IMMUNOLOGY AND BLOOD TRANSFUSION

DEVELOPMENTS IN HEMATOLOGY AND IMMUNOLOGY

Volume 28

The titles published in this series are listed at the end of this volume.

Immunology and Blood Transfusion

Proceedings of the Seventeenth International Symposium on Blood Transfusion, Groningen 1992, organized by the Red Cross Blood Bank Groningen-Drenthe

edited by

C. Th. SMIT SIBINGA and P. C. DAS
Red Cross Blood Bank Groningen-Drenthe, The Netherlands

and

T. H. THE
Professor of Clinical Immunology,
University of Groningen,
The Netherlands

SPRINGER SCIENCE+BUSINESS MEDIA, B.V.

Library of Congress Cataloging-in-Publication Data

Immunology and blood transfusion / edited by C.Th. Smit Sibinga and
P.C. Das and T.H. The.
p. cm.
Includes index.
ISBN 978-1-4613-6352-1 ISBN 978-1-4615-3094-7 (eBook)
DOI 10.1007/978-1-4615-3094-7
1. Blood--Transfusion--Congresses. 2. Immunohematology--Congresses. I. Smit Sibinga, C. Th. II. Das, P. C. III. The, T. H.
[DNLM: 1. Blood Transfusion--congresses. 2. Blood Cells--immunology--congresses. 3. Blood Groups--immunology--congresses.
W1 DE997VZK v. 28 1993 / WB 356 335 1993]
RM171.I455 1993
615'.39--dc20
DNLM/DLC
for Library of Congress 93-5012

ISBN 978-1-4613-6352-1

Printed on acid-free paper

All Rights Reserved
© 1993 Springer Science+Business Media Dordrecht
Originally published by Kluwer Academic Publishers in 1993
Softcover reprint of the hardcover 1st edition 1993
No part of the material protected by this copyright notice may be reproduced or
utilized in any form or by any means, electronic or mechanical,
including photocopying, recording or by any information storage and
retrieval system, without written permission from the copyright owner.

Baxter

Acknowledgement

This publication has been made possible through the support of Baxter, which is gratefully acknowledged.

CONTENTS

Moderators and Speakers XI

Foreword XIII

I. Principles and Fundamentals

Cytokines and Cell Mediated Immunology 3
W.H.A. Dokter

Transfusion-induced Immune Tolerance 11
H.T. Meryman

Potential Involvement of Free Radical Reactions in Inactivating Antigen Presenting Cells by Ultraviolet-B Irradiation 21
T.A. Takahashi

Alloimmunization and Platelet Transfusion Refractoriness 37
A. Brand

Graft-versus-host Disease 45
J. Jansen

Anti-idiotypic Regulation of the Alloimmune Response in Patients Transfused with Platelet Concentrates 57
J.W. Semple

Discussion 65

II. Processing Aspects

Options for Prevention of T Cell Alloactivation by Blood Transfusions 77
F.H.J. Claas

Use of Monoclonal Antibodies for Immune Purging in Bone Marrow Transplantation 85
E. Racadot

Reduction of the Immunogenic Capacity of Blood Components for the Prevention of Alloimmunization to White Cells 99
P. Rebulla

The Effect of Platelets in Red Cell Concentrates on the Leukocyte Depletion Capacity of Leukocyte Depletion Filters 109
I. Steneker

Leukocyte Depletion of Red Cell Preparations: Fourth Filter Generation Leads to 4log Reduction. How Far Can/Should We Go? 121
E. Richter

Immune Targeting of T-lymphocytes 129
L. de Leij

Humoral Functions of Immunoglobulin: Relationship to Purification Technology of Intravenous Immunoglobulin 137
P.L. Yap

Immune Adsorption Chromatography for Protein Purification 151
J.P. Tharakan

Discussion 161

III. Laboratory Aspects

Immunological Principles in Routine Quality Control Immunocharacteristics and Function Tests 173
J.G. Huisman

The Emerging Role of MHC Molecules as Signal Transducing Receptors 187
T.A. Chatila

Flow Cytometry in Transfusion Medicine 195
B. Houwen

New Applications of Molecular Diagnostics in Laboratory Test Systems 219
L.R. Overby

Discussion 231

IV. Clinical Immunology and Blood Transfusion

Prospects and Limitations of Adoptive Immunotherapy and Gene Therapy 239
M. Cottler-Fox

Clinical Strategies for the Prevention of Platelet Alloimmunization 245
E.J. Lee

Immune Mediated Transfusion Reactions: Bystander Immune Cytolysis –
A Frequently Overlooked Mechanism of Hemolysis? 255
B.A. van Dijk

Blood Transfusion and Cancer: Modulation or Tolerance? 261
J.G.A. Houbiers

Blood Product Induced Immunomodulation and Its Clinical Consequences ... 275
H.G. Watson

Transfusion Medicine and Immunological Research: Perspective for the
Future 285
T.H. The

Discussion 289

Index 297

MODERATORS AND SPEAKERS

Moderators

T.H. The (chairman)	– University Hospital Groningen, Groningen, NL
P.C. Das	– Red Cross Blood Bank Groningen-Drenthe, Groningen, NL
L. de Leij	– University Hospital Groningen, Groningen, NL
H.T. Meryman	– Jerome H. Holland Laboratory, Rockville, MD, USA
L.C. Overby	– Alamo, CA, USA
P. Rebulla	– Blood Transfusion Centre, Milan, I
C.Th. Smit Sibinga	– Red Cross Blood Bank Groningen-Drenthe, Groningen, NL

Speakers

A. Brand	– Red Cross Blood Bank Leiden, Leiden, NL
T. Chatila	– Harvard Medical School, Boston, MA, USA
F.H.J. Claas	– University Hospital Leiden, Leiden, NL
M. Cottler-Fox	– National Institute of Health, Bethesda, MD, USA
B.A. van Dijk	– Blood Transfusion Service, University Hospital Nijmegen, Nijmegen, NL
W.N. Drohan	– Jerome H. Holland Laboratory, Rockville, MD, USA
J.G.A. Houbiers	– Red Cross Blood Bank Leiden, Leiden, NL
B. Houwen	– Loma Linda University School of Medicine, Loma Linda, CA, USA
H. Huisman	– Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, NL
J. Jansen	– Methodist Hospital of Indiana, Indianapolis, IN, USA

E.J. Lee – University of Maryland Cancer Center, Baltimore, MD, USA

E. Racadot – Regional Blood Transfusion Centre, Besançon, F

E. Vellenga – University Hospital Groningen, Groningen, NL

H. Watson – Royal Infirmary of Edinburgh, Edinburgh, UK

P.L. Yap – Edinburgh & South East Scotland Blood Transfusion Service, Edinburgh, UK

Prepared discussants

E. Richter – Institute for Transfusion Medicine, Berlin, D

T. Takahashi – Hokkaido Red Cross Blood Center, Sapporo, J

J.W. Semple – Blood Bank St. Michael's Hospital, Toronto, C

I. Steneker – Red Cross Blood Bank Amsterdam, Amsterdam, NL

FOREWORD

Being old has the advantage of having many years of experience. You can look back sometimes astonished, often in wonder on what has been accomplished. It has, however, the disadvantage of not doing research, anymore. What stays is the excitement in reading and talking about the advances in our insight in the biological aspects of life. Looking through the programme it is clear, that there are a number of very interesting developments in blood transfusion. In that respect the theme of this 17th international symposium: "Immunology and blood transfusion" is very well chosen.

Let me look back in wonder and be thrilled by seeing how fast our knowledge has advanced: 35 years ago, when preparing my PhD thesis, I showed that macroglobulins – as they were called at that time – in Waldenström's disease have antigenic determinants in common as well as individual antigenic determinants. We produced antisera of patients with Waldenström's disease and absorbed the antisera with normal serum. We found antibodies, which reacted only with the homologous serum, but also antibodies which reacted with all sera. At that time we differed in opinion with Hässig, chief of the Swiss Blood Transfusion Service. He was of the opinion, that macroglobulins in Waldenström's macroglobulinemia were abnormal proteins specific for the disease: "Krankheitsspezifische Paraproteinen". Our conclusion was that several antibodies are formed against one macroglobulin. Some of the antibodies react only with the homologous macroglobulin (individual specific), but others are capable of precipitating heterologous macroglobulin as well (disease specific). Of course, macroglobulins turned out to be IgM immunoglobulins which has class specific and individual specific antigenic determinants. It is amazing how fast immunology has developed as a discipline of molecular biology. Looking back in wonder.

