo* stimulation with EBV peptides decreased. These data suggest that the immune activation induced by HIV during acute HIV-infection leads to a new EBV viral setpoint.

### Enumeration of virus-specific CD4<sup>+</sup> T-cells

To investigate the role of virus-specific CD4<sup>+</sup> T-cell help in the CTL-mediated control of viruses, we measured CD4<sup>+</sup> T-cell responses against HIV, EBV and CMV. ELISpot assays and FACS analysis were used to enumerate IFN $\gamma$  producing T-cells after *ex vivo* antigenic stimulation.

To perform a detailed longitudinal analysis to investigate HIV-specific CD4<sup>+</sup> T-cell responses during acute infection in individuals, we set up a procedure to measure cytokine production in combination with proliferative responses (CFSE) after stimulation with overlapping peptide pools derived from gag, pol, env and nef. Data from 6 acute infected individuals on HAART show that cytokine production by gag-specific CD4 T-cells can be detected in all individuals analysed (range 0.01-0.55%). In contrast, only 2 out of 6 chronic infected individuals IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> cells were detected (range: 0.01-1%). These data may indicate that treatment during acute infection is a way to preserve cytokine production and proliferation of HIV-specific CD4<sup>+</sup> T-cells.

To study CMV-specific CD4<sup>+</sup> T-cells, PBMC were stimulated with CMV lysate known to 'present' high levels of the pp65 protein, the major target for T-cells. Preliminary studies on 5 HIV- as well as 5 HIV<sup>+</sup> A2<sup>+</sup>DR3<sup>+</sup> subjects showed 0.1 to 7.34% CMV-specific IFN $\gamma$  producing CD4<sup>+</sup> T-cells.

In HIV-infected individuals developing CMV disease or retinitis (n=10), CMV-specific CD4<sup>+</sup> IFN $\gamma$  producers could hardly be detected, whereas in long term non-progressors (n=5) CD4<sup>+</sup> IFN $\gamma$  responses could be detected more easily (0 to 10.2%).

To investigate EBV-specific CD4<sup>+</sup> T-cells, PBMC were stimulated with known epitopes derived from EBV latent proteins EBNA1 and EBNA3C. In about half of both HIV- and HIV<sup>+</sup> individuals, these induced low frequencies of IFN $\gamma$  producing CD4<sup>+</sup> T-cells (0.015 to 0.045% of CD4<sup>+</sup> T-cells). Currently, EBV-specific CD4<sup>+</sup> T-cell responses are investigated using libraries of peptides with a more efficient length, spanning the immunogenic C-terminal region of EBNA1 and the entire BZLF1 protein.

### Development of HLA class II tetramers

To develop reagents that can be used to directly visualise antigen-specific CD4<sup>+</sup> T-cells, we have developed an expression system for HLA class II molecules fused to a peptide epitope sequence in insect cells. For several HLA class II molecules (HLA-DR1, HLA-DR3 and HLA-DR4) we have demonstrated production of the relevant molecules by the cells in tissue culture supernatant. These molecules can be readily purified and after biotinylation, tetramers can be formed. HLA-DR4 molecules complexed with an influenza peptide have been shown to detect antigen-specific T-cell responses in peripheral blood samples of healthy individuals after short-term stimulation with the influenza peptide.

As a model system we complexed HLA-DR3 molecules with and DR3-restricted peptide from the Mycobacterium tuberculosis Ag85. These tetramers were able to stain an Ag85 specific T-cell clone without *in vitro* manipulation. Sequences encoding peptides with proven reactivity for both CMV and HIV have been cloned in the relevant MHC class II beta chains and tetramers will be produced shortly.

## Virological and host genetic aspects of AIDS pathogenesis

### Chemokine receptors and the biological phenotype of HIV-1

HIV variants that induce syncytia in T-cell lines use CD4 and coreceptor CXCR4 whereas non-syncytium inducing (NSI) HIV-1 variants use CD4 and CCR5. In the course of infection in 50% of infected individuals, X4 HIV-1 variants evolve from R5 variants via an R5X4 dualtropic phenotype. We previously demonstrated the subsequent ongoing evolution of X4 HIV-1 in two individuals. Now we demonstrate in 6 individuals that X4 variants that were isolated just after the first emergence of X4 viruses were more sensitive to inhibition by CXCR4 antagonists AMD3100, T22 and four CXCR4 directed monoclonal antibodies than later stage X4 variants. Inhibition of the early R5X4 SI variants by high dose CXCR4 antagonists was complete, also when primary T-cells that express both CCR5 and CXCR4 were used as target cells. This indicated that R5/X4 SI HIV-1 variants have a restricted CXCR4 co-receptor usage in primary cells. These results may point to an increasing affinity of SI HIV-1 for CXCR4 in the natural course of infection.

### Long term asymptomatic HIV infection in the absence of antiviral therapy

We have identified 4 individuals who are seropositive for HIV antibodies but who lack any detectable viral RNA or proviral DNA in the absence of therapy. Three out of four individuals were typed for HLA B57 which has been associated with long term asymptomatic survival. All individuals showed evidence for strong HIV specific cellular immunity by staining with HLA-B57 tetramers folded with gag peptide and ELISpot analysis after stimulation with a gag p17 or gag p24 peptide. HIV specific helper cell responses were demonstrated by intracellular staining for IL2 and IFN- $\gamma$  after stimulation with a gag peptide pool. Strong humoral immunity was demonstrated by virus neutralisation with patient sera. Cells from these individual showed subnormal CD4 and coreceptor expression levels and had a reduced susceptibility to HIV infection *in vitro*.

### Polymorphisms that influence the course of HIV infection

A polymorphism at position -589 in the interleukin 4 (IL-4) promoter region has been associated with the presence of syncytium-inducing CXCR4 using (X4) HIV-1 variants. In a retrospective longitudinal study among 342 HIV-1 infected homosexual men who participated in the Amsterdam Cohort study (ACH), we observed that carriers of the -589T allele (either -589 C/T heterozygotes or -589 T/T homozygotes), showed comparable progression to AIDS (Relative Hazard [RH], 0.94; P=0.71), and survival (RH IL-4 -589 C/T or T/T, 0.94; P=0.69) as carriers of the -589 C/C genotype (the reference group). In contrast to a previous study, we found that the -589T polymorphism was associated with a delayed acquisition of X4 HIV-1 variants (RH, 0.56; P=0.02 for IL-4 -589 C/T or T/T) with no effect on overall disease progression.

A polymorphism in CX3CR1, the fractalkine receptor and a coreceptor for some HIV-1 variants, has been associated with a strong acceleration of HIV-1 disease progression. Within the ACH, the distribution of CX3CR1 280 genotypes was not different from previously reported distributions (T/T280, 70.5% (n=241); T/M280, 27.5% (n=94); M/M280, 2% (n=7). We were however unable to confirm the previously described findings. In addition, we did not find an effect of this polymorphism on the acquisition of SI/X4 HIV variants.

### HIV-1 neutralisation sensitivity

We previously demonstrated the reversal of a neutralisation sensitive towards a neutralisation resistant phenotype of HIV in an accidentally infected US laboratory worker. Sequence analysis revealed a limited number of amino acid changes between the neutralisation sensitive inoculating virus and the neutralisation resistance re-isolated virus. Generation of chimeric viruses and mutagenic analysis have now revealed that mutations at two positions in the envelope protein are responsible for the change in neutralisation sensitivity.

### TSE: Transmissible Spongiform Encephalitis (TSE)

Although the presence of TSE infectivity in human blood has never been demonstrated, recent data in sheep suggest that mad cow disease (BSE) and scrapie – forms of TSE in cattle and sheep, respectively – can be transmitted via blood transfusion. Therefore the focus of the TSE research at Sanquin Research in 2002 remains the development of diagnostic methods for TSE in human and validation methods for plasma-derived products.

To develop a diagnostic method for pre-clinical screening for TSE in human, we used various approaches. Besides setting up an immunological assay to detect the disease-associated form of the prion protein (PrP<sup>Sc</sup>) in blood, we also developed various assays for suggested surrogate markers of TSE i.e. decreased expression of erythroid differentiation related factor (EDRF) in whole blood, increase of normal prion protein (PrP<sup>C</sup>) in patients suffering from various neuro-degenerative diseases including Creutzfeldt-Jakob Disease (CJD) – a form of TSE in human –, and detection of a special form of the prion protein in urine (UPrP<sup>Sc</sup>). For the detection of EDRF, a real-time RT-PCR assay has been established and is currently evaluated for various clinical samples in collaboration with the Academic Medical Centre in Amsterdam. We also are developing antibodies against the EDRF protein to be able to set-up an immunological detection method for decreased expression of EDRF. In addition ELISAs has been developed for detection of PrP<sup>C</sup> in plasma of normal and CJD patients. Preliminary data suggest increased levels of PrP<sup>C</sup> in the patients as compared to normal individuals. Similar ELISAs has been developed for detection of PrP<sup>C</sup> and possibly UPrP<sup>Sc</sup> in urine of normal and CJD patients.

Production processes used for purification of plasma-derived products may contain various steps that could effectively remove the TSE agent. To demonstrate the efficacy of selected steps, we performed a series of prion validation studies. Three steps including nanofiltration steps, were

studied using murine BSE (strain 301v) or hamster scrapie (strain 263K) as spike materials.

The samples of the murine BSE spiking experiment are currently titrated in mice in collaboration with the Institute of Animal Health in Edinburgh and results are expected in July 2003. In addition the developed ELISAs for PrP<sup>Sc</sup> have been adapted to serve as a read-out for the samples of the hamster scrapie spiking experiments. However the current sensitivity of the ELISAs is too low and a concentration step is developed to further improve the suitability of the ELISA as a read-out for such samples.

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### PhD-theses

Ronald van Rij

Chemokine receptors in HIV-1 infection & AIDS pathogenesis.

Promotor: Prof. dr. F Miedema

Co-promotor: Dr. J Schuitemaker

March 21, 2002

University of Amsterdam

Tim Beaumont

HIV-1 sensitivity to neutralization: biological and molecular studies

Promotor: Prof. dr. F Miedema

Co-promotor: Dr. J Schuitemaker

June 14, 2002

University of Amsterdam

Mette Hazenberg

T-cell turnover and thymic function in HIV-1 infection.

Promotor: Prof. dr. F Miedema

Co-promotores: Dr. D Hamann; Dr. RJ de Boer

September 13, 2002

University of Amsterdam

David Kwa

Host and viral factors in AIDS pathogenesis

Promotor: Prof. dr. F Miedema

Co-promotor: Dr. J Schuitemaker

December 12, 2002

University of Amsterdam

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*A Gerritsen MD (Wilhelmina Childrens Hospital, Utrecht)*  
*B Wolach MD PhD (Kfar Saba, Israel, sabbatical guest)*  
*XuQun MD (Jinan, China, NUFFIC fellow)*

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## Allo-immunity against blood group antigens

### Molecular characterisation of the Rhesus complex (in collaboration with Dr. Petra Maaskant-van Wijk, Blood Bank South West Region)

One project aims to characterise the Rhesus (Rh) complex, especially in case of so-called 'weak RhD'. By specific PCR amplification and sequencing we have identified a panel of 'weak-RhD' donors. By Scatchard plot analysis with a panel of antiRhD monoclonal antibodies, we have disproved the hypothesis that in weak D (like in the partial RhD variants) a qualitatively changed RhD antigen is expressed.

Transfection of 'weak-RhD' cDNA into K562 cells provided a tool to study the cause of the diminished expression, and biochemical studies are underway. Another project deals with the characterisation of Rh sequences in donors from various ethnic origin. DNA has been isolated from a large panel of African, Asian and Caucasian ethnic groups and has been investigated for differences in their *RHD* and *RHCE* genes. The frequency of the different RH alleles resulting in RhD negativity in African Negroids was determined. Flegel et al. have developed a PCR specific for the Rh haplotype with a deleted *RHD* gene with primers located in the so-called Rh boxes, which flank the *RHD* gene. Although we could show that in African Negroids the *RHD* gene was deleted in a similar way as in Caucasians, this PCR resulted in this ethnic group in false-negative and -positive results, because of mutations in the Rh boxes. Therefore, we developed a quantitative multiplex PCR to determine the ratio of albumin genes versus *RHD* genes, to determine homo- and heterozygosity for RHD.

### Prenatal molecular Rhesus diagnostics (in collaboration with Dr. Lieve Christiaens, University Medical Centre, Utrecht)

New molecular typing assays have been constructed for foetal genotyping (Kell, Duffy and Kidd) and characterisation of donors and patients with serological problems. Previously, we developed a non-invasive diagnostic method based on

foetal DNA circulating in maternal plasma for RhD typing. This method has now been validated on 100 paired maternal plasma and amniotic fluid samples, with 100% concordant results. Furthermore, we ascertained the presence of foetal DNA to confirm negative bloodgroup genotyping of a female foetus. By means of RQ-PCR (Taqman technology) we can detect human biallelic short insertion/deletion polymorphisms. We technically validated an automated DNA-isolation method for high throughput screening. Next year, this method will be biologically validated.

### Characterisation of RhD antibodies (in collaboration with Dr. Arend Mulder, Leiden University Medical Centre)

The anti-RhD antibodies in allo-immunised donors will be further characterised by phage-display technology. From five donors a cDNA bank has been constructed and anti-RhD, anti-RhC, anti-Rhe and anti-RhG phages have been isolated. From one of these donors, B-cells bearing surface IgG with RhD specificity have been subcloned, and the IgH- and IgL-encoding mRNAs have been sequenced. In the literature, a very restricted  $V_H$  and  $V_L$  gene usage for RhD antibodies has been described; our sequence results, both from the phages and the single B-cells, are in agreement with this concept. Furthermore, in one patient not only a restricted but also a clonal immune response was observed. An artificial library containing only phages expressing  $V_H$  genes from the family most frequently found in anti-D antibodies has been made, to analyse the epitope specificities covered by these antibodies. Epitopes will be identified with K562 transfectants expressing the different extracellular loops of the RhD polypeptide in the background of an RhCE polypeptide. Last year, a method has been developed to produce complete recombinant Ig molecules carrying the variable Ig domains found in the selected phages.

## Fc receptors

The neonatal IgG-Fc receptor (FcRn) is an HLA-class-I related molecule present in placental syncytiotrophoblast cells and in gut epithelial cells, responsible for IgG transport from the maternal circulation to the foetal circulation and from the gut to the blood (from mother milk to the blood of the newborn and decreased loss of IgG from the circulation to the gut in all individuals). FcRn also plays a role in the regulation of plasma IgG levels in adult life. We screened various human cell types for expression of FcRn mRNA and protein. We transfected the choriocarcinoma cell line JAR (placenta epithelium model) and the hepatocyte HEPG2 cell line with FcRn-GFP to facilitate analysis of the intracellular routing of FcRn. The first analysis by confocal laser microscopy showed the presence of fluorescent FcRn in vesicular structures. When the cells were exposed to red-labeled transferrin or IVIG, co-localisation of these proteins with FcRn was noted. Therefore, these transfectants can be used to visualise the different steps in IgG transport by these cells.