Some 40 years ago, when many of you were not born yet, I looked through a microscope at bone marrow smears of patients with Waldenström's disease. There were a great many cells which looked somewhat like lymphocytes. We called them lymphoid cells. Waldenström thought that macroglobulinemia was a disease entity in itself, probably related to chronic lymphocytic leukemia. My theory was that it was a disease closely related to myeloma. In the end I think both of us were right.

The difference between looking at a bone marrow smear stained with May Grünwald-Giemsa and what we now know of the morphological structure of cell biology is hardly understandable.

What will be your future, how will you look back, when you are old? Will you experience the same feeling that science has made such a great leap forward in such a short period of time, your own lifetime! From what direction will come the applications of blood transfusion in the treatment of patients? What are the future trends, where should be our priorities?

As chairman of the Dutch Council of Health Research, a council which advises the government on future research priorities in medicine from a viewpoint of what society needs, it is my opinion that in the meeting of different disciplines, for instance, transplantation, oncology, immunology and transfusion medicine, a very promising field of research is being opened.

I believe you are fortunate doing research at a time, where blood transfusion has developed from a service for patients in a discipline of its own: Transfusion medicine. I am sure that working in that discipline together with other disciplines such as immunology, oncology and transplantation medicine, you will make important contributions to medicine. As I said it is so often that real progress is made at a time, that disciplines meet each other at their borders.

May you all when you are old, look back in amazement with a feeling of joy at what has happened. However, the excitement of doing research and trying to gain insight in basic knowledge is in itself rewarding. It is not always so much the international recognition which is important. It is also rewarding when you have been able to give a high quality of service and care on the basis of sound knowledge that counts.

A truly remarkable and still very active man in medicine is William Kolff, some 50 years ago together with Prof. Tekke Huizinga, chief of the University Hospital Pharmacy, the founder of the blood bank in Groningen. He must be about 80 years old, now. He is a very practical man and he has contributed enormously to medicine. He developed, in commuting between Groningen and a small township south of Groningen, Kampen, the artificial kidney. Coming here as the host of Dr. Smit Sibinga you should know where it all started.

Prof.Dr. E. Mandema

I. PRINCIPLES AND FUNDAMENTALS

CYTOKINES AND CELL MEDIATED IMMUNOLOGY

W.H.A. Dokter, E. Vellenga

Binding of a T cell to an antigen presented by an antigen presenting cell (APC) leads to mutual activation of both cell types and to the release of an array of cytokines. During the last years the increase in knowledge of the involvement of individual cytokines in the interactive process between T cells and monocytes/macrophages has been overwhelming. The fast progress is mainly due to the development of cloning methods, sequencing techniques, and production of cytokines at a large scale. This has given the opportunity to exactly define the biological properties of different factors on the various cell populations.

Interleukin-4 (IL-4) is one of the cytokines produced by activated T cells [1]. Resting T cells do not express IL-4 at mRNA or protein level. In-vitro studies have shown that T-cell activation by the lectin concanavalin-A (con A), which mimics activation of the CD3 receptor complex, results in the induction of IL-4 mRNA expression [2,3]. Maximal accumulation of IL-4 mRNA is observed after 6 h of stimulation. The secretion of IL-4 protein follows the mRNA accumulation. IL-4 protein in the supernatant of con A activated T cells is detected after 24 h of stimulation [3]. In an attempt to elucidate the intra-cellular pathways that control the expression of the IL-4 gene, it became clear that two signals are required for the induction of IL-4 mRNA. This is based on the observation that stimulation of the protein kinase C dependent pathway with the phorbol-ester PMA or stimulation of the Ca^{2+} dependent pathway with the calcium-ionophore A23187 did not result in the expression of IL-4 mRNA. However, IL-4 mRNA was expressed when the combination of synthetic analogues was used [3]. Expression of the IL-4 gene by activation with con A is a process that is dependent on the synthesis of new protein. This is based on the results of experiments using the protein synthesis inhibitor cycloheximide, which was shown to completely block the con A induced IL-4 mRNA expression [3].

IL-4 is pleiotropic in its effect; i.e. it exerts multiple biological effects on a variety of cell types. The ability of cells to respond to a given cytokine and to get modulated by this cytokine is mediated through ligand specific cytokine receptors which are expressed at the cell membrane. Receptor studies with ^{125}I-labelled IL-4 have demonstrated that IL-4 receptors (IL-4R) are present on T-, B-, myeloid- and monocytic cells [4-13]. Initially, studies with ^{125}I-labelled IL-4

identified binding of IL-4 to a trimolecular complex consisting of a 65/70 kDa doublet and a 120 kDa protein with 300 high-affinity binding sites (Kd 100 pM) [14-17]. More recently, it has been demonstrated that the polypeptide chain with a molecular weight (M.W.) of 70,000 (p70) is a breakdown product of p120 [18], confirming the results of the cloned murine IL-4R gene [19]. In addition, an IL-4 binding molecule with a M.W. of 40 kD has geen detected in murine cells [19]. The p40 seems to be the soluble truncated form of the IL-4 receptor [20]. However, the soluble form of the IL-4 receptor has not yet been detected in human cells. mRNA and protein studies in human T cells have further indicated that the induction of IL-4 receptor mRNA is controlled by different intracellular signalling pathways. Stimulation with con A, PMA, or A23187 results in an increased expression of IL-4 receptor mRNA expression after 3-6 h of stimulation [21]. The combination of activators did not further augment the increase in IL-4 receptor mRNA expression compared to the effects of the individual activators. Moreover, evidence is obtained that IL-4 up-regulates IL-4 receptor mRNA after 4-6 h of stimulation.

The degree of stimulation by IL-4 is two fold higher than the effects of the synthetic analogues [21]. By analyzing the intracellular signals induced by IL-4, it appeared that the IL-4 induced IL-4 receptor mRNA up-regulation cannot be due to triggering of one of the known intracellular signalling pathways. This conclusion is based on the results of experiments showing that the induction of IL-4 receptor mRNA by IL-4 could not be blocked by the protein kinase C inhibitor staurosporin. In addition flow cytometric measurements of cytoplasmic free calcium with fluo-3 in IL-4 treated T cells did not demonstrate Ca^{2+} mobilization.

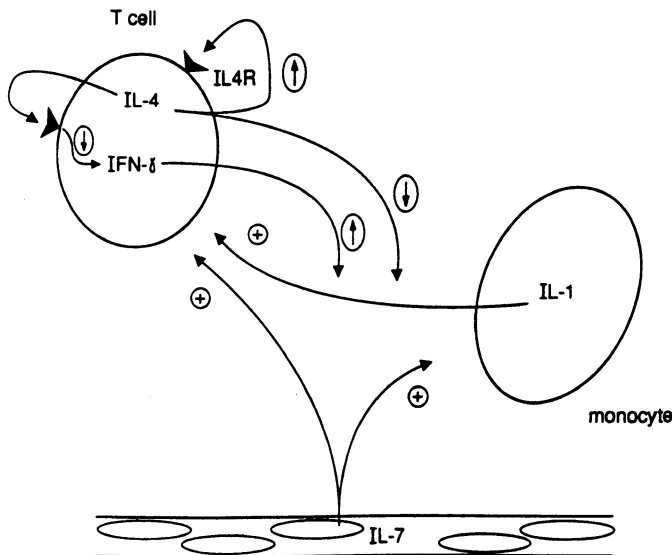


Figure 1. Schematic representation of the interaction between T cells, monocytes and stromal cells through different cytokines. IL-7 produced by stromal cells induces (indicated by +) IL-1 production in monocytes and stimulates (+) the proliferation of T cells. The lymphokine IL-4 down-regulates (↓) IL-1 expression in monocytes, whereas IL-1 expression in monocytes is up-regulated (↑) by IFN-γ. IL-4 enhances (↑) expression of its own receptor on T cells (IL-4 receptor indicated by↓) but inhibits (↑) expression of IFN-γ.

These data indicate that activation of T cells results in an increased expression of IL-4 and IL-4 receptor, demonstrating that the function of the T cell is strictly controlled by IL-4 [21]. Table 1 is a schematic representation of the regulation of the IL-4 and IL-4R gene in human T cells.

Table 1. Schematic representation of the regulation of IL-4 and IL-4R gene expression in human T cells.

	IL-4	**IL-4R**
A23187	–	↑
PMA	–	↑
con A	↑	↑
con A + PMA	↑↑	↑
IL-4	–	↑↑

In addition to its modulatory effects on the expression of the IL-4 receptor expression in T cells, IL-4 protein modulates the function of monocytic cells. IL-4 induces the expression of MHC class II antigen on monocytes and enhances tumoricidal activity [22-25]. However, the tumoricidal activity is confined to selected target cell lines, and only HLA-DR and HLA-DP but not HLA-DQ MHC class II molecules are induced by IL-4 [23]. In addition, IL-4 influences the regulation of cytokine expression in human monocytes. In contrast to endotoxin or IL-1 stimulated monocytes, IL-4 stimulated monocytes do not express cytokines like IL-1, IL-6, tumour necrosis factor (TNF), or granulocyte-colony stimulating factor (G-CSF) [12]. Moreover, endotoxin or IL-1 activated monocytes demonstrate a reduced expression of IL-1, IL-6, TNF, and G-CSF when stimulated with the combination of endotoxin plus IL-4 or IL-1 plus IL-4 compared to the effects of endotoxin or IL-1 alone [12]. The suppressive effect of IL-4 is not restricted to the mRNA level but includes the secretion of protein. Furthermore it appeared that the degree of suppression is dependent on the mode of activation. Cells precultured with IL-4 and subsequently activated with endotoxin demonstrate a stronger reduction in cytokine mRNA expression than cells stimulated with IL-4 plus endotoxin added at the same time [12].

In contrast to the described expression of cytokines which is down-regulated by IL-4, cytokine receptor expression in monocytes can be up-regulated. The IL-1 receptor antagonist which has the property to bind circulating IL-1, is up-regulated by IL-4 and mRNA and protein level [26]. Thus, IL-4 inhibits the IL-1 response of monocytes. Taken together, these data indicate that the T cell product IL-4 can be considered as a negative regulator for monocytes, which limits the degree of activation originating from the interactive process between T cells and monocytes.

γ-Interferon (IFN-γ) is an additional cytokine produced by activated T cells [27]. In the murine model two types of T cells are relevant for the production of cytokines. Activated Th1 cells produce IL-2, IFN-γ, but not IL-4 whereas Th2 cells produce IL-4 and IL-5 but not IFN-γ [27]. In humans this dichotomy of Th cells is not as apparent, since the majority of T cell clones producing IL-4 also secrete IFN-γ and IL-2 [28]. The production and secretion of IFN-γ by human T cells requires stimulation with an activation signal. Activation of the CD3 receptor complex or stimulation with con A or PMA plus A23187 induces the expresion of IFN-γ at mRNA and protein level. These data demonstrate that a strong homology exists with regard to the activation signals required for the induction of IFN-γ and IL-4 mRNA. Moreover, studies have indicated that IFN-γ gene expression is strictly controlled by IL-4. The secretion of IFN-γ by activated T cells is strongly reduced by co-stimulation with IL-4 [29].

Monocytes also express receptors for IFN-γ, and therefore respond to IFN-γ. IFN-γ up-regulates the expression of MHC class II antigens on monocytes/macrophages, which can further promote the interactive process of T cells and monocytes. Moreover, IFN-γ modulates the cytokine expression in human monocytes [30]. IFN-γ by itself is not capable of inducing the secretion of cytokines but instead primes monocytes to a higher degree of activation. IFN-γ pretreated monocytes secrete higher quantities of IL-1 and G-CSF protein after stimulation with endotoxin or IL-1 compared to the effects of endotoxin or IL-1 alone. The increased secretion of cytokines by IFN-γ primed monocytes can trigger T cells and can ultimately result in an augmentation of the cell-mediated immune response. However, this process is also thightly controlled by IL-4. The priming effects of IFN-γ on monocytes can be blocked partially by co-stimulating the cells with IL-4 [30].

The interactive process between T cells and monocytes/macrophages is not only controlled by cytokines produced by circulating blood cells, like IL-4 and IFN-γ, but can also be modulated by factors produced by stromal cells. One of these stromal derived cytokines is IL-7 [31]. IL-7 originally defined as a growth factor for pre-B cells, affects among other T-, null-, and myeloid cells [32-35]. IL-7 stimulates the proliferation of CD4 and CD8 positive T cells and induces lymphokine-activated killer activity by stimulating CD56+ Purified cells [36]. The effect of IL-7 on the different target cell populations seems to be a direct effect since anti-IL-2, anti-IL-4, anti-IL-6 do not abolish the promotive effects-of IL-7 [32,33,36]. However, the supportive effect of IL-7 on eosinophilic progenitors from human bone marrow cells is mediated through the synthesis of IL-5 since it could be blocked by neutralizing concentrations of anti-IL-5 antibodies [37]. In addition, evidence has been obtained that IL-7 influences the expression of cytokines by activated T cells [38]. T cells stimulated by IL-7 alone, do not express IL-3, GM-CSF, and IL-4 mRNA [39,40]. As described above, con A activated T cells express IL-4 at mRNA level after 6 h of stimulation. IL-3 and GM-CSF mRNA are also expressed after 6 h of con A treatment [3]. Co-stimulation with con A plus IL-7 leads to an augmented expression of IL-4, IL-3, and GM-CSF transcripts. The augmented cytokine expression by IL-7 is

due to an increase in the stability of the IL-4 message, i.e. to mRNA stabilization at post-transcriptional level [39,40]. In addition, recent studies have demonstrated that monocyte functions can be regulated by IL-7. IL-7 stimulated monocytes express IL-1, IL-6, and TNF at IL-4 mRNA and protein level [41,42]. These promotive effects of IL-7 on the cytokine expression of monocytes can be blocked by co-culturing the cells with IL-4. The described interactive processes between T cells, monocytes, and stromal cells and the cytokines playing a role in these processes are schematically represented in Figure 1.

These data illustrate that two factors produced by activated T cells (IL-4 and IFN-γ) have opposite effects on monocytic cells. Furthermore, cytokine expression in human T cells can be modulated by stromal derived growth factors. It is conceivable that the resulting network of cytokine effects is a balanced process of stimulation and inhibition of T cells and monocytes.

References

1. Paul WE. Interleukin-4: A prototypic immunoregulatory lymphokine. Blood 1991; 77:1859-70.
2. Lewis DB, Prickett KS, Larsen A, Grabstein K, Weaver M, Wilson CB. Restricted production of interleukin-4 by activated human T cells. Proc Natl Acad Sci USA 1988;85:9743-7.
3. Doktor WHA, Esselink MT, Sierdsema SJ, Halie MR, Vellenga E. Transcriptional and post-transcriptional regulation of the interleukin-4 and interleukin-3 gene in human T cells. Blood 1993;81:35-40.
4. Howard M, Farrar J, Hilfiker M, et al. Identification of a T cell-derived B cell growth factor distinct from interleukin-2. J Exp Med 1982;155:914-23.
5. Rabin EM, Ohara J, Paul WE. B cell stimulatory factor 1 (BSF-1) activates resting B cells. Proc Natl Acad Sci USA 1985;82:2935-9.
6. Yokota T, Otsuka T, Mosmann T. Isolation and characterization of a human clone, homologous to mouse B-cell stimulatory factor-1, that expresses B-cell and T-cell stimulating activities: Proc Natl Acad Sci USA 1986;83:5894-8.
7. Spits H, Yssel Y, Takebe Y, et al. Recombinant interleukin-4 promotes the growth of human T cells. J Immunol 1987;139:1142-7.
8. Peschel C, Paul WE, Ohara J, Green I. Effects of B cell stimulatory factor-1/interleukin-4 on hematopoietic progenitor cells. Blood 1987;70:254-9.
9. Te Velde AA, Hijbens RJF, de Vries JE, Figdor CG. IL-4 decreases FcgammaR membrane expression and FcgammaR-mediated cytotoxic activity of human monocytes. J Immunol 1990;144:3046-51.
10. Wolf de JThM, Beentjes JAM, Esselink MT, Halie MR, Vellenga E. Interleukin-4 suppresses the interleukin-3 dependent erythroid colony formation from normal human bone marrow cells. Brit J Haematol 1990;74:246-50.
11. Vellenga E, de Wolf JThM, Beentjes JAM, Esselink MT, Smit JW, Halie MR. Divergent effects of interleukin-4 (IL-4) on the granulocyte colony-stimulating factor and IL-3 supported myeloid colony formation from normal and leukaemic bone marrow cells. Blood 1990;75:633-7.
12. Vellenga E, Dokter WHA, de Wolf JThM, van der Vinne B, Esselink MT, Halie MR. Interleukin-4 prevents the induction of G-CSF mRNA in human adherent monocytes in response to endotoxin and IL-1 stimulation. Brit J Haematol 1991;79:22-6.
13. Wagteveld AJ, van Zanten AK, Esselink MT, Halie MR, Vellenga E. Expression and regulation of IL-4 receptors on human monocytes and acute myeloblastic leukaemic cells. Leukemia 1991;5:782-8.