## Stem-cell transplantation research

### Migration of haematopoietic progenitor cells

In previous studies, it has been established that SDF-1 and its receptor CXCR-4 play an important role in the homing of haematopoietic progenitor cells (HPC). We hypothesise that a discrepancy exists between the real number of (potential) repopulating stem cells and the actual number of cells that reach the bone marrow and contribute to haematological reconstitution. New strategies are being developed to optimise engraftment of stem cells. For this purpose, cell-permeable peptides, consisting of the protein transduction domain of the HIV-Tat protein coupled to the C-terminus of various small GTP-ases (Rac1, Rac2, RhoA and Cdc42), have been constructed. These peptides were shown to be selective inhibitors of GTPase signalling in various cell types. Analysis of leukocyte and CD34+ cell migration has shown that this migration largely depends on Rac1 and, to a lesser

extent, on RhoA. Additional analysis has shown that these peptides can be used to delineate intracellular signalling: they show selective intracellular localisation and associate with specific cellular proteins. The nature of these proteins and the stimulus-dependence of these interactions are currently under investigation.

In a complementary approach, we have transduced HL-60 cells with the activated variant of Rac1 to test modulation of chemotaxis. In contrast to our expectation, this protein reduced rather than stimulated SDF-1-induced migration, indicating that constitutive inhibition, as well as activation of the Rac GTPase interferes with the cell's migratory capacities.

An alternative strategy that was initiated to promote cell migration involved the loading of cells with the lipids that are normally post-translationally attached to the C-terminus of Rho-like GTPases, to enhance membrane targeting. These lipids, in particular geranyl-geranyl pyrophosphate, appear to enhance SDF-1-induced chemotaxis, although the specificity, cell-type dependence and mechanism of this finding remain to be determined.

To develop a model cell line for HPC, we generated a KG1a leukaemic cell line that expresses the human receptor for SDF-1, CXCR4, tagged with GFP at its C-terminus. This receptor fully induces SDF-1 responsiveness in a variety of assays in these originally CXCR4-deficient cells. Studies in which the CXCR4-GFP was detected with an antibody to the extracellular part of the receptor showed that, although CXCR4-GFP appeared to be distributed relatively random along the plasma membrane, staining with the antibody indicated that SDF-1 treatment induced polarisation of the receptor. Real-time analysis of migrating cells furthermore suggest that CXCR4 distributes to the leading edge when cells are adherent to cytokine-activated endothelium in the presence of SDF-1. Future studies with this model will include analysis of the *in vivo* behaviour of this cell line in NOD-SCID mice to assess the contribution of CXCR4 to tissue-specific homing and cell survival of transplanted cells.

## As blood banking organisation, Sanquin is ideally equipped to implement new developments in the clinic. Our stem cell laboratory is a good example.

The studies on the role of VE-cadherin as an important factor in the efficiency of HPC transendothelial migration have been continued. Recent work has focussed on intracellular signalling that is initiated upon loss of cadherin function. Our data suggest that this leads to modulation of Rho-like GTPase activity and accompanying changes in the endothelial actin cytoskeleton. Current work centres on the molecular details of these events and their relation to transendothelial migration of HPC. To this end, a wide range of GFP-tagged proteins, relevant to this issue, have been constructed or obtained from other labs. Expression of these constructs in primary endothelial cells, as well as in bone-marrow endothelium, has been optimised and emphasis currently lies on the live-cell analysis by real time fluorescence confocal microscopy.

### Ex-vivo expansion of stem cell derived progenitor cells for therapeutic purposes

Previous work has shown that stem cells can be expanded *ex vivo* into megakaryocytes by the combination of thrombopoietin (Tpo) and IL-1. The purpose of this work was to study the feasibility of *ex-vivo* expansion of stem cells and subsequent transfusion of the autologous megakaryocytes in patients treated with myeloablative therapy. In this way, the occurrence of chemotherapy-induced thrombocytopenia might be prevented. In the past year, the expansion procedure was optimised. Furthermore, because of limited availability of recombinant Tpo and fear of its immunogenicity, a peptide mimicking Tpo activity was produced that proved equally effective in *ex-vivo* expansion of stem cells to megakaryocytes. For the clinical phase-I trial, which will start in 2003, the effect of the cryopreservation of the purified CD34+ cells was studied. Experiments on the use of tissue culture bags in comparison with the culture in plate wells are underway. As blood banking organisation, Sanquin is ideally equipped to implement these new developments in the clinic. In this context, it is important to note that the Stem Cell Laboratory is part of our department and has developed GMP procedures by which it will be possible to treat the first patients with expanded

megakaryocytes in a MEC-approved trial (in collaboration with Prof. Rodenhuis and Dr. Baars, AvL/NKI, Amsterdam). Additionally, in animal models we will test the homing of the *ex-vivo* expanded megakaryocytes to bone marrow, and we will try to optimise this essential process. Finally, the fact that stem cells can also be differentiated into non-haematopoietic progenitor cells has led to collaboration with the University of Utrecht (Dept. of Vascular Medicine, Prof. Rabelink). In this collaboration, we will study the potential of adult stem cell-derived CD34+ endothelial progenitor cells (EPC) for therapeutic neovascularisation. Because monocytes additionally enhance EPC-mediated vasculogenesis, we hypothesise that monocytes might be transformed by angiogenic factors into an endothelial and more proangiogenic phenotype. In this respect, we will study whether such manipulated monocytes can be used as a cellular therapy, enhancing post-ischaemic neo- and revascularisation.

In the near future we will start a 3-fte project to culture and expand a recently identified stem cell that can be isolated from bone marrow samples. This stem cell, called the multipotent adult progenitor cell (MAPC), has been shown to differentiate into various tissues, such as bone, cartilage, neurons, vascular cells, etc. The use of these cells as autologous transfusion product enabling formation of new or repair of damaged tissue is an exciting hope for the future. Furthermore, this MAPC as progenitor of stromal cells in the bone marrow might be used to facilitate haematologic stem cells to form new blood cells.

### Studies on thrombocytopaenic patients

In previous work, we identified in patients with congenital amegakaryocytic thrombocytopaenia (CAMT) various mutations in the *MPL* gene, which encodes the Tpo receptor. Stem cells from these patients differentiated nor proliferated in response to exposure to Tpo *in vitro*. Four of the *MPL* mutations predicted amino-acid replacements. To define the causal relationship between the *MPL* mutations and the lack of response to Tpo by the stem cells, we have started to express all four mutants in BAF/3 cells and

K562 cells. At this moment, a full set of transfectants has been obtained and studies on expression levels of mutant MPL, Tpo binding and response to Tpo are under way.

### Detection of minimal residual disease

The tumour load in leukaemic patients can be measured by PCR with leukaemia-specific targets. By following the decrease in the number of disease-specific cells during therapy, an impression is obtained about the resistance of the leukaemic clones to this therapy, as well as an early sign of a relapse. Previously we have set up, in collaboration with the group of Prof. van Dongen (Erasmus Medical Centre, Rotterdam), real-time quantitative PCRs for Ig heavy chain, kappa-deleting elements and TCR rearrangements. With these PCRs the Amsterdam and Rotterdam groups have tested all blood and bone-marrow samples obtained from patients included in a uniform treatment protocol in 1997 and 1998. In September 2003, a new clinical trial will start; in this study patients will be stratified according to the PCR results. In collaboration with Prof. Caron (Academic Medical Centre/Emma Children's Hospital, Amsterdam) and Dr. Bierings (Wilhelmina Children's Hospital, Utrecht) the use of real-time PCR for the detection of bone-marrow infiltration in neuroblastoma patients has been evaluated. A panel of 34 candidate target genes for MRD detection were selected, based on high expression in neuroblastoma tumours and low expression in haematological cells according to SAGE-library analysis. Quantitative RT-PCR assays (Taqman based) have been developed for all these gene products, and these assays have been tested on panels of normal bone marrow and primary tumours. The results have been compared with the sensitivity and specificity of previously defined RT-PCRs. We have been able to develop at least 5 new assays that are more sensitive (at least  $10^{-4}$  up to  $10^{-6}$ ), and more specific than the assays available so far. Next year these PCRs will be tested on bone marrow follow-up samples of neuroblastoma patients.

## Leukocyte migration and adhesion-mediated signalling in transendothelial migration

### General remarks

Similar to the studies on the interactions between stem cells and endothelium of the bone marrow, related projects within the department investigate the adhesion of leukocytes to and subsequent transmigration of leukocytes across endothelial cell mono-layers in the context of inflammation. We focus on the interactions of granulocytes and monocytes with primary human endothelial cells (i.e. HUVEC) as well as with lung epithelial cells

### Leukocyte adhesion and transmigration and endothelial signalling

Two projects are focussed on monocytes. In the first project, the role of platelet-expressed adhesion molecules is studied in enhancing the adhesion of monocytes with platelets on their surface (as encountered under platelet-activating conditions, such as myocardial infarction and extra-corporeal circulation) to the endothelium under physiologic flow conditions. The second project involves the endothelial response to the adhesion of monocyte-platelet complexes. In this project we will focus on defining and manipulating the intracellular signalling that regulates monocyte transmigration.

Recently, an interesting paracrine stimulation between monocytes and endothelial cells was found that seems to facilitate migration of monocytes. Co-operations have been initiated with the Utrecht group of prof. De Groot and dr. Lenting to study interactions between leukocytes and the for cellular haemostasis important matrix and plasma glycoprotein von Willebrand Factor. Together with the group of professor Preissner in Germany, we investigate the role of u-PAR in signalling between and activation of various integrin adhesion receptors.

### Epithelial and endothelial cell-cell adhesion

In the context of a project on asthmatic inflammation, we have initiated a more detailed analysis of the modulation of epithelial cell-cell adhesion by cytokines. In addition, recent data indicate asymmetric distribution of the adhesion molecule CD31/PECAM-1 during neutrophil migration. This suggests that CD31 has additional, previously unidentified functions in cell migration and leukocyte-endothelium interactions. GFP-tagged and mutated versions of CD31 are available to approach this topic in more detail. The research on endothelial cell function has in the past year been boosted by the acquisition of an 'Electrical Cell Impedance System', which measures endothelial integrity (i.e. mono-layer electrical resistance) in real time with extreme sensitivity. This equipment allows us to monitor in detail not only the kinetics and characteristics of loss of cell-cell adhesion, but also of its restoration and of wound healing/cell migration under a variety of conditions and stimuli.

With this new type of analysis, the role of microtubules (MT) in the control of endothelial integrity has been firmly established. Microtubules are required for maintenance of endothelial cell-cell adhesion and MT repolymerisation drives formation of cell-cell contacts. The role of the Rac and Rho GTPases in the connection between MT's and VE-cadherin is currently under investigation. Also the role of MT's in the effects of established stimuli such as thrombin is subject of current studies.

In earlier work, we identified a Rac-mediated signalling pathway that modulates endothelial cell-cell adhesion, in part through the release of Reactive Oxygen Species. We have now started the analysis of the type of ROS-generating enzyme(s) that is (are) expressed in primary endothelial cells and have unambiguously identified the NOX2 and NOX4 enzymes as being expressed in HUVEC. GFP- and HA-tagged versions of these proteins have been generated, and localisation as well as functional analysis

are ongoing. These studies should provide us with more insight in the mechanism by which these enzymes and their products couple to cadherin function and the way in which they are controlled by extracellular stimuli.

### Granulocyte activation

#### NADPH oxidase

Phagocytic leukocytes generate reactive oxygen species as a defence against pathogenic micro-organisms. The enzyme responsible for this reaction is an NADPH oxidase located in plasma membranes of these cells. Patients with defects in this system suffer from chronic granulomatous disease (CGD), characterised by recurrent, severe infections with bacteria, yeasts and fungi. The leukocyte NADPH oxidase is composed of several subunits, each of which, when deficient, can give rise to CGD. Our laboratory is a reference laboratory for the detection of mutations in CGD patients. The results are used for prenatal diagnosis and genetic counselling. Moreover, in collaboration with colleagues from Great Britain, we are collecting clinical data from CGD patients all over Europe for better characterising the clinical expression of the disease. The central, enzymatic subunit of leukocytic oxidase is a *b*-type flavocytochrome, with two haem groups and FAD as prosthetic groups. Mutations in the *CYBB* gene for this protein cause the X-linked form of CGD in two-thirds of the CGD patients. To study the activation process of the NADPH oxidase we have created GFP-tagged p67-phox (one of the cytosolic subunits of the oxidase) and GFP-tagged Rac2, the small GTPase involved in the activation process of the leukocyte NADPH oxidase. Transient transfection of K562 cells proved the functionality of these proteins, and stable retroviral transduction of PLB985 cells with these proteins was then used to study their translocation to flavocytochrome *b*<sub>558</sub> in the plasma membrane after cell activation. We found that the presence

of the flavocytochrome was not needed for the initial translocation, but was essential for maintenance of the GFP-tagged cytosolic proteins around the phagosomes. In addition, we have also studied the expression and the role of Toll-like receptors on human neutrophils in the oxidase activation process. We found that these cells express TLR-1, -2, -4 and -6. TLR-2 and -4 are involved in the priming and activation of the oxidase by peptidoglycan and LPS, respectively. With bacteria that express various amounts and different types of LPS, we are now studying the localisation of these receptors before and after phagocytosis of these bacteria. In collaboration with Prof. Van Dissel (Leiden University Medical Centre), we are investigating mechanisms used by *Salmonella* bacteria to escape oxidative killing by leukocytes. One non-pathogenic *Salmonella* mutant was found to be much more sensitive for reactive oxygen species, whereas another mutant was found to have lost the ability to prevent NADPH oxidase activation of leukocytes.

### Opsonisation

For efficient uptake into phagocytic cells, most micro-organisms need to be covered with antibodies and/or complement components, a process called opsonisation. We have started a systematic analysis of the serum components involved in this process. First, we have developed a method to measure the rate and extent of phagocytosis of these opsonised micro-organisms by neutrophils by means of FACS analysis. With that system, we are now investigating the importance of mannose-binding lectin (MBL) in the opsonisation of zymosan (yeast particles) and various bacteria species. We found that MBL is absolutely required for zymosan opsonisation, but in its absence, anti-zymosan antibodies can partially take over the opsonisation function. In case of *Staphylococcus*, both MBL and Ig mediated complement activation are necessary for proper opsonisation. We also found that amplification of complement activation by the positive feedback loop of the alternative pathway is essential for adequate opsonisation of zymosan. We obtained evidence that mutations in MBL

predispose patients with Kawasaki Disease to coronary complications. In collaboration with Japanese colleagues we have also started to isolate and study the properties of ficolin/p35 species, lectins with a similar function as MBL.

### Apoptosis

Apoptosis of neutrophils is an important mechanism of regulating the duration of an inflammatory response. Several cytokines are known to affect this process, e.g. G-CSF, GM-CSF and interferon- $\gamma$  (IFN- $\gamma$ ) prolong neutrophil survival, whereas TNF $\alpha$  enhances neutrophil apoptosis. To investigate in more detail the effect of the proinflammatory cytokines, we investigated the effect of G-CSF on several phenomena involved in spontaneous neutrophil apoptosis. We found that G-CSF prevents the translocation of the Bcl-2-like protein Bax to the mitochondria, the activation of caspase-3 and the phosphatidylserine (PS) exposure on the outside of the plasma membrane. Neutrophil-derived cytoplasts, which lack nucleus, granules and mitochondria, spontaneously underwent caspase-3 activation and PS exposure, without Bax redistribution. G-CSF had no effect on either of these processes in the cytoplasts. These data demonstrate that at least two routes regulate neutrophil apoptosis: one via Bax-to-mitochondria translocation, and a second mitochondria-independent pathway, both linked to caspase activation. G-CSF exerts its anti-apoptotic effect in the first (mitochondria-dependent) route, but has no impact on the second (mitochondria-independent) pathway. A further distinction was made in the mitochondria-dependent regulation of apoptosis. When the general caspase inhibitor z-VAD-fmk was present during incubation of neutrophils with the apoptosis-inducing cytokine TNF $\alpha$ , cell death was not abolished, but instead, nonapoptotic cell death was induced. This phenomenon was characterised by PS exposure, nucleus condensation into strange fragments, lack of DNA fragmentation, and mitochondrial clustering without Bax translocation. We found that these morphological changes develop already a few hours after incubation and are mediated by reactive oxygen species generated by

the mitochondria. It thus seems that caspases are not only instrumental in inducing apoptotic events but also protect cells against other forms of cell damage. We also investigated the functional capacities of neutrophils undergoing apoptosis. In general, the decline of the various neutrophil functions (chemotaxis, phagocytosis, superoxide generation) correlated with PS exposure, and G-CSF and GM-CSF protected both against apoptosis as well as against functional decline. However, G-CSF preserved chemotaxis and phagocytosis better than superoxide-generating capacity, and GM-CSF had the opposite effect. We found that cells that expose PS on their outer surface were no longer capable of chemotaxis or phagocytosis, and showed a lack of signal transduction from surface receptors.

## Red-cell research

In the past year, we have started a project in collaboration with investigators from the Centre of Human Genetics of the University of Leiden to detect a large number of haemoglobin mutations by DNA array techniques. Several methods of mutation detection were tested, such as single nucleotide primer extension, allele-specific oligonucleotide amplification and oligonucleotide ligase amplification. In all cases, the oligonucleotides were carrying a tag, for detection of the labelled product by arrays spotted with probes hybridising to the tags. Both glass arrays and three-dimensional flow-through arrays (PamArrays from PamGene, Den Bosch, The Netherlands) were tested. The flow-through arrays have the advantage that different hybridisation conditions can be used to achieve optimal hybridisation. An array comprising 6 platelet antigens and 13 clinically relevant red cell blood group systems was designed. In pilot experiments, the PamArray was tested, but the results were too variable to continue to invest time in the development of this costly set-up. Future experiments with glass arrays are planned. A multiplex PCR amplifying 16 gene fragments is now operational, and this will be extended to cover 21 gene fragments.

As part of a cost-effectiveness analysis of the new Dutch policy on screening for irregular red cell antibodies and antenatal RhD immunoprophylaxis, we started with a case-control trial including two-year cohorts of pregnant women with a positive screening test for clinically relevant irregular red cell antibodies. This is a co-operative study together with prof. Bonsel from the University of Amsterdam. Furthermore, this year a study will start to elucidate whether the antenatal RhD immunoprophylaxis has led to a reduction in RhD immunisations. The outcome of these studies may lead to changes in the screening policy.

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## PhD thesis

Dominique Reumaux

Anti-Neutrophil Cytoplasm Autoantibodies (ANCA): clinical and functional studies

Promotores: Prof. dr. D Roos; Prof. dr. P Duthilleul

November 1, 2002

University of Amsterdam

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Most of the research of the Department of Immunopathology is centred around the theme 'humoral immune response against non-infectious antigens'.

The research is organised in four subthemes, i.e. immunoglobulins, auto-immune diseases, inflammation and allergy.

## Immunoglobulines

Immunoglobulin Research is focussed around two themes, the biological and the biochemical and structural aspects of intravenous immunoglobulin (IVIg) preparations. Regarding the biological effects previously it was shown that IVIg could modulate immune complex-mediated inflammation in a rat model. It was hypothesised that this was due to an increased number of monomeric IgG levels to Fc $\gamma$ -receptors (Fc $\gamma$ R) thereby preventing the binding of immune complexes. Last year a study was initiated to analyse the interaction of monomeric IgG with the low affinity Fc $\gamma$ RII and Fc $\gamma$ RIII. Using Fc $\gamma$ RIIIa transfected cells it was indeed shown that high concentrations of monomeric IgG are able to displace dimeric IgG from Fc $\gamma$ RII or III. To what extent signal transduction is mediated via this interaction of monomeric IgG is unknown. Research on the biochemical and structural aspects of immunoglobulins and IVIg has been dedicated to the proteolytic breakdown of IgG in IVIg, on the presence of pepsin, and on the aggregation process of IgG molecules.

## Auto-immune Diseases

Auto-immune Diseases Research has been investigating the interaction of various plasma proteins with apoptotic cells. The hypothesis is that impaired clearance of these cells may result from defects in the proteins contributing to the clearance, and lead to an increased risk for systemic lupus erythematosus (SLE). A main finding last year was the identification of clusterin as a major protein binding to apoptotic cells. An assay for this protein has been developed, studies on the concentrations of clustering in various diseases are underway. A main treatment of rheumatoid arthritis (RA) is administration of Infliximab (anti-TNF monoclonal antibody). Clinical responses may decrease in time in some

## An interesting finding was that the well-known immunosuppressive drug mycophenolic acid had contrasting effects on cytokine products; production of interleukin-1 $\beta$ was enhanced.

RA patients. Last year assays were set up for Infliximab as well as human antibodies against Infliximab. In co-operation with the department of Rheumatology of the VU Medical Centre (Dr GJ Wolbink a.o.) studies were initiated regarding Infliximab and anti-Infliximab levels in patients. Several other new assays for antibodies were developed last year as well, including one for antibodies against de-aminated fibrinogen and one for antibodies against tissue transglutaminase. Also an assay for mannan binding lectin (MBL) was further explored. This assay was used to study activation of complement via MBL, classical or alternative pathways by micro-organisms. During the work on these new assays it was noted that in some monoclonal antibody sandwich assays a high background occurred when human plasma was tested at high concentrations. It was found that this background was due to the presence contaminating antibodies from foetal calf serum.

In the Auto-immune Diseases group work on immunosuppressive and anti-inflammatory drugs in the whole blood system as an *ex vivo* model, has been continued. Part of this work was done in co-operation with pharmaceutical companies. An interesting finding was that the well-known immunosuppressive drug mycophenolic acid had contrasting effects on cytokine production. Whereas the production of most cytokines was suppressed, that of interleukin-1 $\beta$  was enhanced. The molecular basis for this phenomenon is currently under investigation. To allow studies on the role of Toll-like receptors in the whole blood system, it was attempted to raise monoclonal antibodies against this class of important receptors. Progress was made last year in this field since three of such antibodies were obtained. The functional activity of these will be studied this year.

### Allergy

The Allergy Research group has continued its work on major allergens implicated in rhinitis and asthma as well as in food allergy. Most of the work on inhalant allergens is carried out in collaboration with allergen manufacturers. The aim is to develop novel allergen-specific strategies to treat and

diagnose type I allergies. At present, diagnosis and allergen-specific immunotherapy are performed with almost crude allergen extracts. The Allergy Research group has cloned and expressed the most important inhalant allergens and evaluates their potential to replace allergen extracts. Production of recombinant allergens is being done in several different expression systems like *E. coli*, the yeast *P. pastoris* and tobacco.

Food allergy research is mainly performed in the frame of two EU-funded projects under the fifth framework program. These projects focus at the characterisation of allergens in fruits and nuts. Several of these allergens were purified as well as cloned and expressed as recombinant molecules. Differences in recognition profiles of these allergens in various EU member states are used to elucidate the relation between allergen exposure and sensitisation. An important aspect of the food allergy research is the study of specific IgE antibodies against foods that lack clinical relevance. These antibodies negatively influence the quality of existing diagnostic tests. IgE antibodies without clinical relevance are usually highly cross-reactive antibodies directed to very conserved epitopes in plants. Examples of such epitopes are plant-derived N-linked glycans and the ubiquitous protein profilin. An answer to the question why IgE antibodies against such structures are biologically inactive will result in badly needed improvement of diagnostic tests for food allergy.

In 2002, a large EU-funded project with 29 partners in 8 countries, and co-ordinated by our group, had its kick-off. The initiative for this project was taken by the IUIS and the WHO. The aim is the development of new WHO-certified reference materials for allergens. Candidate references have been successfully produced in the first year of the project. These purified natural and recombinant major allergens are now being evaluated as candidate references.

A great advantage of the availability of a broad spectrum of purified natural and recombinant allergens is that they are ideal reagents to be used in large epidemiological studies on allergy. Over the past year, our group has been involved in highly successful studies into the relation between parasite

infections in developing countries and the development of allergy. Observations that sensitisation to inhalant allergens was less frequently accompanied by clinical allergy than in the developed world were studied in depth in collaboration with the Department of Parasitology of the LUMC (Dr. Maria Yazdanbakhsh). These studies have revealed that an anti-inflammatory network induced by chronic helminth infections suppresses the effector phase of an allergic response. IL10 has been shown to play an important role in this anti-inflammatory process.

### Inflammation

The Inflammation group has continued the research on novel activation products of the classical pathway. It was found that covalent fixation of activated C4 and C3 to C1q occurred during classical pathway activation and not during other activation processes. A differential antibody sandwich ELISA was developed for these C1q-C4 and C1q-C3 complexes. Currently this assay is being optimised. In addition the biological activity and the biochemical composition of these complexes is investigated. In another project the structure and function of the classical pathway inhibitor C1-inhibitor was investigated. A patient with a genetic deficiency was identified that lacked part of the aminoterminal domain of C1-inhibitor, which domain is unique for C1-inhibitor and is not shared by other inhibitors. Yet, the molecule produced by this patient is dysfunctional. Studies using recombinant forms of this mutant as well as several other amino-terminal mutants revealed that in spite of its unique sequence the aminoterminal domain exerts an important function for the serpin domain, i.e., it stabilises this domain via 2 disulfide bridges. Studies on the molecular mechanisms of complement activation by ischemia-reperfusion (I/R) have shifted last year to studies of the molecular mechanisms of complement activation by apoptotic cells. Several mechanisms involving immunoglobulins and pentraxins, have been identified. The contribution of these mechanisms is currently under investigation.

In another project, in co-operation with the department of rheumatology of VU Medical Centre (Dr A. Voskuil), studies were initiated regarding the role of complement activation in the response to Infliximab in RA patients. It was found that Infliximab decreased complement activation, in particular in patients who responded well to the therapy. These studies once again demonstrate the complex interaction of inflammatory systems *in vivo*.

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## PhD thesis

Laure Ribeiro-de Couto

Control of cytotoxic responses on human neonates: naivity or tight regulation

Promotor: Prof. dr. LA Aarden

Co-promotor: Dr. CJP Boog

July 4, 2002

University of Amsterdam

# Department of Plasma Proteins

The research portfolio of the Department of Plasma Proteins addresses a variety of blood plasma proteins, with particular reference to constituents of the haemostatic system. Within this field of interest, research is multidisciplinary, comprising protein chemistry and enzymology, vascular biology and immunology. The programme is closely related to the pharmaceutical plasma proteins that Sanquin produces from human plasma.

In 2002 research has continued to focus on the coagulation factors VIII and IX, as well as on von Willebrand factor. As in previous years, studies have covered the entire lifespan of these proteins, ranging from intracellular events involved in biosynthesis and secretion, to molecular structure and function, and mechanisms that contribute to clearance from the circulation. Recent developments within this programme are summarised in the following sections.

## Biosynthesis of the factor VIII-von Willebrand factor complex

While factor VIII and von Willebrand factor (VWF) circulate in plasma in a non-covalent complex, it has remained controversial whether or not cells exist that are capable of expressing the combination of both proteins. It has been generally accepted that the liver is a major site of factor VIII synthesis. Employing a murine model, we have previously observed that factor VIII mRNA synthesis occurs both in the liver and in non-hepatic tissues such as kidney and brain. Immunohistochemical studies together with quantitative gene expression studies revealed that hepatic factor VIII synthesis is primarily confined to cells lining the sinusoids. These cells, however, are apparently devoid of VWF mRNA. The lack of co-localisation of factor VIII and VWF synthesis in these and other liver cells supports the view that complex assembly occurs after the constituent proteins have entered the circulation. This issue is currently further explored in hepatic tissue of patients with severe liver disease. As for VWF, it has been established that it is synthesised in vascular endothelial cells, where it is stored in typical

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organelles, the Weibel-Palade bodies. The regulated secretion of VWF, and other constituents of the Weibel-Palade bodies, involves the translocation of the Weibel-Palade bodies from the cytoplasm towards the plasma membrane and the fusion of these vesicles therewith. Limited information is available concerning the mechanisms underlying these processes. Increased levels of cytosolic free calcium have been implicated in the mechanism of exocytosis of a number of agonists, including thrombin and histamine. Regulated secretion of VWF can also be induced by secretagogues such as epinephrine, adenosine and the vasopressin analogue DDAVP. These agents are known for their ability to activate cAMP-dependent signalling independently of cytosolic calcium levels. Calcium-raising agents and agents that activate the cAMP pathway may act in a synergistic manner. In our laboratory we are exploring the intracellular pathways that drive the trafficking of Weibel-Palade bodies which ultimately leads to VWF secretion. We previously reported that the small GTP-binding protein Ral is associated with Weibel-Palade bodies in endothelial cells. Activation of endothelial cells by thrombin results in transient cycling of Ral from its inactive GDP-bound to its active GTP-bound state. The significance of Ral and a variety of effector molecules in the trafficking of Weibel-Palade bodies is currently under investigation. Because Ral has also been reported to be involved in cytoskeleton dynamics and rearrangement in other cell types, it seems well conceivable that this small GTP-ase indeed plays a key role in regulated secretion of VWF from endothelial cells.