15. Galizzi J-P, Zuber CE, Cabrillat H, Djossou O. Banchereau J. Internalization of human interleukin-4 and transient down-regulation of its receptor in the CD23-inducible Jijoye cells. J Biol Chem 1989;264:6984-9.
16. Foxwell BMJ, Woerly G, Ryffel B. Identification of interleukin-4 receptor associated proteins and expression of both high- and low-affinity binding on human lymphoid cells. Eur J Immunol 1989;19:1637-41.
17. Galizzi J-P, Castle B, Djossou OT, et al. Purification of a 130k-Da T cell glycoprotein that binds human interleukin-4 with high affinity. J Biol Chem 1990;265:439-44.
18. Keegan AD, Beckmann MP, Park LS, Paul WE. The IL-4 receptor: Biochemical characterization of IL-4-binding molecules in a T cell line expressing large numbers of receptors. J Immunol 1991;146:2272-9.
19. Mosley B, Beckmann P, March CJ, et al. The murine interleukin-4 receptor: Molecular cloning and characterization of secreted and membrane bound forms. Cell 1989; 59:335-48.
20. Idzerda RL, March CJ, Mosley B, et al. Human interleukin-4 receptor confers biological responsiveness and defines a novel receptor superfamily. J Exp Med 1990; 171:861-73.
21. Dokter WHA, Borger P, Hendriks D, van der Horst I, Halie MR, Vellenga E. Interleukin-4 receptor expression on human T cells is affected by different intra-cellular signaling pathways and by IL-4 at transcriptional and post-transcriptional level. Blood 1992;80:2721-8.
22. Te Velde AA, Klomp JPG, Yard BA, de Vries JE, Figdor CG. Modulation of phenotypic and functional properties of human peripheral blood monocytes by IL-4. J Immunol 1988;140:1548-54.
23. Crawford RM, Finbloom DS, Ohara J, Paul WE, Meltzer MS. B cell stimulatory factor-1 (interleukin-4) activates macrophages for increased tumoricidal activity and expression of Ia antigens. J Immunol 1987;139:135-41.
24. Stuart PM, Zlotnik A, Woodward JG. Induction of class I and class II MHC antigen expression on murine bone marrow-derived macrophages by IL-4 (B-cell stimulatory factor-1). J Immunol 1989;140:1542-7.
25. Zlotnik A, Fischer M, Roehm N, Zipori D. Evidence for effects of interleukin-4 (B-cell stimulatory factor-1) on macrophages: Enhancement of antigen presenting ability of bone marrow-derived macrophages. J Immunol 1987;138:4275-9.
26. Orino E, Sone S, Nii A, Ogura T. IL-4 up-regulates IL-1 receptor antagonist gene expression and its production in human blood monocytes. J Immunol 1992;149:925-31.
27. Mosmann TR, Coffman RL. Two types of mouse helper T cell clone. Immunol Today 1987;8:223-8.
28. Yokota T, Arai N, de Vries JE, et al. Molecular biology of interleukin-4 and interleukin-5 genes and biology of their products that stimulate B cells, T cells and hemopoietic cells. Immunol Rev 1988;102:137-43.
29. Peleman R, Wu J, Fargeas C, Delespesse G. Recombinant interleukin-4 suppresses the production of interferon-gamma by human monoclear cells. J Exp Med 1989;170: 1751-6.
30. De Wit H, Dokter WHA, Esselink MT, Halie MR, Vellenga E. Interferon-gamma enhances the LPS-induced G-CSF production in human adherent monocytes which is regulated at transcriptional and post-transcriptional level. Exp Hemat 1993; in press.
31. Namen AE, Schmierer AE, March CJ, et al. B cell precursor growth-promoting activity. Purification and characterization of a growth factor active on lymphocyte precursors. J Exp Med 1988;167:988-1002.
32. Goodwin RG, Lupton S, Schmierer A, et al. Human interleukin-7: Molecular cloning and growth factor activity on human and murine B-lineage cells. Proc Natl Acad Sci USA 1989;86:302-6.

33. Londei M, Verhoef A, Hawrylowicz C, Groves J, de Berardinis P, Feldmann M. Interleukin-7 is a growth factor for mature human T cells. Eur J Immunol 1990;20: 425-30.
34. Armitage RJ, Namen AE, Sassenfeld HE, Grabstein KH. Regulation of human T cell proliferation by IL-7. J Immunol 1990;144:938-41.
35. Morrissey PJ, Conlon P, Charrier K, et al. Administration of IL-7 to normal mice stimulates B-lymphopoiesis and peripheral lymphadenopathy. J Immunol 1991;147: 561-8.
36. Naume B, Espevik T. Effects of IL-7 and IL-2 on highly enriched CD56+ natural killer cells. A comparative study. J Immunol 1991;147:2208-14.
37. Vellenga E, Esselink MT, van Straaten J, et al. The supportive effects of interleukin-7 on eosinophil progenitors from human bone marrow cells can be blocked by anti-interleukin-5. J Immunol 1992;149:2992-5.
38. Herbelin A, Machavoine F, Schneider E, Papiernik M, Dy M. IL-7 is requisite for IL-1-induced thymocyte proliferation. Involvement of IL-7 in the synergistic effects of granulocyte-macrophage colony-stimulating factor or tumor necrosis factor with IL-1. J Immunol 1992;148:99-105.
39. Dokter WHA, Sierdsema SJ, Esselink MT, Halie MR, Vellenga R. Interleukin-7 enhances the expression of IL-3 and GM-CSF mRNA in activated human T cells by post-transcriptional mechanisms. J Immunol 1993;150:in press.
40. Dokter WHA, Sierdsema SJ, Esselink MT, Halie MR, Vellenga R. Interleukin-4 mRNA and protein expression is enhanced by interleukin-7 in activated human T cells. (Submitted for publication).
41. Alderson MR, Tough TW, Ziegler SF, Grabstein KH. Interleukin-7 induces cytokine secretion and tumoricidal activity by human peripheral blood monocytes. J Exp Med 1991;173:923-30.
42. Ziegler SF, Tough TW, Franklin TL, Armitage RJ, Alderson RM. Induction of macrophage inflammatory protein-1β gene expression in human monocytes by lipopolysaccharide and IL-7. J Immunol 1991;147:2234-9.

TRANSFUSION-INDUCED IMMUNE TOLERANCE[1]

H.T. Meryman, M. Mincheff

As early as 1946, Medawar [1] reported delayed rejection of skin homografts in rabbits following donor-specific blood transfusions, an observation subsequently confirmed in animals by a number of other investigators [2-6]. The impact of these reports on clinical practice was negligible since the data were collected mainly in rodents and since it was well known that transfusions could also immunize patients and thereby jeopardize the success of or even the opportunity for a subsequent transplant [7]. However, in 1973, Opelz et al [8] published data indicating that allogeneic pre-transplant transfusions significantly reduced the incidence of rejection episodes following renal transplants in humans and improved the 1-year kidney survival rates. This report was followed by more extensive data collected by Opelz et al [9] as well as confirmations by a number of other investigators which have been summarized in at least three major review articles [10-12]. Alloimmunization continued to be a hazard but the introduction of immuno-suppressive agents, particularly azathioprine [13,14], greatly reduced this threat and both allogeneic and donor-specific transfusions have become a standard component of pre-transplant therapy.