### Structure and function of enzyme-cofactor complexes

One characteristic of the coagulation cascade is that it comprises several serine proteases that act in combination with a non-enzymatic cofactor. We focus on the complex of activated factor IX (factor IXa) and its cofactor factor VIII, and in particular on the relation between cofactor binding and enhancement of factor X activation by factor IXa. By protein engineering studies of the factor IXa protease domain (the heavy chain), we previously demonstrated that

multiple surface-exposed loops in the protease domain limit the accessibility of the substrate binding groove. This makes enzyme activity strictly dependent on the presence of its cofactor. Upon factor VIII binding, intramolecular rearrangements facilitate substrate binding and cleavage by a so far unrecognised mechanism. Our previous studies have demonstrated that factor VIII binding involves two helical structures in the factor IXa protease domain (residues 301–303 and 333–339). In addition to these factor VIII-interactive sites in the factor IXa heavy chain, also the factor IXa light chain contributes to assembly with factor VIII. We have observed that in particular the interface between the two Epidermal Growth Factor-like (EGF) domains is involved in factor VIII-dependent rate enhancement of factor IXa. This was demonstrated by a mutagenesis study employing recombinant factor IX chimeras. In these variants, the interconnection between the two EGF modules was exchanged by its larger counterpart from factors X or VII. While these substitutions did not affect factor IXa activity in the absence of factor VIII, their response to factor VIII was severely reduced. Surprisingly, chimeras containing an elongated EGF-linking segment displayed reduced response to both complete activated factor VIII and its isolated A2 domain. This is an intriguing observation, because the same mutants still displayed normal association with the factor VIII A2 domain in surface plasmon resonance studies. Because the A2 domain is thought to interact with the helix regions in the factor IXa heavy chain, a direct role of the EGF-linking region in A2 domain binding seems unlikely. We previously proposed that this region in the factor IXa light chain provides an interactive site for the A3 domain in the factor VIII heavy chain. While our current data are fully compatible with this view, we propose that the EGF-linking region in the factor IXa light chain also contributes to the enhancement of factor IXa catalytic activity that occurs upon assembly with activated factor VIII. In collaboration with a variety of other groups, we have further addressed the 3-dimensional structure of membrane-bound factor VIII. In this study, the factor VIII molecule has been modelled within a 3-dimensional density map

It was particularly surprising to find endothelial cells mediating factor X activation in an apparently quiescent, non-apoptotic state.

obtained by electron crystallography. The factor VIII heterodimer of the heavy chain (domains A1-A2) and the light chain (domains A3-C1-C2) probably has a compact spiral organisation with the A3 domain in close association with the C1 and C2 domains near the phospholipid surface. Four loops of the C2 domain are embedded within the lipid membrane at about 0.7 to 1.0 nm depth. In this orientation the factor VIII molecule can be docked onto the factor IXa molecule in a manner that includes all putative factor VIII-interactive sites in the factor IXa molecule. Although this model has relatively low resolution (1.5 nm), it greatly contributes to our understanding the assembly of factor VIII with factor IXa at the lipid surface.

We further have studied the assembly the factor VIII with factor IXa on the surface of endothelial cells. We previously reported factor X activation studies suggesting that the interaction of factor VIII with the endothelial cell membrane differs from that with artificial lipid membranes. In view of the notion that many cells display pro-coagulant activity after cell activation and concomitant disruption of the natural lipid asymmetry, it seems particularly surprising that we found endothelial cells to mediate factor X activation in an apparently quiescent, non-apoptotic state. This led us to explore the structural requirements for endothelial membrane binding. Employing synthetic factor VIII-derived peptides and monoclonal antibodies against several sites in the factor VIII light chain region, we found that the carboxy-terminal region of the factor VIII C2 domain (residues 2318-2332) contributes to assembly with factor IXa on the endothelial membrane. The same region has previously been demonstrated to bind to synthetic lipid membranes, and includes one of the four membrane-embedded loops of the C2 domain in the above-mentioned model of membrane-bound factor VIII. We conclude that quiescent endothelial cells are capable of supporting the assembly of the factor IXa/factor VIII complex, possibly by the exposure of small, but potentially significant amounts of pro-coagulant phospholipids at the endothelial surface.

find that the isolated factor VIII light chain displays the same high-affinity LRP binding as the intact heterodimer of heavy- and light chain. Although LRP binding is inhibited by a monoclonal antibody against the C2 domain, the isolated recombinant C2 domain displays only low affinity for LRP in surface plasmon resonance studies. Thus, one or more high-affinity LRP binding sites are located in the A3-C1 moiety of the factor VIII light chain. By using a combination of synthetic peptides, recombinant antibody fragments, and factor VIII/factor V hybrid molecules, we found that LRP binding involves the residues 1811-1818 in the A3 domain. This suggests that the LRP binding site is partially overlapping with a factor IXa interactive region, which has previously been shown to involve the same surface-exposed part of the factor VIII A3 domain. We propose that multiple factor VIII light chain regions contribute to LRP binding, some of which may represent interactive sites involved in assembly with factor IXa. Whether or not the interaction of LRP with factor VIII and other coagulation factors has physiological implications remains unclear. This issue is currently under investigation.

## Journals

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# Department of Transfusion Technology

**Research in the Department of Transfusion Technology aims to improve storage conditions, cryopreservation and safety of cellular blood products. Details of unwanted side effects of new technologies to inactivate pathogens are investigated in order to determine a safe window between pathogen inactivation and collateral cell damage.**

**The department consists of two laboratories, the Laboratory of Blood Transfusion Technology and the Laboratory of Cryobiology.**

## Blood Transfusion Technology

Research on the effect of photodynamic treatment (PDT) for the inactivation of pathogens in red cell concentrates (RCC) and platelet concentrates (PC) has continued. An unwanted side effect of PDT of red cells has been characterised in detail, namely the loss of phospholipid asymmetry of the plasma membrane resulting in the exposure of phosphatidylserine (PS) on the outer leaflet. It has been shown previously that PS exposure results in rapid clearance of red cells from the circulation, thereby compromising the efficacy of a RCC transfusion.

PS exposure can most easily be observed by measuring the binding of fluorescent AnnexinV in a flow cytometer. Illumination of red cells in the presence of DMMB as photosensitiser induced about 10% AnnexinV-positive cells. Scavengers of singlet oxygen (e.g. dipyrindamole) prevented this effect.

Experiments with various inhibitors of phospholipid transport indicated that activation of phospholipid scramblase might be responsible for PS exposure and hence lipid transport was measured more directly by analysing the inward trans-location of NBD-labelled phospholipids by flow cytometric analysis. Only red blood cells subjected to PDT showed translocation of NBD-phosphatidylcholine (NBD-PC), indicative for scramblase activity. Double colour FACS analysis indicated that AnnexinV-positive cells are much more active in the uptake of NBD-PC than the AnnexinV-negative cells, supporting a role of scramblase activation in the loss of phospholipid asymmetry.

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*J Lagerberg PhD*

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## We found that phospholipid scramblase in red blood cells can be activated by exposure to singlet oxygen, thus revealing a new mechanism of activation.

Scramblase activation was found to be independent of changes in  $\text{Ca}^{2+}$ , so far the only known activator of scramblase. The results of this study showed that the phospholipid scramblase in red blood cells can be activated by exposure to singlet oxygen, thus revealing a new mechanism of activation for this important enzyme. Two approaches based on light have been investigated for pathogen inactivation of platelet concentrates. In the first approach (in collaboration with Gambro BCT), pathogen inactivation relies on the use of Riboflavin as photosensitiser in combination with light. In the second approach, pathogen inactivation relies on illumination with very intensive Broad Spectrum Pulsed Light (BSPL) without the need for an exogenous photosensitiser. As an aid to determine the limits of light intensities that can be used, we developed a direct test of mitochondrial function in platelet concentrates, based on the accumulation of the fluorescent dye JC-1 inside the mitochondrial matrix. In the presence of a high membrane potential ( $\Delta\psi$ ), this dye accumulates in the mitochondria with a concomitant increase in red fluorescence. Thus, after JC-1 loading, the ratio of red (FL2) to green (FL1) fluorescence (as determined in a flow cytometer) is an indicator of mitochondrial integrity. Indeed, the FL2/FL1 ratio of platelets showed a dose-dependent decrease in response to the uncouplers FCCP and DNP, and to TTFa, an inhibitor of the respiratory chain. At higher dye concentrations (or at lower cell concentrations) the FL2/FL1 ratio was significantly lower, suggesting uncoupling by the dye itself. Plasma concentrations above 3% interfered with optimal JC-1 loading. We now use an optimised JC-1 protocol for measuring  $\Delta\psi$  under various conditions of platelet storage, with or without pathogen inactivation treatment. As part of contract research activities, the laboratory played a pivotal role in the development of a new blood warming device to be used in emergency medicine. The warming of a red cell concentrate in this device is based on illumination with infrared light in a flow cell, that ensures proper mixing and avoids local rises in temperature.

Another part of contract research activities involved the testing of prototype whole blood platelet saving filters. Both recovery of components (plasma, erythrocytes and platelets) and *in vitro* quality parameters were studied in detail and resulted in improvements of the prototype. In addition, the laboratory was involved in testing the *in vitro* quality of platelets collected by the so-called 'dry-platelet-apheresis' protocol, resulting in a platelet concentrate in a relatively small volume of plasma, to be diluted with platelet additive solution.

The collaborative project with the department of Physiology of the Amsterdam Medical Centre on the development of useful test models to determine red cell function *in vivo*, has resulted in three different rat models in which part of the rat blood cells are replaced by human red cells. In all models, the microvascular oxygen tension is measured to determine local oxygen delivery. In one of these models (haemodilution of the rat to an haematocrit of 15%, followed by transfusion of human red cells), a clear effect of storage of the RCC was observed. Further studies into the underlying mechanism of this deficiency are ongoing.

### Cryobiology

The collaborative study with the Sanquin Blood Bank North West Region on a suitable cryoprotectant for human platelets was completed. It was concluded that a synthetic medium, in which 10% DMSO is present, yields satisfactory results, provided it is added in a 1:1 ratio to platelets suspended in plasma. Plasma exerts important protective effects, resulting in quality parameters (swirling, morphology, activation antigens) that stay within acceptable limits even after 24h of storage of the thawed platelets. To provide a theoretical model for optimal cryopreservation protocols, a collaborative project was started with ID-Lelystad (Dr. H.Woelders) that aims to measure the permeability of different blood cell types for water and for various cryoprotective agents. The difference in these permeabilities determines the cell damage inflicted by the freezing process and knowledge on these parameters will result in refinement of current freezing protocols.

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# Blood Bank North West Region

The attention of the department of Research, Development & Education of the Blood Bank North West Region was focused on: (I) transmission of infectious diseases by blood products and bacteria in specific and (II) technological aspects of blood bank processing and storage of blood components, transfusion efficacy and safety.

Part of the investigations were performed as contract research which resulted in an active input in the development of improved blood bank technology. The collaborations with a number of institutes, including (academic) hospitals, universities, (research) institutes and pharmaceutical companies, fellow blood banks in The Netherlands and abroad, the Sanquin divisions Research and Diagnostic Services (former CLB), was continued. As will be illustrated below, these collaborations resulted in fruitful exchange of ideas and techniques to pursue our goals.

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## Academic support staff

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*M Eijzenga-Demmendal*

*M Habibuw*

*LAE Liefiting*

*L Scholtalbers*

## Research and development

### Universal leukoreduction

Two members of our staff (MJ Dijkstra and PF van der Meer) participated in 2001 in the working party 'Universal Leukoreduction Low Level Leukocyte Counting', that had the aim to determine the optimal method to count low numbers of leukocytes. Based on a comparison of eligible methods, the flow cytometer was selected to be used in the blood banks. Margriet Dijkstra evaluated in 2002 the results of the validation of the flow cytometer performed according to a national protocol. Based on the results, the conclusion was that the protocol had not been concisely followed leading to differences in interpretation and acceptance of the validation. This question is currently being discussed in the Laboratory Advisory Group (LABAS) whether or not the validation protocol should be rewritten and the validations repeated.

## Methods to optimise isolation of DNA from platelets and measure free DNA in plasma, will be applied to detect very low numbers of leukocytes.

### Detection of low numbers of leukocytes

Although the currently used flow cytometer method is sensitive enough to give a 'pass or fail' result for the residual leukocyte number of  $10^6$  per component, ongoing improvement of blood processing and filtration technology requires the development of more sensitive leukocyte counting methods. Leukocytes are thought to be the only sources of DNA in platelet concentrates, a real time PCR technique may be therefore be the solution to count low numbers of leukocytes. Methods to optimise isolation of DNA from platelet concentrates and measure free DNA in plasma have been investigated and will soon be published. Together with Sanquin Diagnostic Services, these methods will further be refined and validated to apply the real time PCR technique to detect very low numbers of leukocytes. This study has been accepted by the Research Programming Committee of Sanquin and has obtained a grant for 1 year; pilot experiments started late 2002 and the study will continue in 2003.

A cell imaging instrument has been evaluated for counting low numbers of leukocytes and red cells in plasma media but the results revealed that the sensitivity and specificity could not meet those of the flow cytometer. This project has therefore been abrogated.

### Platelet storage

A platelet concentrate from one buffy coat for paediatric purposes has a volume ranging between 40 and 70 mL. The introduction of screening of platelet concentrates for bacterial contamination requires a large sample volume (about 25 mL), leuko-reduction by filtration further reduces the volume. Therewith preparation of paediatric single donor platelet units from a buffy coat becomes obsolete. To solve this problem a leuko-reduced platelet concentrate obtained by apheresis can be sampled for culturing and next be divided into 4 portions and stored as such for paediatric transfusions. Studies were performed to find a platelet storage bag to store these platelets in plasma for at least 5 days. The results indicated that platelet concentrates can

be stored for a maximum of 5 days after blood collection in a 600 mL polyolefin bag. The results will be published soon. The method has been introduced in all blood bank divisions in 2003.

Platelet storage by freezing with 5% DMSO and a controlled freezing protocol has been studied earlier in collaboration with Sanquin Research dept. of Transfusion Technology. The aim of this study was to find an optimal medium for storage after thawing, i.e. 100% plasma, mixtures of plasma and additive solutions, and mixtures of additive solution and GPO (pasteurised protein solution). Pilot studies revealed that in plasma morphology of the platelets was maintained best with the lowest activation up to 24 hours after thawing.

Platelets require glucose to maintain mitochondrial metabolism. Earlier investigations suggested that absence of glucose and/or drugs and/or low temperatures could bring platelets to 'hibernation' thus inducing low metabolism and slower ageing of the platelets finally allowing longer storage *in vitro*. A project to first investigate conditions *in vitro* to mimic hibernation and eventually extending the results to routine storage of platelet concentrates and *in vivo* studies was granted by Sanquin until 2005. The study is performed in collaboration with the Department of Thrombosis and Haemostasis of the Utrecht Medical Centre (Head Prof. Dr. J.W.Akkerman).

### Increments following platelet transfusion

A protocol was developed in collaboration with the Haematology Department of the VU medical centre to measure the increments and adverse reactions following transfusion of platelet concentrates derived from buffy coats and stored in plasma up to 7 days after blood collection. Data have been collected from April 2001 through August 2002. Platelet increments were measured in clinically stable patients after transfusion of platelet concentrates of various storage days. Good clinical results were obtained for platelets stored for up to 7 days.

### Detection of bacterial contamination of blood products

Bacterial screening of platelet concentrates was introduced in the blood centres in 2001. The data from Amsterdam and Utrecht of one year screening, time to detection, species detected and the follow up of the blood products has been reviewed for publication.

To investigate other, quicker, methods of bacterial detection a collaborative study was started late 2001 to use an eubacterial PCR technique developed in the Microbiology Department of the VU medical centre. The primers detect a highly conserved region of the 16-S DNA. The pilot studies consisted of a number of experiments to optimise the conditions of the PCR technique for the application for platelet concentrates in plasma. Extraction of DNA and 'background' in reagents and plasma were first investigated until satisfactory results were obtained. The PCR will further be validated with samples of platelet concentrates spiked with various species of bacteria. Next various methods of bacterial detection will be compared with this PCR technique in 2003. This project received a grant from Sanquin until 2004.

Already in 2000 a proposal to develop and evaluate an impedance measurement for detection of bacteria in platelet concentrates was submitted to the European Commission in the Fifth Framework Programme for demonstration projects. The proposal was submitted by 3 (Associate) members of the European Union: Israel (a small company and the Magen David Adom blood centre in Tel Aviv), Germany (a company) and The Netherlands (the department of microbiology of the Slotervaart Hospital and our Blood Bank). The project received a grant until November 2005. Following the development of the equipment, the blood centres and microbiology laboratories will validate the measurements with samples of platelet concentrates spiked with various species of bacteria, starting late 2003.

### Infectious disease transmission, epidemiology

In collaboration with the department of Clinical Virology of the AMC and Ortho Diagnostics a new prototype Hepatitis C core protein (HCV-Ag) assay has been extensively evaluated and validated for quantitative measurement of HCV-Ag in chronic HCV-patients treated with antiviral therapy. The decline of HCV-RNA measured by the bDNA assay correlated well with the HCV-Ag assay. Therefore the diagnostic HCV-Ag assay can replace the costly and time consuming nucleic acid technology based test systems for monitoring HCV patients.

In collaboration with dr P Simmonds from the University of Edinburgh department of Clinical Virology studies were carried out for transmission of Hepatitis G virus (HGV)/ GBV-C by blood products to recipients. HGV/GBV-C is efficiently transmitted by donor blood and patients with anti HGV/GBV-C antibodies were protected.

In collaboration with Sanquin Diagnostic Services (M Koppelman) the Blood Bank North West region participated in the evaluation of a sensitive NAT donor screening test for HBV-DNA (grant from Sanquin). The expectation is that within a few years this test will be implemented in routine in most high-income countries in the world.

### Factor VIII in apheresis plasma

The Sanquin Guideline for blood products (2002) requires that apheresis plasma intended for Factor VIII preparation should be frozen within 12 hours to designate it fresh frozen. A further requirement is the presence of at least 0.7 IE Factor VIII clotting activity (Factor VIIIc) per mL, after freezing and thawing. Because the 12 hour limit introduces many logistical problems and the need for plasma for Factor VIII preparation is high the head of Sanquin Plasma Products on behalf of the Board of Sanquin has appointed the Blood Bank North Holland (R Pietersz) to investigate the loss of Factor VIII in apheresis plasma during storage prior to freezing. In collaboration with Sanquin Plasma Products (H van Raalte) the Factor VIIIc of the apheresis plasma was measured during storage up to 24 hours (3 hour intervals) and after freezing at 12 and 24 hours after collection,

respectively. The results revealed an average Factor VIIIc well above 0.70 IE/mL even after 24 hour storage at ambient temperature, provided the plasma is immediately cooled to 20–24°C and frozen within 1 hour to -30°C and stored at -25°C or below. The faster the plasma will be frozen the better, but extending the time to freezing will allow more plasma for fractionation. The Guideline will be adapted in 2003.

### Immuno Hematology Diagnostics

The Blood Bank North West Region participates in the micro array determination of blood group typing in collaboration with Sanquin Diagnostics (M de Haas) and the group in Rotterdam (Blood Bank South West Region).

### Rheumatology

A study to retrospectively follow up donors who later developed a rheumatic disease was started in 1999 on a grant from the 'Nationaal Rheuma Fonds' in collaboration with the Jan van Breemen Institute in Amsterdam, the Free University Medical Centre and the Leiden University Medical Centre. The aim of the study was to evaluate the value of auto-antibodies to predict the development of rheumatoid arthritis (RA). Serum samples of donors who were later diagnosed with RA were studied for the presence of specific auto-antibodies (IgM-RF and CCP). Samples from a panel of matched control donors were also studied. About 50% of the RA patients had specific auto-antibodies several years before the first symptoms. The auto-antibody tests enable early detection of RA in high risk populations.

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### PhD Thesis

John Jongerus

Detection of blood transmissible viral agents: implications for blood safety, Promotor: Prof. dr. WG van Aken

Co-promotors: Dr. EF van Leeuwen; Dr. CL van der Poel

June 21, 2002

University of Amsterdam

# Blood Bank South West Region

The research programme of the Blood Bank South West Region is divided into four themes: (I) Molecular Blood Group Studies, (II) Cord blood, (III) Photodynamic sterilisation of blood products and (IV) Clinical Studies.

## Molecular Blood group Studies

The aim is to evaluate the application of molecular blood group typing of red cell, platelet and HLA genes for blood bank purpose and for clinical purpose in case of complicated genetic backgrounds such as post-transplantation, in case of patients with a non-Caucasian origin or for transfused patients with (multiple) antibodies and to understand the relationship between expression of blood groups and organisation at the molecular level.

### Molecular biological backgrounds of the RhD blood group

Research to the molecular background of weak D expression confirmed that weak D expression is not caused by decreased amounts of RHD mRNA transcripts. In the erythroleukemic cell-line model, K562, transfection of weak RhD type 1 and 3 lead to decreased expression of RhD with comparable amounts of RHD mRNA transcripts. This indicates that the T809G (weak D type 1) and the C8G (weak D type 3) mutations cause weak D expression at a post transcriptional level. Scatchard plot analysis and flow cytometry confirmed lower epitope densities but did not indicate qualitative alteration of RhD in weak D expression.

### Consequences of a multiracial society on the prevention of Rhesus immunisation

Recently, a proposed mechanism of RHD deletion has been described. Therefore, it is now possible to determine RHD zygosity with a PCR-RFLP assay. This is important for RhD-negative pregnant women with an allo anti-D. In case the father is homozygous for RHD, the child will be affected, whereas when the father is heterozygous there is a 50% chance the child will not be affected. Research question

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## A real time PCR assay on foetal DNA isolated from maternal plasma was developed to predict foetal RhD status, taking non-Caucasian RhD-negativity into account.

is whether this assay can be used in a multiracial society. Preliminary results indicate that there are different RH-boxes in people from different ethnic backgrounds. In Caucasians, the most common cause of RhD negativity is RHD deletion. In non-caucasians three forms of RhD negativity exist; r's gene (RHD(1-3)-RHCE(3-7)-RHD(8-10) hybrid gene), RHD pseudogene and the RHD deletion. A real time quantitative PCR assay on foetal DNA isolated from maternal plasma has been developed to predict foetal RhD status with these forms of RhD negativity taken into account. The assay will be validated on blood samples from RhD negative pregnant women from Curacao (high frequency of RHD pseudogene).

A collaboration with the Blood bank Shangdong from China has started to investigate the cause of RhD negativity in the presence of an RHD gene in the Chinese population.

### Validation of blood-group genotyping as part of daily practice in blood transfusion

Pyrosequencing assays were developed for the detection of SNPs (Single Nucleotide Polymorphisms), which are the molecular basis of most blood groups. The pyrosequencing technique is a 'middle-throughput' technique suitable for a blood bank setting. In a pilot-study 485 donors were typed for KEL, 385 donors were typed for JK and FY/GATA FY and 112 donors were typed for HPA-1, -2, -3 and -5.

In 2003 a research project (4 year program Sanquin) will start in which serology will be compared to genotyping using the pyrosequencing technique on a large scale. For the development and implementation of a real 'high-throughput' system, the transfusion medicine DNA micro-array, there is a collaboration with Sanquin Research/Diagnostics, the Blood Bank North West Region and JT den Dunnen (Human and Clinical Genetics, Leiden). A European Consortium, of which the Blood Bank South West Region is a subcontractor, has been formed to demonstrate the use of molecular genetic techniques to genotype a large cohort of individuals drawn from across the EU in order to demonstrate the accuracy and improvement of this technology over standard serological testing.

### Cord blood

The purpose of research is to increase the applications of placental blood for transplantation and transfusion. This includes expansion of stem cells and improvement of engraftment in order to facilitate transplantation in adult patients. In addition, expansion of progenitor cells, immunological profile of cord blood mononuclear cells and placental red cells are evaluated for transfusion purposes.

### Megakaryocyte progenitor cell expansion

Research includes 3 subprojects a) characterisation of optimal precursor in CB for in-vitro expansion of megakaryocytes b) in-vivo engraftment in NOD/SCID mouse of the distinct subpopulations of *in-vitro* expanded megakaryocyte progenitors and c) effect of co-culture with mesenchymal stem cells (MSC's). Previous research showed that in-vitro culture of CD34<sup>+</sup>CB with thrombopoietin (Tpo) as single growth factor results in a 40 fold expansion of > 85% pure megakaryocytes (MK). In the current year the precursor cell in CB was further characterised. We showed that 99% of MK precursors from CB in-vitro were derived from a CD34<sup>+</sup>/CD41<sup>-</sup> subset. This contrasts with bone-marrow and peripheral blood mobilised stem cells in which a considerable proportion of MK's is derived from the CD 34<sup>+</sup>/41<sup>+</sup> subset. Ad b) During expansion and maturation with Tpo, the CD34<sup>+</sup> CB cell shows 3 sequential cycles, which can be distinguished in CD34<sup>+</sup>/CD41<sup>-</sup>, CD34<sup>-</sup>/Lin<sup>-</sup> en CD34<sup>-</sup>/CD41<sup>+</sup> fractions. This pattern of differentiation is unique for CB and is not observed with bone marrow or PBSC CD34 cells. We addressed the question which of these subpopulations resulted in MK engraftment and platelet production in the NOD/SCID mouse model. To detect human platelets in the NOD/SCID mouse a method was validated. After injection of human platelets and human CB-CD34<sup>+</sup> cells, human platelets can be detected and their survival time can be established. Currently transplantation of the three subsets in the mouse is performed. Ad c) *In-vitro* conditions of co-cultures of MCS's (derived

from embryonic pulmonary tissue) and CD34<sup>+</sup>CB-cells is evaluated for the behaviour of expansion, maturation and homing receptors of the megakaryocyte lineage in the presence and absence of Tpo. This work is performed in collaboration with the Dept of Exp Haematology LUMC (WE Fibbe and WA Noort).

### Autologous cord blood transfusions

An inventarisation of transfusions to premature children was undertaken and methods to increase the volume of blood from premature placenta's are studied. Of prematures, less than 36 weeks 37% receive red cell transfusions (68% of prematures < 29 weeks) and the majority receive 1–2 'units' of 10–20 ml. From almost 80% of premature placenta's volumes > 20 ml can be collected. Preliminary results show that the red cells can be stored for at least 2 weeks. A method was validated for reliable bacterial culture from waste plasma. Future work will focus on development of placenta extraction systems and optimal blood processing techniques and suitable disposables. Second, the need and possibilities for usage of expanded autologous erythropoietic progenitor cells for autologous support of red cells and eventually granulocytes and platelets will be investigated. The autologous cord blood project is integrated in a medical student project who participate in a 24 hour on call system for cases of premature delivery (Collaboration Leiden University Medical Centre: HHH Kanhai, S Scherjon, Dept of Obstetrics, F Walther, dept of Neonatology).

### Photodynamic sterilisation of blood products

The aim of this research explores the inactivation of undesired micro-organisms in red cells, platelets and stem cell products and the consequences for cell functions, reduction of cell damage and effect on immuno-modulation.

### Red cell and stemcell sterilisation

Previous research resulted in a very promising photosensitiser Sylsens-B CL showing limited red cell damage in-vitro and sufficient inactivation of non-enveloped viruses and gram – and + bacteriae. Sylsens-BCI has also been shown to be suitable for sterilisation of cord blood stem cells without affecting viability and differentiation ability of progenitor cells (manuscript in preparation).

Further development of the application of Sylsens in routine practice is performed by a commercial company PhotoBiochem which performed a human volunteer study in 2002. After establishing a good recovery and survival in Rhesus monkeys, we further focus on the observed increase in IgG binding on Sylsens treated red cells, immuno-modulation and stem cell viability. The effect of photodynamic treatment (PDT) on white cells was studied. Interestingly proliferative lymphocyte functions are not affected by PDT, while the proliferative response on allogeneic cells and stimulator capacity in mixed lymphocyte cultures is impaired. The level of this defect seems located at the cell membrane and its nature is further studied. Collaboration: LUMC dept of IHB (D Roelen, E van Beelen); PhotoBiochem (TMAR Dubbelman).

### S-59 Platelet inactivation

In collaboration with Baxter Healthcare and Cerus Corporation *in vitro* and *in vivo* research has been conducted to the safety and effectiveness of transfusions with S59 photochemically treated platelets (euroSPRITE study). Photochemical treatment inactivates the DNA/RNA present in the blood product and this inactivates the viruses, bacteria, protozoa and leukocytes. The results are promising and efforts to investigate further usage of S-59 platelet transfusions in clinical practice will be continued. Collaboration: Erasmus Medical Centre, Rotterdam and Baxter Health Care Corp, Cerus Corp.

## Clinical studies

Evidence-based improvement of transfusion therapy or for alternatives for blood products

### Multiple Organ Failure and blood transfusions

The former Bloodbank Leiden-Haaglanden and the LUMC have a long-standing collaboration as TACTICS group aiming at evidence-based improvement of transfusion therapy.

In 2001 a collaborative study with the Amsterdam Medical Centre in heart valve surgery was finished. The study, like a previous study, pointed into the direction of a role of leukocytes in blood transfusions and mortality from Multiple Organ Failure (MOF) in addition to a transfusion dose association and MOF. In contrast to heart surgery, in another study, the role of leukocytes in red cell transfusions in case of large aortic aneurysm or gastro-intestinal surgery was less obvious. Although in the latter study for almost all end-points, leukoreduction scored favourably, most remarkably was a shorter hospital stay because a few (~1%) patients, who received standard transfusions stayed longer than 30 days in hospital.

From most patients who participated in both studies blood was collected at several time points around surgery. Genetic variability to respond to environmental factors are presumed to contribute to development, severity and recovery of MODS and are subject for research in forthcoming years.

### Optimal transfusion triggers in orthopaedic surgery

Orthopaedic surgery is confronted with a plethora of new approaches to reduce blood transfusions and to improve wound healing. In order to make evidence based choices on the usage of epoietin, several forms of autologous transfusions, wound blood, one of the black boxes for topical buffy-coat applications, large multi-arm and multicenter studies are needed. The basic requirement for such studies is a strict transfusion protocol. End 2001 a trial was started in three hospitals (Leiden University Medical

Centre, Leyenburg Hospital, and Reinier de Graaf Hospital) to compare a fixed restrictive transfusion trigger with common practice in the hospital in patients undergoing hip and knee surgery. The results may reveal a generally accepted transfusion trigger, which can serve as control arm for new studies. Finally it is necessary to compare the use of all alternatives for allogeneic transfusions in one study.

### Optimal transfusion trigger in patients with MDS

Myelodysplastic syndromes (MDS) are clonal disorders characterised by dysplasia in at least two myeloid cell lines. Fatigue, assumed to be anaemia-related, is one of the most important symptom. MDS patients are treated with blood transfusions to improve health-related quality of life (HRQoL). Although improving HRQoL is the major goal of blood transfusion in MDS, the HRQoL have not been investigated empirically. In a cross-sectional design HRQoL and chronic anaemia is measured.