Although the introduction of cyclosporin and other advances in the management of transplant patients have improved transplant survival to the point where the benefits of transfusion are less apparent [15,16], the weight of evidence is overwhelming that transfusions do induce some degree of tolerance to allografts. Recognition of these has more recently led to concern regarding the possibility of other, less desirable results of immunosuppression and a number of studies have been conducted in an effort to determine whether immuno-modulation by transfusions could adversely affect patient responses to such immune-dependent challenges as bacterial infection or tumour growth.

A number of studies linking transfusion to an increase in infectious complications in surgical patients have now been published [17-28] and most found a positive relationship. At least three investigators [24-26] have found that autologous transfusions fail to correlate with infections. The difficulties of discriminating between transfusions as an independent variable and transfusions as an

1. Supported in part by a grant from the G. Harold and Leila Y. Matchers Charitable Foundation and MIH BSRG 2 507RR05737.

Table 1. Alloimmunization by leukocyte-depleted red cells and platelets.

		Mean residual leukocytes in		Incidence of alloimmunization	
Author	**Ref.**	**Platelets**	**Red cells**	**Control**	**WBC depleted**
Sirchia et al	31	no platelets	$< 2\times10^7$	8/13 (61%)	0/11 (0%)
Schiffer et al	34	1×10^7	3×10^7 (frozen) $1\text{-}5\times10^8$ (washed)	4/12 (33%)	4/15 (27%)
Murphy et al	35	$1\text{-}2\times10^8$	$< 8\times10^6$	15/31 (48%)	3/19 (16%)
Eernisse & Brand	36	5×10^6	$< 1\times10^8$	26/28 (93%)	16/68 (24%)
Andreu et al	37	$0.5\text{-}1.5\times10^8$	6×10^7	11/35 (31%)	4/34 (12%)
Sniecinski et al	38	6×10^6	5×10^7	10/20 (50%)	3/20 (15%)
Fisher et al	39	1.5×10^7 $< 5\times10^6$	no red cells no red cells	–	5/12 (42%)
Myllylä	40	1×10^5	1×10^5	1/18 (6%)	0/21 (0%)
Saarinen et al	41	4×10^4	1×10^5	12/17 (71%)	0/18 (0%)
Kooy et al	42	$< 5\times10^6$	$< 5\times10^6$	11/26 (42%)	2/27 (7%)
Handa et al	43	4.3×10^6	2×10^7	9/22 (41%)	2/33 (6%)
Oksanen et al	44	4×10^4	1×10^5	2/15 (13%)	2/16 (12%)
Braine et al	45	1×10^4	5×10^5	10/20 (50%)	3/13 (23%)

This table summarizes clinical data using leukocyte-depleted platelets and red cells. Sirchia et al selected only those filtered red cell units containing no leukocytes in a 1.8 μl sample and is the only investigator transfusing only red cells. Schiffer et al reported only that red cells were either frozen or washed. Residual leukocyte figures given for his experiments are literature values for those procedures. The patients of Fisher et al were previously non-transfused and received three transfusions at bi-weekly intervals. In the series of Myllylä et al, control and experimental patients received an average of 77 and 83 units of platelets respectively. The incidence of alloimmunization in their control group is surprisingly low. The publication by Oksanen et al builds on the data presented in the abstract of Myllylä but with the deletion of some patients because of deviations from the study protocol. Saarinen et al and Eernisse and Brand used refractory state as evidence of alloimmunization, the others used antibody formation. The data of Andreu et al and of Handa et al were from multi-institutional studies. In the latter study, "leukocyte-poor red cells" of unspecified depletion were used and, when not available, Sepacell filtration at the bedside was used with approximately 2×10^7 residual leukocytes in the filtered units. The data of Braine et al were collected in bone marrow transplant patients, the majority of whom were never transfused or pregnant. Leukocyte depletion of platelets was by elutriation, red cells were filtered twice through two Pall RC100 Filters. The figures for residual leukocytes in all cases are, of course mean values, the counting techniques vary in their sensitivity and individual units can vary widely in their leukocyte counts.

indicator of the severity of the surgical procedure continue to cloud the relationship [23].

Several investigators have also looked for linkage between transfusions and malignancies. Retrospective studies in humans have yielded mixed conclusions, some finding a strong correlation, some no correlation, others a negative correlation. The subject has been reviewed by Meryman [12] and Blumberg and Heal [27]. A subsequent paper by Ness et al [28] failed to find a correlation.

Although the clinical significance of immuno-suppression by transfusions remains to be adequately defined there is consensus that it does occur and that blood transfusions probably will either alloimmunize or immuno-suppress [22]. However, despite a great deal of interest in this question, the mechanisms of both immunization and suppression by transfusions remain speculative. Techniques for either preventing or maximizing immuno-suppression by transfusions have yet to be demonstrated and will be difficult to develop in the absence of an understanding of the mechanism.

Evidence to date indicates that alloimmunization by transfusions is largely, although perhaps not wholly, dependent on the presence of leukocytes [11,29, 30]. Leukocyte depletion of red cells [31] and platelets [32,33] has uniformly resulted in a reduction in the incidence of HLA alloimmunizations although depletion to as few as 10^4 to 10^5 residual leukocytes has still not entirely eliminated immunizations, implying that a second source of immunization may be involved. Clinical studies of alloimmunization by leukocyte-depleted red cell and platelet transfusions are summarized in Table 1.

Both laboratory and clinical investigation of immuno-suppression by transfusions are difficult since there is as yet no specific assay that can define the presence or absence of an effect. The primary evidence for tolerizing comes from statistical analyses of kidney transplants, primarily those performed during the 1970's prior to the advent of cyclosporin. These data do tend to indicate leukocytes as the responsible agent, although not unequivocally. The use of frozen red cells, for example, which are moderately leukocyte-depleted, was reported by Poleskey [46,47] to be associated with a higher 2-year cadaver kidney survival (90%) than a mix of frozen and fresh (62%), whereas Opelz and Terasaki [48] found frozen blood transfusions to be as ineffective as no transfusions.

Whereas Betuel et al [49] reported that graft survival in patients transfused pre-operatively with leukocyte-depleted platelets was comparable to results with whole blood transfusions, Pallardo et al [50] found transfusions of leukocyte-rich platelets to be no more effective than no transfusions.

Animal studies have been both dramatic and contradictory. Foster et al [51], Faustman et al [52] and Wood et al [53] have all demonstrated that transfusions of rat red cells or purified liver plasma membrane, both of which, like human platelets, express only class I MHC, induced sufficient immuno-suppression to permit allograft acceptance, although comparable procedures in dogs [54,55] were without benefit.

Perhaps the most unsettling experiments were those performed by Oh and McClure [56]. These investigators reported that of 20 Rhesus monkeys receiving

2nd party whole blood transfusions, 19 were alloimmunized whereas of 20 monkeys first receiving two bi-weekly transfusions of leukocyte-depleted platelets from 2nd party donors only four were alloimmunized by subsequent whole blood transfusions from the same donors, and of eight of those non-immunized monkeys, none were immunized by whole blood from 3rd party donors, highly significant evidence for immuno-suppression by platelet transfusions. In a subsequent report [57], however, these same authors confirmed their previous observations using 2nd party platelets but also found that autologous platelet transfusions prevented subsequent alloimmunization by 2nd party whole blood, raising entirely new questions regarding the immuno-suppressive effects of liberated platelet factors, of blood bag plasticizers, anticoagulants or even of transfusion-related stress.

In summary, the conclusion that transfusions can be immuno-suppressive is wholly dependent on evidence that alloimmunization or allograft rejection can be reduced or prevented by prior transfusions but the evidence remains inconclusive as to which component of the blood is responsible and whether, as the experiments of Oh and McClure suggest, a major component of the mechanism may be non-specific.