Fifty randomly chosen MDS patients (RA, RARS, RAEB en CMMoL) completed the SF-36 (generic HRQoL), the Multi-dimensional Fatigue Inventory (MFI), and a Visual Analogue Scale for self-rated health within 24 hours after a regular hospital visit. The questionnaires have a high feasibility and MDS patients have a worse HRQoL than age-related persons. There is a correlation found between haemoglobin level and HRQoL, especially the physical domain. Both haemoglobin value as HRQoL seem to be relevant for evaluation of the severity of chronic anaemia. This will be evaluated in a prospective randomised clinical trial.

### Optimal transfusion trigger in patients with MDS

The HRQoL questionnaires, validated in the pilot study, will be used in a prospective randomised clinical trial (Temple study: transfusion Effects in Myelodysplastic Patients: Limiting Exposure) to analyse the effect of red cell transfusion. The Temple study started in September 2001. 200 Patients with Myelodysplastic Syndromes (MDS) will be included: 100 patients will receive red cell transfusion when  $Hb \leq 4.5$  mmol/l and 100 patients will receive red

cell transfusion when  $Hb \leq 6$  mmol/l. Primary outcome is fatigue, measured with the MFI questionnaire. The goal of the Temple study is to develop a new HRQoL driven transfusion policy.

### Optimal transfusion trigger in patients with AML

Red-cell transfusions are the cornerstone of supportive care for haematological patients treated with intensive chemotherapy. Concerns about the transfusion-related complications, such as infections, tumour behaviour and immunomodulatory effects, and the costs, necessitated a re-evaluation of the transfusion practice. In a retrospective study 84 patients with Acute Myeloid Leukemia (AML), who were treated with intensive chemotherapy, were included. We determined whether a restrictive policy of red-cell transfusion (4.5-5.5 mmol/l, dependent on patient age and symptoms, n=38) had led to a diminished use of red-cell transfusions compared to a liberal transfusion policy (i.e. transfusion if  $Hb \leq 6$  mmol/l, n=46). We also investigated if both transfusion policies were comparable in terms of preventing signs and symptoms of anaemia and other chemotherapy related. It may be concluded that a restrictive transfusion policy, which led to a decreased use of red-cell transfusions, might be safely applied in the supportive care of AML patients treated with intensive chemotherapy.

### Optimal transfusion policy after delivery

Improving HRQoL is one of the most important goals of therapy for women after delivery. Red cell transfusion is an important tool therefore. The optimal transfusion policy is yet unknown. In a pilotstudy three internationally used HRQoL questionnaires (SF36, EuroQoL and MFI) are validated in fifty patients. Also the relationship HRQoL and anaemia will be measured in this cross-sectional design. The questionnaires will be used in a prospective randomised clinical trial to develop a more objective HRQoL driven transfusion policy.

### Optimal transfusion policy after delivery

In a multicenter randomised clinical trial 200 patients will be included: 100 patients will receive a red cell transfusion with a restrictive transfusion trigger and 100 patients will receive red cell transfusion with a more liberal transfusion trigger. Primary outcome is HRQoL. The goal of the study is to develop a more HRQoL based transfusion policy based on the HRQoL questionnaires, validated in a pilot study.

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# Blood Bank

## North East Region

The research programme of the department focuses on megakaryocyte and haematopoietic progenitor cells and on medical sociology in relation to customer service. Also flowcytometric analyses with respect to leukoreduction was done.

### Ex vivo expansion of megakaryocyte progenitor cells

In cancer patients receiving high-dose chemotherapy and haematopoietic stem cell (HSC) transplantation the period of profound cytopenia generally lasts between 1 to 6 weeks, depending on the number and source of HSCs infused. Experimental transplantation models in mice have demonstrated that long-term engraftment is supported by undifferentiated stem cells, while short-term (transient) engraftment is mediated by more differentiated progenitor cells. Therefore, supplementing stem cell transplants with *ex vivo* expanded progenitor cells may be an approach to accelerate the haematopoietic recovery. The principle of this cell-based therapy has been demonstrated in mice and non-human primates, although the recovery is slower than predicted from the large number of progenitors infused. In the human setting, multiple studies have now demonstrated that the *ex vivo* expansion process can be used to generate large quantities of more mature progenitor cells, and a number of clinical studies have shown promising results. In our *ex vivo* expansion studies we have focused on expansion of the megakaryocytic lineage, as the period of thrombocytopenia following stem cell transplantation is a major problem for many patients. During the *ex vivo* expansion process, CD34<sup>+</sup> cells are stimulated with recombinant cytokines *in vitro* to generate partially differentiated progenitor cells. A prerequisite for *ex vivo* expansion protocols to be feasible in the autologous setting, is that patients who could potentially benefit from this treatment do supply stem cell grafts which can be expanded successfully. Therefore, we questioned if patients with a delayed platelet reconstitution *in vivo* are able to generate megakaryocyte progenitors *in vitro*. The megakaryocyte expansion of CD34<sup>+</sup> cells selected from stem cell grafts of patients with strongly delayed platelet engraftment after

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autologous transplantation was examined. A homogeneous group of patients was selected: with the same disease (multiple myeloma), all received high amounts of CD34<sup>+</sup> cells (>2 x 10<sup>6</sup> c/kg) so that this was not a limiting factor, and all showed rapid myeloid engraftment indicating there was no general engraftment defect. However, the time to platelet recovery (>20 x 10<sup>9</sup> /L) varied widely from 7 to 115 days. Our results demonstrate that patients with a strongly delayed platelet recovery did not show impaired *in vitro* expansion of CD61<sup>+</sup> cells when compared to the rest of the study group and compared to normal bone marrow samples. In the patient group studied, four of the five patients with strongly delayed platelet recovery received CFU-Mk doses in the lower range and might benefit from infusion of additional megakaryocytic progenitors; however, a similar CFU-Mk dose gave rapid platelet recovery in other patients. It is possible that the delayed platelet recovery is not caused by insufficient megakaryocyte progenitors in the graft, but e.g. by a defect in expression of adhesion molecules on the progenitors or by a suboptimal micro-environment in the myeloablated bone marrow.

### Factors controlling expansion and maturation of haematopoietic progenitors

Dissecting the interaction (cross talk) between cytokines involved in proliferation (to generate many progeny) and differentiation (to generate cells at a specific maturation stage with the appropriate homing properties) should prove to be valuable in uncoupling the proliferation and differentiation processes during expansion culture. We have analysed how cytokines interact at the cellular and molecular level by investigating their effects on proliferation, differentiation and signal transduction processes in megakaryocyte and erythroid progenitor cells. In a recent study we described the cross talk between Erythropoietin (Epo) and Prostaglandin E-2 (PGE<sub>2</sub>)-activated signal transduction pathways by analysing their effects on STAT5 (signal transducer and activator of signalling) signalling. *In vitro* erythroid colony assays have shown that PGE<sub>2</sub> increases the number of colony-forming units of erythroid

cells (CFU-e), and up-regulates erythroid differentiation by increasing haemoglobin synthesis. Epo is crucial for proliferation and differentiation of erythroid progenitor cells. Several signalling cascades are activated upon Epo-stimulation, including the PI 3-kinase pathway, the MAP kinase ERK pathway and STAT proteins. STAT5 is ubiquitously expressed in haematopoietic cells and is involved in erythroid differentiation and survival. Epo-mediated STAT5 transcriptional activity in erythroid AS-E2 cells was enhanced 6-fold by PGE<sub>2</sub>, without modulating the STAT5 tyrosine or serine phosphorylation, or STAT5 DNA binding. One of the signalling pathways that are activated by PGE<sub>2</sub> is the cAMP pathway, involving activation of adenylyl cyclase and protein kinase A (PKA). We demonstrated that the co-stimulatory effect of PGE<sub>2</sub> on Epo-mediated STAT5 transactivation was mediated by PKA and its downstream effector cAMP-responsive element binding (CREB) protein. In addition, the CREB-binding protein (CBP) was shown to play an essential role in Epo-mediated STAT5 transcriptional activity. As a result of PGE<sub>2</sub> co-stimulation, the STAT5 target genes Bcl-X, SOCS2 and SOCS3 were upregulated *in vivo*. These results indicate that the stimulatory effects of PGE<sub>2</sub> on erythroid proliferation and differentiation might in part be regulated by STAT5 and are mediated by activation of the PKA/CREB pathway.

In ongoing studies we are investigating the effects of stem cell factor (SCF) and transforming growth factor β (TGF-β) during megakaryocyte expansion. A number of studies in megakaryocytic cells have now established that activation of the Jak/Stat and PI 3-kinase pathways play an important role in cell survival and proliferation, while activation of the MAP kinase/ERK pathway is critical for differentiation and endomitosis in megakaryocytes. *In vitro*, these Tpo-activated signalling routes can be positively and negatively modulated by additional growth factors and inhibitors.

At a physiological level, the growth factors SCF and TGF-β are known to affect Tpo-regulated processes in, respectively, a stimulating and a down-regulating way. As SCF is widely used in expansion protocols and TGF-β is synthesised, stored and secreted by megakaryocytes themselves, it will

## Patients with strongly delayed platelet recovery receiving CFU-Mk doses in the lower range might benefit from additional megakaryocytic progenitors.

be of interest to study the effects of these growth factors on activation or down-regulation of specific signalling pathways linked to proliferation or differentiation. These projects are performed in collaboration with Prof. Dr E. Vellenga of the Department of Haematology, University Hospital Groningen.

### Flowcytometric analysis of leukocytes

In 2002, studies were done to investigate unexplained results during flow cytometric leukocyte counting. Since the introduction of universal leukoreduction of blood components, the residual number of leukocytes is determined using flow cytometric methods. It was observed that in units that had been stored overnight and subsequently filtered, events were observed that fell outside the set gate, and therefore not counted as leukocytes. The origin and nature of this 'extra' population was unknown, but further analysis revealed that, if these events were included and counted as leukocytes, a large fraction of the blood components would have to be rejected as being leukoreduced. Therefore, in co-operation with Blood Bank North West Region, a study was designed to determine the nature of this population. The study was performed by a Laboratory High School student. In this study, various blood components (whole blood, red cell concentrates in additive solution and in plasma, and in platelet concentrates) were stored and sampled at various time points up to three days after blood collection. Standard leukocyte counting procedures were performed using counting kits and flow cytometers from BD Biosciences and Beckman Coulter. The samples were either counted immediately, or stored overnight and then counted. In addition, the influence of EDTA anticoagulant on the final counting result was determined.

The study showed that already 24 hours after collection, extra populations were visible, and increased with longer storage. The increase in extra events was associated with a decrease in the number of leukocytes counted, most notably in whole blood and red cell concentrates. The populations were predominantly detected using the BD Biosciences counting method. Storage of the sample tube resulted in

lower numbers of leukocytes, again mostly in whole blood and red cell concentrates. Finally, it was shown that use of EDTA resulted in dramatically lower residual numbers of leukocytes. These results suggest that the extra populations are probably disintegrating granulocytes, because this is the major fraction of the total number of leukocytes present in whole blood and red cell concentrates. Further studies are currently carried out to prove this hypothesis.

### Quality Assessment and Improvement Programme

In 2002 the Quality Assessment and Improvement Programme, focussing on customer service was continued. The assessment objective is to ensure that: (a) the interaction between Blood Bank (personnel) and customers (donors, hospital laboratories, etc.) is positive, and (b) that it promotes good customer service. As assessment method was chosen for satisfaction surveys. In 2002 we evaluated the results of two studies carried out in 2001 and implemented policy improvement.

A study focussing on the preference for donation schedules and preference for call up (telephone or card) should lead to a decision whether or not to adapt donation schedules. A second study is focussing on the interaction between the blood bank departments Quality Control, Production, Storage and Distribution and the Hospital Laboratories. In 2002 a new study focussing on the experience of the blood donors on the overall performance of the Blood bank was carried out: donor satisfaction survey.

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# Blood Bank South East Region

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The Sanquin Blood Bank South East Region collaborates with several departments of University Hospitals of Nijmegen and Maastricht, of the Universities of Nijmegen, Maastricht and Wageningen and Institute of Cellular Signalling, Cardiovascular Research Institute Maastricht and Synaps BV.

Major research themes at the Blood Bank South East Region concern three different areas (I) Research on cellular products, (II) Donor related research, and (III) Clinical oriented research

The cellular area focuses on two lines of research: Red cell ageing and survival, and Platelet activity and viability.

Donor related research involves two different themes Cardiovascular risk profile of donors, and Donor motivation Clinical oriented research is focussed on Autologous blood donation and transfusion, treatment of haemochromatosis patients, and Clinical evaluation en cost efficacy of double portion single donor platelet products.

Bone Bank Gelderland intensively co-operates with the Blood Bank in producing human highly concentrated platelets, prepared from autologous and homologous whole blood. These preparations are subsequently mixed with bone chips and used as implantation material in mandibular reconstructions. Measurement of growth factors will be helpful in understanding the clinical results. We have performed similar studies in which highly concentrated platelets and bone chips prepared from goat and rabbit was used.

In 2002 two studies have started concerning quality improvement of different methods of testing and sampling of bone tissue.

## Red cell ageing and survival

### Improvement of blood processing and storage conditions on ageing and *in vivo* survival of red blood cells

Detailed knowledge of the basic mechanisms that cause red blood cell ageing *in vivo* or *in vitro* is still lacking, in spite of the importance of such knowledge for determination of optimal conditions for handling and storage of blood products in general and of red blood cells in particular. More is known about the effect of storage and red cell preservation solutions. So decreases prolonged storage of red blood cells the *in vivo* survival after transfusion. It is clear that the critical event in the ageing process of red blood cells is the recognition and subsequent removal of old cells by the immune system. The binding of autologous IgG to old red blood cells, which leads to their recognition and phagocytosis by macrophages, is one of the first documented examples of a physiological autoimmune process. The identity of the molecule(s) involved is still subject to controversy. There are various theories on the molecular identity of the antigen that is recognised by the immune system, the senescent cell-specific antigen (SCA). The anion exchange protein gene family band 3 plays a central role in regulation of removal of old and pathological cells by providing SCA. A decrease in membrane stability is also probably involved in the ageing-related shedding of vesicles *in vivo*. Vesicle formation, which is an integral but little understood part of the rbc ageing process, is probably responsible for the loss of at least one fifth of the total haemoglobin from the normal rbc in the final stage of its life span. Our own, recently obtained data in human red blood cells and vesicles formed *in vivo* indicate that ageing-related cleavage of band 3 may be part of the mechanism by which vesicles are formed. The different ageing parameters of red cells will be related to the *in vivo* survival after transfusion, which can be measured by a sensitive flowcytometric assay. Last year we have developed in our blood bank a sensitive flowcytometric method for the measurement of the *in vivo* survival of transfused red cells. We were able to measure

the presence of a 0.1% positive donor population in artificial mixtures. Currently we are designing a protocol to measure the *in vivo* survival of transfused donor red cells in patients. Recently we have developed an enzyme linked antiglobulin test for the measurement of autologous IgG bound to red cells of different storage times (1-36 days). At the moment we are ending these experiments and it seems that the amount of red cell bound IgG doesn't increase with the storage time but stays stable. Furthermore are we adopting existing methods for the measurement of ATP (by chemoluminescentie) and of 2,3-DPG (by spectrophotometry) of red cells with different storage times. The next step will be the examination of the *in vitro* sensitivity of red blood cells with different storage times to phagocytosis by a monocyte monolayer assay or flow cytometry to study the relationship to the *in vivo* survival of red cells. This research is performed in collaboration with the University Medical Center, St. Radboud Nijmegen, dept. Transfusion services (JM Werre, MD, PhD; B de Pauw, MD, PhD); dept. of Biochemistry, Institute of Cellular Signalling (G Bosman, PhD) and Rijnstate Hospital Arnhem (FLA Willekens).