A variety of mechanisms for the putative immuno-suppression by transfusions has been proposed, including the development of a suppressor cell network [58-60], clonal deletion [61], anti-idiotypic antibodies [62-64], prostaglandin release [65,66] and blocking antibodies against the Fc receptor [67]. None of these explanations has been accompanied by indisputable evidence. The most prevalent assumption is that leukocytes are most probably involved in the development of an immuno-suppression sufficient to influence the acceptance of allografts and the superiority of donor-specific transfusions over allogeneic transfusions [68] would appear to support this conclusion.

We [69] have investigated the possibility that immuno-suppression may be induced by donor leukocytes incompetent to provide the complete signalling required for an immune response. It has been well established that antigen-presenting cells must not only express class II MHC carrying an antigen-derived peptide but must also provide a second, accessory signal, in order to fully activate the CD4 T cell to secrete IL-2 and to proliferate [reviewed in 70]. It has also been established, at least in vitro, that presentation of the MHC-peptide signal in the absence of the accessory signal can induce a state of non-responsiveness, or anergy, in the CD4 cell [71, reviewed in 72].

Peripheral-blood lymphocytes expressing the class II MHC consist primarily of monocytes and B cells. Monocytes can express the accessory signal and are therefore competent to induce a complete immune response leading to alloimmunization. B cells, on the other hand, are constitutively unable to provide the accessory signal and, in the absence of monocytes, would be expected to tolerize. Our experiments showed a progressive loss of the ability of monocytes to provide the accessory signal as a result of blood storage, with complete incom-

petence by day 13. UVB irradiation was also shown to inhibit expression of the accessory signal by blood monocytes.

On this basis, fresh blood transfusions containing leukocytes could induce alloimmunization through activation of recipient CD4 cells by donor monocytes. If alloimmunization by monocytes fails to occur, donor B cells presenting MHC-peptide in the absence of accessory signal could induce non-responsiveness against the specific MHC-peptide complexes being presented. The extent to which this would ameliorate the response to a subsequent transplant would depend on the extent to which the MHC-peptides presented were comparable to those subsequently presented by the transplant. This would be consistent with the observation that increasing the number of allo-transfusions improves kidney survival [73] and that donor-specific transfusions are best.

The loss of the ability of monocytes to induce alloimmunization after 13 days of refrigerated blood storage is consistent with the report of Light et al [74] that stored blood failed to alloimmunize while fresh blood did. Since our experiments showed that the lymphocytes in blood stored more than 13 days continue to express MHC-peptide in the absence of the accessory signal, this should result in immuno-suppression and it is probable that this kind of modulation of the cellular immune system would be necessary to influence allograft rejection.

The prevention of transfusion-induced alloimmunization, on the other hand, may be achieved by other, perhaps non-specific mechanisms. It does appear that immunization by transfusion is a borderline phenomenon, easily influenced by numbers of leukocytes, recipient immune status, concurrent medication and degree of HLA mismatch [69] and it is probably useful to differentiate between the extent of suppression necessary to inhibit alloimmunization and that required for the curtailing of rejection or the enhancement of infection.

If transfusions can indeed alloimmunize or immuno-suppress through the mechanisms proposed above, storing red cells for at least two weeks will provide the most economical way to avoid alloimmunization but immuno-suppression would remain a side-effect of transfusion. The same would be true for UVB irradiated platelets. Where immuno-suppression is clinically undesirable, leukocyte depletion may be the only option.

Acknowledgements

We wish to express our appreciation to Tish Alsop and Veena Kapoor for their competent assistance in the collection of data for this study.

References

1. Medawar PB. Immunity to homologous grafted skin. II. The relationship between the antigens of blood and skin. Brit J Exp Pathol 1946;27:15-24.
2. Castermans A. Effect of pre-treatment by homologous splenic cell suspension and whole blood transfusion on homograft survival in mice and rabbits. CR Soc Biol 1956;150:2042-6.
3. Peer LA. Behavior of skin grafts exchanged between parents and offspring. Ann NY Acad Sci 1958;73:584-9.
4. Marino H, Benaim F. Experimental skin homografts: Effect of homohemotherapy on their survival time. Am J Surg 1958;95:267-73.
5. Stark KB, Dwyer E. The enhancement of homografts of skin in the adult rabbit using elements of homologous whole blood. Surgery 1959;46:227-87.
6. Halasz NA, Orloff MJ, Hirose F. Increased survival of renal homografts in dogs after injection of graft donor blood. Transplantation 1964;2:453-8.
7. Hume DM. Kidney transplantation. In: Rappaport FT, Dausset J (eds). Human Transplantation. Grune and Stratton, New York 1968:110-50.
8. Opelz G, Sengar DPS, Mickey MR, Terasaki PI. Effect of blood transfusions on subsequent kidney transplant. Transplant Proc 1973;5:253-9.
9. Opelz G, Terasaki PI. Improvement of kidney-graft survival with increased numbers of transfusions. N Engl J Med 1978;299:799-803.
10. Kahan B. Donor-specific transfusions – 2 balanced view. In: Morris PJ, Tilney NL (eds). Progress in transplantation. Vol 1. Churchill Livingstone, New York 1984:115-48.
11. Perkins HA. Transfusion-induced immunological unresponsiveness. Trans Med Rev 1988;2:196-203.
12. Meryman HT. Transfusion-induced alloimmunization and immunosuppression and the effects of leukocyte depletion. Transf Med Rev 1989;3:180-93.
13. Anderson CB, Sicard GA, Etheredge EE. Pretreatment of renal allograft recipients with azathioprine and donor-specific blood products. Surgery 1982;92:315-21.
14. Glass NR, Miller DT, Sollinger HW, et al. Comparative analysis of the DST and Imuran-plus-DST protocol for live donor renal transplantation. Surgery 1983;36:636-41.
15. Opelz G. Improved kidney graft survival in non-transfused recipients. Transplant Proc 1987;19:149-52.
16. Groth CG. There is no need to give blood transfusions as pretreatment for renal transplantation in the cyclosporine era. Transplant Proc 1987;19:153-4.
17. Tartter PI. Blood transfusion and infectious complications following colorectal cancer surgery. Brit J Haematol 1988;75:789-92.
18. Tartter PI, Driefuss RM, Malon AM, et al. Relationship of postoperative septic complications and blood transfusions in patients with Crohn's disease. Am J Surg 1988; 155:43-7.
19. Preiksaitis JK, Brown L, McKenzie M. The risk of cytomegalovirus infection in seronegative transfusion recipients not receiving exogenous immunosuppression. J Infect Dis 1988;157:523-9.
20. Waymack JP. The effect of blood transfusions on resistance to bacterial infections. Transplant Proc 1988;20:1105-7.
21. George CD, Morello PD. Immunologic effect of blood transfusion upon renal transplantation, tumor operations, and bacterial infection. Am J Surg 1986;152:329-37.
22. Graves TA, Cioffi WG, Mason AD Jr, McManus WF, Pruitt BA. Relationship of transfusion and infection in a burn population. J Trauma 1989;29:948-54.
23. Nichols RL, Smith JW, Klein DB, et al. Risk of infection after penetrating abdominal trauma. N Engl J Med 1984;311:1065-70.
24. Fernandez Mc, Gottlieb M, Menitove JE. Blood transfusion and post-operative infection in orthopedic patients. Transfusion 1992;32:318-22.