## Platelet activity

### Excitability of platelets during prolonged storage

In collaboration with Synaps BV (P Giesen, University Maastricht) and the departments of Biochemistry (J Heemskerk, University Maastricht) and Haematology (L van Pampus, University Hospital Maastricht) research concerning the activity and excitability of platelets in different blood bank products has continued. In addition to the *in-vitro* experiments (flowcytometry and thrombin generation assays) we have adapted a specific *ex-vivo* flow system that mimicks the adhesion of platelets to sub cellular structures under physiological flow rates. Results obtained in this system support the earlier findings that platelets stored in plasma gradually lose their ability to become active upon stimulation. Moreover, there is no difference in platelet excitability between buffy coat pooled

and aphaeresis products in plasma. In light of the recent discussion concerning prolongation of the storage time of platelet products from five to seven days, the specific need for patients should be evaluated.

### Platelet quality in donors

Platelet quality in products presumably depends on the quality of platelets in donors. Therefore we have made an inventory on aspirin (like) drug intake amongst whole blood donors in South Limburg. We have also evaluated platelet function in these donors by means of aggregometry and measurement of a closure time with an *in vitro* bleeding time assay. An unexpected high number of whole blood donors (23 from a total of 109) reported the intake of painkillers within seven days before donation or on the day of donation. Of these donors, only 3 from 109 reported this intake on the Sanquin questionnaire. The question remains, to what extent this intake of aspirin (like) drugs leads to an impaired function of platelets in products. This study is a collaboration with J van Wersch (Science and Education, Heerlen).

## Donor related research

### Blood donation and atherosclerosis in the general Dutch population

Blood donation has been associated with a reduced risk of myocardial infarction in a number of epidemiological studies. The protective effect could be mediated by a reduction in body iron load, as iron may promote atherosclerosis by oxygen radical formation. Hereditary hemochromatosis (HH) is characterized by iron overload in the body, due to the Cys282Tyr mutation of the human hemochromatosis-associated (HFE) gene. Carriers of a HFE gene mutation have an increased risk of myocardial infarction. Recently, elevated non-transferrin-bound iron has been demonstrated in HH homozygote and heterozygote subjects, but little is known about its role in atherosclerosis.

In this project the relationship between blood donation, iron status and atherosclerosis is studied.

The project is embedded in a large epidemiological study of folic acid, vitamin E and atherosclerosis (FACIT study; project leader: Dr. P Verhoef) that is conducted at Wageningen University. For the FACIT study, 838 blood donors and their spouses aged 50-70 years have been recruited from Sanquin Blood Bank South East Region. At baseline (year 2000-2001), detailed information was obtained on blood donation history (including plasma donation), medical history (including anemia), medication, anthropometry, and lifestyle (e.g. smoking, alcohol use). Blood sampling was performed. Thickness of the intima-media layer of the common carotid artery, as a measure of generalized atherosclerosis, was assessed by B-mode ultrasonography. Within the FACIT project there is an unique opportunity to examine the health effects of blood donation, an area that is not well studied but of major importance in the blood transfusion chain. For this purpose, laboratory analysis of iron parameters in blood will be performed, i.e. hemoglobin, hematocrit, serum ferritin, serum iron, total iron-binding capacity and non-transferrin-bound iron. C-reactive protein will be assessed as an inflammatory marker for adjustment of serum ferritin levels. Mutations in the HFE gene (e.g. Cys282Tyr) will be assessed in DNA isolated from whole blood. The project will generate scientific data on the relationship between blood donation and atherosclerosis, and provide insight into the potential intermediary role of iron. More generally, it will contribute to knowledge about the health status of blood donors. In line with the hypothesis, less thickening of the carotid vessel wall is to be expected in frequent blood donors. This study may (for the first time) reveal an adverse effect of non-transferrin-bound iron on atherosclerosis in the general population. Furthermore, examining the relation between blood donation and atherosclerosis by HFE genotype may indicate a larger beneficial effect of blood donation in Cys282Tyr carriers. This is a collaborative project of Sanquin Blood Bank

## Within the FACIT project there is a unique opportunity to examine the – as yet not well studied – health effects of blood donation.

South East Region, the Division of Human Nutrition & Epidemiology, Wageningen University and the Department of Clinical Chemistry, University Medical Center 'St. Radboud', Nijmegen. Executive staff: MF Engberink, Wageningen University (junior researcher); Dr. JM Geleijnse, Wageningen University (senior investigator); Prof. dr. EG Schouten, Wageningen University (professor of epidemiology); J Durga, Wageningen University (coordinator FACIT study); Dr. DW Swinkels, University Medical Centre 'St. Radboud', Nijmegen.

### Clinical oriented research

#### Use of autologous erythrocytes products collected with component collection (double portion erythrocytapheresis)

The use of autologous blood is lower in The Netherlands than it is in other European countries and the United States of America. In the province of Limburg we have observed an increased demand for autologous products.

This increase might be the result of legal information duty of the physician about benefits and risks associated with the administration of homologous blood products. Thus, patients receive information about alternatives for transfusion and the possibility for autologous donation/transfusion. We have evaluated the use of autologous blood from January 1999 to December 2002. Analysis of a questionnaire sent together with the autologous products showed that the number of homologous products used in addition to the collected autologous units was high, especially in urological and vascular surgery. Moreover, the autologous plasma units were not used and therefore discarded in all cases. These results lead to the start of a new project on Component collection of double portion erythrocytes for the use in autologous transfusion.

Objectives of this study are: (I) to analyse the cost effectiveness of collection of autologous erythrocytes by means of whole blood donation versus cytapheeresis, (II) to evaluate whether the collection of double portion erythrocytes

reduces the use of additional homologous blood products, and (III) to evaluate the number of donor complications during this type of autologous blood collection. This project is a collaboration of Sanquin Blood Bank South East Region, University Hospital Maastricht, Atrium Hospital Heerlen, Maasland Hospital Sittard, Laurentius Hospital Roermond and Vicuri Hospital Venlo.

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# Department of Product and Process Development

## Sanquin Plasma Products

The product development strategy of Sanquin's Plasma Products division aims primarily at maintaining the state-of-the-art level of its blood products portfolio and production processes. To that end the product and process development program is regularly evaluated and updated if needed. Besides this, 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.

### Product development

In 2002 product development focussed heavily on the liquid intravenous immunoglobulin project, in which the Plasma Products division is co-operating with the Finnish Red Cross Blood Transfusion Service in Helsinki. Main items concerned robustness studies of the virus reducing capacity of several process steps, ongoing shelf-life studies, and the clinical evaluation of the new product (Nanogam<sup>®</sup>) in hypogammaglobulinemia and ITP patients. These studies have been completed in 2002 and showed good efficacy and excellent clinical tolerance. In close collaboration with the Finnish Red Cross Blood Transfusion Service the registration dossier was compiled and the registration application for the Nanogam<sup>®</sup> will be filed in Finland in 2003.

Another project concerns a new potential anti-HIV agent, the so-called negatively charged albumin. The three clinical grade batches produced so far have shown excellent stability on storage. A proof-of-principle trial in a limited number of terminally ill AIDS patients in whom standard therapy has failed is now scheduled to take place in 2003. This development project is executed in co-operation with the University Centre for Pharmacy, State University of Groningen and the International Antiviral Therapy Evaluation Centre (IATEC) of the Academic Medical Centre of Amsterdam.

Besides these development projects, evaluation of newly emerging and promising technologies for reducing the viral risk of plasma products in general is taking place

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## A proof-of-principle trial in a limited number of terminally ill AIDS patients in whom standard therapy failed, is now scheduled to take place.

continuously. To this end, virus validation studies are carried out, in line with assessing the effect of these technologies on the products concerned. In 2002 irradiation with concentrated visible light (PureBright™ of PurePulse Technologies, Inc.), treatment with UVC, and evaluation of new virus filtration membranes have been topics of investigation.

### Process development

In 2002 technologically oriented projects comprised the following:

- The production line for the new liquid formulated intravenous immunoglobulin has been installed and is now in the phase of technical validations.
- The department for aseptic filling of non-albumin products has been reconstructed and a new filling line has been installed. Validations are now taking place.
- New freeze-dryers, both for intermediate product and final product drying have been installed and are now in process validation phase.
- Engineering of a new, small-scale, process for the production of anti-*Varicella zoster* immunoglobulin was completed. The project is now in its preconstruction phase.
- Engineering of an extension of the production capacity for albumin was completed. Implementation, commissioning and validation are now taking place.
- An alternative procedure for freezing i.v. immunoglobulin product prior to lyophilisation has been developed and validated.
- A non-destructive, full batch test for vacuum assessment of all lyophilised products has been implemented and validations are ongoing.

# CAF-DCF Brussels, Belgium

## Central Fractionation Department of the Belgian Red Cross

The R&D strategy of the CAF-DCF focuses on both the efficacy of plasma derivatives and their safety as regards pathogens and environmental pollutants. New virus inactivation technologies (like UVC irradiation) have been fully studied; their implementation in different plasma-protein production processes is under continuous evaluation. Methodologies for state-of-the-art characterisation of therapeutic proteins and their excipients in plasma or concentrates, both immunological and biochemical, are developed and exploited in industrial applications.

### Hypoxia and B19 infectivity model

B19 displays remarkable tropism for red blood cell progenitors. It multiplies poorly in erythropoietin-differentiated established cell lines. Because local oxygen pressure influences viral replication, an infectivity assay for B19 was developed using the pluripotent erythroid cell line KU812F infected under low oxygen concentration (hypoxia). Because it is possible to carry out several rounds of productive B19 infection in KU812F cells subjected to hypoxia, this model is a promising one for studying B19 biology, identifying neutralising antibodies, and evaluating existing or new virus inactivation methods in plasma protein manufacturing processes.

### Screening of specific anti-B19 antibodies

By means of dedicated algorithms, about 10 linear epitopes (8 to 20 amino acids) located in the B19 nonstructural protein (NS1) were identified. Epitopic peptides were synthesised and used as coating antigens in an ELISA quantification test or as tools for purifying specific epitope-restricted immunoglobulins. The method was validated with clinical and healthy individual plasma. We demonstrated anti-NS1 IgM and IgG in parallel with antibodies against coat proteins VP1 and VP2. This encouraged us to extend the epitope identification programme to these capsid proteins VP1 and VP2. We thus identified about 17 epitope sequences in VP1 and 20 in VP2, including those described already in the literature. Besides the fundamental interest of identifying B19-neutralising antibodies, particularly with

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### Graduate students

*A Dang*

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### Secretary

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## Hypoxia enables evaluation of B19 infectivity neutralisation by specific antibodies and of B19 inactivation in a pluripotent cell line.

B19 in established cell lines cultured under hypoxia, these analytical tools will be used to study the therapeutic quality of immunoglobulins produced from plasma screened or not for B19 by NAT and their subsequent removal from the starting fractionation plasma pool.

### B19 and HBV screening by NAT

To reduce the risk of virus contamination in plasma pools, we used sensitive NAT, adapted to the starting plasma first homogenate, to detect the presence of HBV and B19. All 116 pools tested were found negative for HBV-DNA, but 39 tested positive for B19 DNA. The viral load was quantified in each case by real-time PCR.

A low-cost in-house NAT was developed to screen plasma donations by mini-pools of 576 donations. An algorithm was used to resolve each minipool identified as positive for B19-DNA first to pools of 96 donations, then to pools of 8 and 12 donations. This last step enabled us to identify the contaminated donations. The samples were produced by the Blood Transfusion Services (Service Francophone du Sang and Dienst voor het Bloed) in the format of multiwell plates containing 96 individual donations or pools of 8 donations. The in-house PCR test (95% sensitivity: 4800 IU/ml) contains an internal control and an in-house working standard (low and high viral concentrations). To ensure the traceability during NAT processes, all starting, intermediate, and final samples and reagents are encoded by code bar, in addition to final amplicon detection. The test was validated according to guidelines including robustness testing and inter-laboratory validations with the Belgian Public Health Institute and Academic Virology Laboratory, and in a National Insurance study. Since October 2002, a total of 234,185 donations have been analysed. 28 donations have been found positive (prevalence: 1.2/10,000). Viral loads (up to 1014 IU/ml) and anti-B19-coat-protein and anti-NS1 antibodies were determined in each contaminated donation. The genomes will be sequenced in 2003.

### Development of biochemical methodologies for assessing plasma derivative quality

In 2002, the new methods included quantitative determi-

nation of plasmin and plasminogen in Cohn fractions, of total and specific anti-pneumococcal antibodies, and of intrinsic and extrinsic protein fluorescence. Fluorescence measurements on the indole group of tryptophan can reveal local conformational changes of the protein. Many different processes, including collisions with quenchers, formation of complexes with specific ligands, or local conformational changes can affect the fluorescence anisotropy, quantum yields, ( $\lambda_{max}$ , or lifetime of the fluorophore. We notably found fluorescence to be one of the rare powerful tool for monitoring the effects of UV irradiation on fibrinogen.

### Biochemical equivalence of two FVIII concentrates

A new twice-inactivated FVIII concentrate (Factane) was marketed by the DCF-CAF in 2002. The inactivation treatments applied were solvent-detergent treatment (SD) and nanofiltration (35 and 15 nm). The product's biochemical properties were analysed and compared with those of the former FVIII-SD. A battery of tests (clotting and chromogenic assays, antigen assays using a panel of monoclonal antibodies, identification before and after thrombin activation and subsequently proteolysis by western-blotting) demonstrated that both products were equivalent. FVIII activation and proteolysis by thrombin was shown to be slower in Factane. Further analyses showed that the excipient lysine is the agent reducing FVIII proteolysis by thrombin *in vitro*.

### Journals

Covaci A, Laub R, Di Giambattista M, Branckaert Th, Hougardy V, Schepens P – Polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) are eliminated from therapeutic Factor VIII and immunoglobulin concentrates and severely reduced in albumin by plasma fractionation – *Vox Sang* 2002; 83: 23-28

Laub R, Strengers P – Parvoviruses and blood products – *Pathol Biol* 2002; 50: 339-48

### Patents

Caillet-Fauquet PC, Di Giambattista M, Laub R – Parvovirus B19: procédé de répliation in vitro de virus. Patent delivered in Belgium. 2002

# Department of Biotechnology

**The Department of Biotechnology is a business unit specialised in a broad array of pharmaceutical services aimed at the development of biologicals intended for therapeutical application in humans. These services include the contract production of monoclonal antibodies and r-DNA products as well as safety testing and validation of such products.**

## Contract production

The Department of Biotechnology has ample experience in designing production strategies and scaling up of production in accordance with GMP-guidelines. The department can perform large scale fermentation, purification and sterile filling of monoclonal antibodies and other biologicals as research-grade reagents, diagnostics or clinical grade pharmaceuticals.