25. Mezrow CK, Bergstein I, Tartter PI. Postoperative infections following autologous and homologus blood transfusions. Transfusion 1992;32:27-30.
26. Triulzi DJ, Vanek K, Ryan DH, Blumberg N. A clinical and immunological study of blood transfusion and postoperative bacterial infection in spinal surgery. Transfusion 1992;32:517-24.
27. Blumberg N, Heal JM. Transfusion and host defenses against cancer recurrence and infection. Transfusion 1989;29:236-45.
28. Ness PM, Walsh PC, Zahurak M, Baldwin ML, Piantadosis S. Prostate cancer recurrence in radical surgery patients receiving autologous or homologous blood. Transfusion 1992;32:31-6.
29. Mincheff MS, Meryman HT. Induction of primary mixed leukocyte reactions with ultraviolet B or chemically modified stimulator cells. Transfusion 1989;48:1052-6.
30. Marrack P, Kappler J. T-cells can distinguish between allogeneic major histocompatability complex products on different cell types. Nature 1988;332:840-3.
31. Sirchia G, Rubulla P, Mascaretti L, et al. The clinical importance of leukocyte depletion in regular erythrocyte transfusion. Vox Sang 1986;51 (suppl 1):2-8.
32. Schiffer CA. Prevention of alloimmunization against platelets. Blood 1991;77:1-4.
33. Nemo GJ, McCurdy PR. Prevention of platelet alloimmunization. Transfusion 1991;31:584-5.
34. Schiffer CA, Dutcher JP, Aisner J, Hogge D, Wiernik PH, Reilly JP. Randomized trial of leukocyte-depleted platelet transfusions to modify allo-immunization in patients with leukemia. Blood 1983;62:815-20.
35. Murphy MF, Metcalfe P, Thomas H, et al. Use of leukocyte-poor blood components and HLA-matched-platelet donors to prevent HLA alloimmunization. Brit J Haematol 1986;62:529-34.
36. Eernisse JG, Brand A. Prevention of platelet refractoriness due to HLA antibodies by administration of leukocyte-poor blood components. Exp Hematol 1981;9:77-83.
37. Andreu G, Dewailly J, Leberre C, et al. Prevention of HLA immunization with leukocyte-poor packed red cells and platelet concentrates obtained by filtration. Blood 1988;72:964-9.
38. Sniecinski I, O'Donnell MR, Nowicki B, Hill LR. Prevention of refractoriness and HLA-alloimmunization using filtered blood products. Blood 1988;71:1402-7.
39. Fisher M, Chapman JR, Ting A, Morris PJ. Alloimmunization to HLA antigens following transfusion with leukocyte-poor and purified platelet suspensions. Vox Sang 1985;49:331-5.
40. Myllylä G, Ruutu T, Kekokmaki R, Oksanen L, Rasi V. Prevention of HLA-alloimmunization by using leukocyte-free blood components. Abs XX Cong Int Soc Blood Transf, London 1988:203.
41. Saarinen UM, Kekokmaki R, Siimes MA, Myllylä G. Effective prophylaxis against platelet refractoriness in multitransfused patients: Use of leukocyte-free blood components. Blood 1990;75:512-7.
42. Kooy MvM, Prooijen HCv, Moes M, Bosma-Stants I, Akkerman JWN. Use of leukocyte-depleted platelet concentrates for the prevention of refractoriness and primary HLA alloimmunization: A prospective randomized trial. Blood 1991;77:201-5.
43. Handa M, Ikeda Y, Kurata Y, et al. Clinical examination of the effectiveness of platelets transfusion using a leukocyte-depleted filter. Jap J Transf Med 1991;36:752-6 (in Japanese).
44. Oksanen K, Kekokmaki R, Ruutu T, Koskimies S, Myllylä G. Prevention of alloimmunization in patients with acute leukemia by use of white cell-reduced blood components – randomized trial. Transfusion 1991;31:588-94.
45. Braine HG, Meryman HT, Fuller AK, et al. Alloimmunization in bone marrow transplant patients receiving red cells and platelets with very low residual leukocyte counts (submitted for publication).

46. Polesky HF. The Emily Cooley Lecture: Leukocyte-poor blood, a study in the evolution of component therapy. In: Seminar on Blood Components. American Association of Blood Banks, Washington DC 1977:53-73.
47. Meryman HT. The cryopreservation of blood cells for clinical use. In: Brown EB (ed). Progress in Hematology. Vol XI. Grune and Stratton, New York 1979:193-227.
48. Opelz G, Terasaki PI. Poor kidney transplant survival in recipients with frozen blood transfusions or no transfusions. Lancet 1974;ii:696-701.
49. Betuel H, Cantarovitch D, Robert F, et al. Platelet transfusions preparative for kidney transplantation. Transplant Proc 1985;17:2235-7.
50. Pallardo LM, Montoro J, Moll Jl, et al. Platelet transfusions do not improve cadaveric renal allograft survival. Transplant Proc 1985;17:2338-9.
51. Foster S, Wood RJ, Morris PJ. Suppression of renal allograft rejection in the rat using purified plasma membrane preparations expressing donor class I major histocompatibility antigens. Int Congr Transplant Soc, Helsinki 1986:74 (abstract).
52. Faustman D, Lacy P, Davie J, et al. Prevention of allograft rejection by immunization with donor blood depleted of 1a-bearing cells. Science 1982;217:157-8.
53. Wood KJ, Evins J, Morris PJ. Suppression of renal allograft rejection in the rat by class I antigens on purified erythrocytes. Transplantation 1984;39:56-62.
54. Gores PF, Sitges-Serra A, Hesse UJ, et al. Transfusion of donor class I MHC antigen prior to kidney transplantation in dogs. Transplantation 1986;41:648-50.
55. Bijnen AB, Heineman E, Marquet RL, et al. Lack of beneficial effect of thrombocyte transfusions on kidney graft survival in dogs. Transplantation 1984;37:213-4.
56. Oh JH, McClure HM. Platelet-induced immunosuppression in Rhesus monkeys. Transplant Proc 1985;17:1113-5.
57. Oh JH, McClure HM. Nonspecific immunosuppressive effect of platelet transfusions in Rhesus monkeys. Transplantion 1987;43:304-5.
58. Wood ML, Okazaki H, Monaco AP. The effects of blood transfusions on the immune response. Transplant Proc 1988;20:1200-3.
59. Lenhard V, Mytilineos J, Hansen B, et al. Immunoregulation after blood transfusion in the rat model – anti-idiotypic antibodies or suppressor cells? Transplant Proc 1985; 17:2393-6.
60. Champagne E, Chapuis F, Klatzman D, et al. Allosensitization-induced suppressor cells in humans. Transplant Proc 1985;17:2397-2400.
61. Terasaki PI. The beneficial transfusion effect on kidney graft survival attributed to clonal delation. Transplantation 1984;37:119-25.
62. Singal DP, Fagnilli L, Joseph S. Blood transfusion induces anti-idiotypic antibodies in renal transplant patients. Transplant Proc 1983;15:1005-8.
63. Singal DP, Ludwin D, Joseph S, et al. The blood-transfusion effect: Characterization of T-cell alloantigen-specific receptors by sera from transfused mice. Transplant Proc 1987;19:1429-31.
64. Burlingame WJ, Sollinger HW. Anti-idiotypes and transfusion. Transplant Proc 1988;20:1197-9.
65. Lenhard V, Gemsa D, Opelz G. Tranfusion-induced release of prostaglandin E_2 and its role in the activation of T suppressor cells. Transplant Proc 1985;17:2380-2.
66. Roy R, Lanchane JG, Beaudoin R, et al. Prostaglandin-dependent suppressor factor induced following 1-5 blood transfusions: Role in kidney graft outcome. Transplant Proc 1985;17:2383-6.
67. Kotlan B, Gyodi E, Szabo T, et al. Comparative study of alloimmune reactions induced by leukocyte and platelet transfusions in humans. Characteristic changes of activation markers, gamma interferon, and FcR blocking antibody production. Human Immunol 1988;22:19-29.
68. Salvaterria O, Melzer J, Potter D, et al. A seven-year experience with donor-specific blood transfusions. Transplantation 1985;40:654-9.

69. Mincheff MS, Meryman HT. Blood transfusion and immunomodulation. A possible mechanism. Vox Sang 1993;(in press).
70. Schwartz RH. Acquisition of immunologic self tolerance. Cell 1989;57:1073-81.
71. Jenkins MK, Schwartz RH. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. J Exp Med 1987;165:301-19.
72. Nossal GJV. Immunologic tolerance: Collaboration between antigen and lymphokines. Science 1989;245:147-53.
73. Opelz G, Terasaki PI. Improvement of kidney graft survival with increased numbers of blood transfusion. N Engl J Med 1978;299:799-803.
74. Light JA, Metz S, Oddenino K, et al. Fresh versus stored blood in donor specific transfusion. Transplant Proc 1982;14:296-301.