## Biosafety testing

The Department of Biotechnology is also experienced in biosafety testing of biotechnological products and the regulatory issues involved and offer a complete battery of safety tests designed to satisfy both EC- and FDA guidelines. For this purpose, all assays involved have been GCLP (Good Control Laboratory Practice) accredited. Furthermore, the department performs process validation studies in order to demonstrate the reduction of (model) viruses or DNA during purification as well as the validation of client dedicated assays.

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# Medical Department

## Sanquin Plasma Products

**The main activity of the Medical Department is designing and performing of clinical trials with (new) plasma products aiming for application for marketing authorisation, for new indication(s) or for new therapeutic modalities. To safeguard the clinical use of plasma products, providing medical information and advice on the usage of plasma products in clinical care and pharmacovigilance is in place.**

Clinical studies are designed in close collaboration with study co-ordinators in the Netherlands and abroad, and in particular with the Inter-University Working Party on the Study of Immune Deficiencies and with the Haemophilia Treatment Centres. The department is involved in pharmacovigilance: a system of activities to monitor the occurrence of adverse events of medicinal products in regular medical care in order to prevent their occurrence or recurrence. The system consists of two sub-systems: passive pharmacovigilance based on ad random received reports, and active pharmacovigilance by performing post marketing surveillance clinical studies in ad random patient groups. The data of pharmacovigilance are presented in Periodic Safety Update Reports (PSURs) and in scientific papers. The Drug Safety Officer has prepared PSURs for submission to regulatory authorities for Cetor<sup>®</sup>, Cofact<sup>®</sup>, Haemocomplettan<sup>®</sup> P, Hepatitis B Immunoglobuline, Varicella Zoster Immunoglobuline for a five years period and required for re-registration. Regular PSURs were submitted for Nonafact<sup>®</sup>, twice, and for Ivegam<sup>®</sup>.

The Medical Department provides medical information and advice to physicians, nurses and pharmacists on the use (e.g. dosage, indications, administration) of blood and plasma products to safeguard the clinical use of plasma products, and gives oral presentations on the clinical use and indications of these products. In the provision of specific source plasma from plasmapheresis donors for the fractionation of Anti-rhesus (D) Immunoglobulin, Sanquin blood banks receive assistance with the selection of specific units of erythrocytes for immunisation purposes.

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## Sanquin provides medical information and advice to physicians, nurses and pharmacists on the use of both blood and plasma products.

### Clinical Trials ongoing in 2002

'C1-esterase-HP (Cetor<sup>®</sup>) after acute myocardial infarction for the reduction of infarct size': The purpose of the study was to verify if late treatment with Cetor<sup>®</sup> (6–9 hours after onset of acute myocardial infarction) reduces the infarct size in patients with acute myocardial infarction. The end report of this study in 22 patients has been finalised. The results showed that Cetor<sup>®</sup> can be used safely in the treatment of these patients.

Two studies have been performed on the Factor IX product Nonafact<sup>®</sup>. The study 'Follow-up of haemophilia B patients using Factor IX-M' has been set up for evaluating Nonafact, in long term clinical treatment. The 48-month end report of this study in 10 patients has been finalised. The results showed that Nonafact is an efficacious and safe plasma derived factor IX concentrate, which is well tolerated by the haemophilia B patients.

The purpose of the second study 'Post marketing study in haemophilia B patients using Nonafact<sup>®</sup> 100 IU/ml powder and solvent for solution for injection (human coagulation factor IX) (human plasma derived factor IX product, freeze dried)' is to follow up the safety of Nonafact<sup>®</sup> for 24 months in 20-40 patients. The study protocol has been approved by the Institutional Review Board of University Medical Centre of Utrecht.

A new liquid intravenous immunoglobulin product, IVIG-L, has passed several clinical trials. These studies have been performed according to the 'Note for guidance for clinical trials with intravenous immunoglobulin products' of the CPMP. The purpose of the clinical study 'Kinetics, efficacy and safety of IVIG-L (human normal immunoglobulin for intravenous use) in hypogammaglobulinaemia patients' was to obtain clinical data on the efficacy and safety of this new liquid intravenous immunoglobulin product during treatment of patients with primary immune deficiency on basis of the number of infections and the plasma trough level of IgG. The first interim report shows the excellent efficacy and safety of the product in 15 patients.

The objective of the clinical study 'Efficacy and safety of IVIG-L (human normal immunoglobulin for intravenous use) in Idiopathic Thrombocytopenic Purpura (ITP) patients' was to obtain clinical data on the treatment of patients with ITP. In the final report, the outcome of the study in 24 patients shows that treatment with IVIG-L is safe and effective. Further for this goal, a literature dossier for the indication of Guillan Barré syndrome, and a literature dossier for the indication of Kawasaki disease was finalised.

'Study on the efficacy of PPSB Solvent Detergent<sup>®</sup> and VP-VI in patients using oral anticoagulant therapy and undergoing acute cardiac surgery with a cardiopulmonary by-pass':

The purpose of this study, which has been set up in collaboration with the medical department of CAF-DCF cvba, the alliance partner of Sanquin, is comparing the efficacy of treatment with PPSB Solvent Detergent with the efficacy of the standard treatment with SD treated Fresh Frozen Plasma (FFP) in 40 patients. The study started at the Academic Hospital of the Catholic University of Leuven (Belgium).

### Journals

De Zwaan C, Kleine AH, Diris JH, Glatz JF, Wellens HJ, Strengers PF, Tissing M, Hack CE, van Dieijen-Visser MP, Hermens WT – Continuous 48-h C1-inhibitor treatment, following reperfusion therapy, in patients with acute myocardial infarction – *Eur Heart J* 2002 Nov; 23(21): 1670-7\*

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# Research and Development Services

## Quality assurance

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## Flow cytometry facility

*B Hooibrink (head)*

## HLA-typing facility

*J Lardy PhD (head)*

## Radiochemistry

*ACT Debast (head)*

## Labotatory Animal facility

*P van Mourik (head)*

## Library

*EB Sputneset*

## Quality Assurance

The external audit carried out annually by the Dutch Council for Accreditation (Raad voor Accreditatie) has led to renewal of the accreditation of the departments KVI, Virus Safety Services and Blood Transfusion Technology according to the ISO 17025 criteria. An external audit carried out by the CCKL, the Dutch Accreditation Board for Medical Laboratories has also led to the accreditation of these departments according to the ISO15189 criteria. The laboratory of Blood Transfusion Technology has also become re-accredited according to the SC03 criteria of the Dutch Council for Accreditation, which describes the criteria for research and development. A number of procedures specific for Research purposes have been established. By coaching of the staff members of Sanquin Research the quality awareness of the employees has been improved. The departments have made a start to implement the quality system of the division into their daily work. The internal audits executed in most departments, has led to a number of points for improvements which will be continued in consultation with the department of Quality Assurance.

## Flow cytometry facility

The flow cytometry core facility at CLB premises is located at the department of Immunobiology. Two systems are installed: analysers and cell sorters. This central flow cytometry facility serves all division serves all Sanquin divisions at location CLB.

The analysers are used for phenotyping of cells, apoptotic studies, interleukine studies, determination of autoantibodies against thrombocytes and leukocytes etc. The diagnostic department uses one instrument for diagnostic assays, the research departments use two other analysers. One instrument is separated physically from the others and is used for analysis of HIV infected material only. The facs analysers are operated by the users them self. They are well supported by user manuals and the assistance of a facs-operator in case of problems occurs.

The second group of instruments is the cell sorter. Two machines are installed. A facstar-plus 3-colour normal speed

cell sorter and a Moflo 5-colour high speed cell sorter. The cell sorter systems are only operated by the facsoperators, due to the complexity, daily alignment and adjustments that are need for operation. Every researcher can bring his samples for cell sorting. Daily quality control and calibration schemes guarantee the stability and reproducibility of the facs systems. Data acquired on each machine is stored for a minimum of 5 years using the latest technology available.

### HLA-typing facility

At the laboratory of HLA-diagnostics serological and molecular typing techniques are employed for Histocompatibility testing in the field of solid organ and allogeneic bone marrow transplantation, paternity testing and HLA and disease association. A not so well known aspect of Histocompatibility testing is the HLA antibody screening programme in which all transplant candidates are analysed for the presence of HLA antibodies. Alloantibodies directed against mismatched HLA antigens in an HLA incompatible donor-recipient combination have a deleterious effect on graft survival. Thus far, routine HLA antibody screening is performed in the conventional microcytotoxicity test. A major draw back of this technique is that it does not allow the distinction between HLA antibodies and other irrelevant cytotoxic antibodies. Thus, much effort was placed in the evaluation of an enzyme-linked immunosorbent assay that would allow detection and quantitation of IgG and IgM HLA specific antibodies in sera.

### Radiochemistry

The radio-isotope facility is available to Sanquin investigators for assisting those who are frequent users of radioisotopes in training individuals to use radioactive material in accordance with the provisions of safety authorities. The facility also provide personnel and laboratory monitoring, requires record keeping and waste disposal options. The facility accommodates central counting equipment and waste disposal storage.

### Laboratory Animal facility

The laboratory animal facility is responsible for the supply and husbandry of laboratory animal used by the various research and R&D programmes at location CLB. They adhere the highest standards of laboratory animal care and monitor the health of the animals. They provide animals that are genetically uniform and free from diseases with the interpretation of research result. Isolators and a pathogen-free barrier facility are present. Special trained staff members of the facility provide technical expertise to various investigators on a daily basis. Research protocols involving laboratory animals are reviewed by approved by a special governmental committee.

### Library

The library serves all research and development departments within at location CLB, and – more and more other Sanquin divisions. The library maintains an up-to-date electronic and printed collection of publications covering all aspects of blood transfusion research. The librarian gives advice and offers help for literature and patent searches and is responsible or other (electronic) information services relating to research and development. A large number of journals are available through the internet for full text accessibility.

# PhD theses

## Ronald van Rij

Chemokine receptors in HIV-1 infection & AIDS pathogenesis.

Promotor: Prof. dr. F Miedema

Co-promotor: Dr. J Schuitemaker

March 21, 2002

University of Amsterdam

## Tim Beaumont

HIV-1 sensitivity to neutralization: biological and molecular studies

Promotor: Prof. dr. F Miedema

Co-promotor: Dr. J Schuitemaker

June 14, 2002

University of Amsterdam

## John Jongerius

Detection of blood transmissible viral agents: implications for blood safety

Promotor: Prof. dr. WG van Aken

Co-promotores: Dr. EF van Leeuwen; Dr. CL van der Poel

June 21, 2002

University of Amsterdam

## Laure Ribeiro-de Couto

Control of cytotoxic responses on human neonates: naïvity or tight regulation

Promotor: Prof. dr. LA Aarden

Co-promotor: Dr. CJ Boog

July 4, 2002

University of Amsterdam

## Mette Hazenberg

T-cell turnover and thymic function in HIV-1 infection.

Promotor: Prof. dr. F Miedema

Co-promotores: Dr. D Hamann; Dr. RJ de Boer

September 13, 2002

University of Amsterdam

## Dominique Reumaux

Anti-Neutrophil Cytoplasm Autoantibodies (ANCA): clinical and functional studies

Promotores: Prof. dr. D Roos; Prof. dr. P Duthilleul

November 1, 2002

University of Amsterdam

## David Kwa

Host and viral factors in AIDS pathogenesis

Promotor: Prof. dr. F Miedema

Co-promotor: Dr. J Schuitemaker

December 12, 2002

University of Amsterdam

# Miscellaneous publications

Beckers EAM, Van Houtum WH, Korver CRW, Overbeeke MAM, Van Rhenen DJ – Ernstige hemolyse door auto-antistoffen type IgA: een diagnostische valkuil – Ned Tijdschrift Klin Chem 2002; 27: 131-135

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Kuijpers TW, de Kraker J – Histiocytose en Hemofagocytose, In: Kinderen en Kanker – Eds. H Behrendt, H van den Berg, MD van de Wetering 2002; 138-148

Kuijpers TW, Weening RS, Roos D – Clinical laboratory work-up of patients with a neutrophil defect. In: Growing up in pediatric immunology – Eds. CJ Heijnen, A Kavelaars, GT Rijkers 2002; 99-118

Kuijpers TW – Diagnostiek en behandeling van de Ziekte van Kawasaki. In: De rol van intraveneuze immunoglobulinen bij autoimmuunziekten – Eds. AJ Meulenbroek, PFW Strengers 2002; 25-28

Rijnders RJ, Christiaens GC, Bossers B, van der Schoot CE – Congenital adrenal hyperplasia: clinical aspects and neonatal screening – Ned Tijdschr Geneesk 2002 Sep 7; 146(36): 1713-4; author reply 1714

Ruitenbergh EJ – Xeno transplantation and xenozoonoses – in: Smit Sibinga CT, Dodd RY – Transmissible diseases and blood transfusion: proceedings of the twenty sixth International Symposium on Blood Transfusion, Groningen, The Netherlands. Kluwer Academic Publishers 2002: 93-97

Ruitenbergh EJ, Houweling H – Universal vaccination against group-C meningococci and pneumococci; summary of the advice from the Health Council of the Netherlands – Ned Tijdschr Geneesk 2002 May 18; 146(20): 938-40

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Smeenk RJ – Methodological update detection of antibodies to dsDNA: current insights into its relevance – Clin Exp Rheumatol 2002 May-Jun; 20(3): 294-300

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# Sponsors of research projects

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

Abbott

Academic Dental Centre, University of Amsterdam and Vrije Universiteit Amsterdam

AKZO/intervet

Amcell Corp.

American Red Cross

ASAC

A-Viral ASA

Baxter BioScience

Baxter Oncology

Bekales

Bentley/Baxter

Berna Biotech

Biogen

BioMérieux Nederland

Bioplex

Biosafe

Biotest Pharma GmbH

Boehringer Ingelheim Pharmaceuticals Inc.

British Biotechnology Ltd.

Cellgenix

Centeon

Centocor

Cerus Corporation

Chiron corporation

Chromogenetics

Cypress Bioscience Inc.

Deutsche Forschungsgemeinschaft

Deutsche Rote Kreuz

Diaclone

Diagnos Biochemical Cattle Management

DSM Biologics

Dutch AIDS fund (SAF)

Dutch Cancer Fund /KWF

Dutch Cancer Society

Dutch Heart Foundation

Dutch Kidney Foundation

Dutch Medical Research Council, ZON/MW)

Dutch Thrombosis Foundation

European Commission

Eurovet

EVA/NBSB

Finnish Red Cross

Foundation Jan Kornelis de Cock

Foundation for Pediatric Cancer Research

Fresenius HemoCare

Friends of Research on MS

Fuji

Gambro BCT

Genentech

Genmab

GlaxoSmithKline

Haemonetics

HAL/Madaus

Harimex

Hellsinn

Human RT

Immulogic

Immunotoko

Innogenetics

Kamada

Langsteiner Foundation for Blood Research (LSBR)

Leiden University Medical Centre

Macopharma

Microsafe BV

Miltenyi Biotech

Ministry of Public Health, Welfare and Sport

Municipal Health Services Amsterdam (GG&GD)

Natec

Natal Bioproducts Institute

National AIDS Therapy Evaluation Center

National Foundation for Rheumatism

Nedalco

Nefkens Foundation

Netherlands Asthma Foundation

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