POTENTIAL INVOLVEMENT OF FREE RADICAL REACTIONS IN INACTIVATING ANTIGEN PRESENTING CELLS BY ULTRAVIOLET-B IRRADIATION

T.A. Takahashi[1], K. Nakai[1], Y. Mogi[1], M. Hosoda[1], C. Yakushiji[1], J. Akasaka[1], N. Kamo[2], N. Kuwabara[3], S. Sekiguchi[1]

Introduction

Repeated transfusions of platelet concentrates (PC) lead to alloimmunization and platelet refractoriness was subsequently observed in about 40% of recipients [1]. Contaminating leukocytes in platelet concentrates are known to play an essential role in alloimmunization. Prevention of alloimmunization by reducing the number of leukocytes contaminating PC has been achieved using leukocyte removing filters with encouraging results [2]. However, introduction of leukocyte removing filters into clinical transfusion meets with some problems such as complexity in filtration procedures, increase in cost, and loss of platelets during filtration.

Recently, ultraviolet-B (UV -B) irradiation onto PC has been tried to prevent alloimmunization [3,4]. Inactivation of leukocytes by UV-B irradiation is a complex phenomenon [5,6]. Membrane markers affected by UV-B irradiation include class II major histocompatibility complex [7], ICAM-1 [8], and CD14 [Andeu, 1992], which are important molecules responsible for cell adhesion and antigen presentation. Interleukin-1 (IL-1) production is severely reduced by the irradation of UV-B [9]. But, the addition of exogenous IL-1 is not sufficient to restore the cell function. Expression of membrane IL-1 by rat macrophages is also inhibited by UV-B irradiation [10]. UV irradiation induces disruption of intracellular calcium metabolism [11], and overall protein synthesis [12]. However, the mechanisms by which UV-B irradiation induces the above phenomena is poorly understood.

In the present study, the hypothesis that free radical reactions generated in human peripheral blood mononuclear cells by irradiation of UV-B may be involved in the inactivation of these cells is examined. By developing a new assay method for the detection of hydrogen peroxide, the dehydrorhodamine 123 method, we show that UV-B irradiation generated hydrogen peroxide, concomitant with production of lipid peroxide and consumption of endogenous anti-

1. Hokkaido Red Cross Blood Center.
2. Faculty of Pharmaceutical Sciences, Hokkaido University.
3. Hokkaido University School of Veterinary Medicine, Sapporo, Japan.

oxidants. Furthermore, exogenously provided free radicals could induce the inhibition of mixed lymphocyte reactions (MLR) and cap formation, and the augmentation in intracellular free calcium concentration ($[Ca^{2+}]i$), which are the distinctive phenomena in the irradiation of UV-B. In conclusion, free radical reactions are apparently induced by irradiation of UV-B, and may be involved in inactivating antigen presenting cells.

Materials and methods

Isolation of leukocytes from human peripheral blood – Buffy coats were prepared from whole blood of healthy human donors. Mononuclear cells were obtained by a procedure in which buffy coat was subjected to Ficoll-Paque (Pharmacia, Piscataway, NJ) density sedimentation and then washed twice with $Ca^{2+}Mg^{2+}$ free Hanks' balanced-salt solution (HBSS(-)). If necessary, contaminated red blood cells were lysed by a hypotonic shock treatment. Monocytes and lymphocytes were purified from mononuclear cell fraction using a counterflow centrifugal elutriation method (J2-21M/E and JE-6 rotor, Beckman, Fullerton, CA) described previously [13].

Ultraviolet and gamma-ray irradiation – Mononuclear cells (1×10^7 cells/ml) were irradiated either with 2 to 100 Gy from a ^{60}Co source at 4°C or various doses of UV-B having a peak intensity at 306 nm (FL15E UV-320, NIS) at room temperature. Cells were suspended in HBSS(-) otherwise noted, and plated in a disposable polypropylene dish or tube. The UV irradiation density was measured by UVX DIGITAL RADIOMETER (UVP Inc., San Gabriel, CA). UV-B irradiation was also performed in the presence of superoxide dismutase (SOD, 30 U/ml at final concentration, Sigma) as a superoxide scavenger, catalase (100 U/ml, Sigma) and peroxidase (100 U/ml, Sigma) as hydroxy peroxide scavengers, and dimethylsulfoxide (DMSO, 100 mM) as a hydroxy radical scavenger.

UV-B of platelet concentrates and platelet function tests – PC obtained by platelet apheresis were transferred to a Stericell bag (Dupont, Wilmington, DE) designed for UV irradiation. After the irradiation was performed with a UV-B irradiation (Platelet Treatment System, Haemonetics, Braintree, MA), mononuclear cells in the platelet concentrates were isolated by a Ficoll-Paque gradient centrifugation and examined in MLR. Hypotonic shock response (HSR) and platelet aggregability were tested as described previously [14]. Platelet aggregation studies were performed with 200 µl of PC in a platelet aggregometer (HEMA TRACER 801, Nikko Bioscience, Tokyo). Aggregation inducer used was a pair of ADP (5 µM, Boehringer, Manheim, Germany) and collagen (1 µg/ml, Hormon-Chemie, München, Germany).

Mixed lymphocyte reaction – After mononuclear cells were treated with irradiation or exogenously provided free radicals, they were washed with HBSS(-), resuspended in RPMI 1640 at 2×10^6 cells/ml, and used as the stimulator. All stimulator cells were treated with mytomycin C (MMC, 25 µg/ml) for 30 min at 37°C prior to MLR. The responder cells were prepared from another

donor at 2×10^6cells/ml in RPMI 1640 containing 20% AB serum. Triplicate cultures (200 μl/well) were established in 96-well microtiter trays. After 6 days of culture at 37°C in a 5% CO_2 humidified atmosphere, each well was pulsed with 0.5 μCi [^{3}H]thymidine (Dupont, Wilmington, DE) and incubated for a further 24 h. Incorporated [^{3}H] was measured by a scintillation counter (BECKMAN LS 5000 TD, Beckman, Fullerton, CA).

Treatment of cells with exogenously provided free radicals – Mononuclear cells (1×10^7 cells/ml) were treated with exogenous free radicals, superoxide and singlet oxygen. Cells were incubated at 37°C for 10 min with a superoxide generating system consisting of xanthine oxidase (0.4 U/ml, Sigma) and xanthine (200 μM, Sigma). Singlet oxygen was produced by methylene blue at 50 μM with a irradiation of visible light for 15 min, in which singlet oxygen is produced intra- and extracellularly as methylene blue is permeable to the cell membrane.

Detection of free radicals in UV-B irradiated cells – Hydrogen peroxide was detected with the dehydrorhodamine 123 method. After monocytes and lymphocytes were treated with irradiation of UV-B, they were incubated with 6 μM of dehydrorhodamine 123 (DHR 123, Sigma) and 6.4 U/ml of horseradish peroxidase (Sigma) for 30 min at 37°C. DHR 123 was oxidized by hydrogen peroxide to fluorescent rhodamine 123 (R 123) in the presence of peroxidase, and the fluorometric intensity of R 123 accumulated in cells was determined by flow cytometry (Ortho Cytron, Ortho Diagnostic Systems, Tokyo). A preliminary study indicates that the molar ratio in the reaction between DHR 123 and hydrogen peroxide is to be 1:1.

The generation of superoxide by UV-B irradiation was determined by the cytochrome c reduction method [15].

Cap formation assay – Mononuclear cells treated either with UV-B, gamma-ray, or exogenously provided free radicals, were washed twice and resuspended in phosphate buffer saline, pH 7.4 (PBS(-)). Cell suspension was incubated with an equal volume of monoclonal anti-HLA-DR antibody (HU-4, a generous gift of Dr. A Wakisaka, Hokkaido University School of Medicine, Japan) at 37°C for 30 min. The incubation was also carried out at 0°C as the negative control. After cells were washed and resuspended in PBS(-) again, an equal volume of FITC-conjugated monoclonal anti-mouse IgG antibody (Ortho Diagnostic Systems) at 37°C for 1 h. The negative control was incubated at 0°C. cap formation was detected with a fluorescent microscope, after cells were washed twice with PBS(-) containing 30 mM NaN_3 and then resuspended in 20 μl of PBS(-) containing 50% (v/v) of glycerol.

Intracellular calcium concentration measurement – Monocytes were adhered onto a glass dish by a procedure in which mononuclear cells were incubated on the dish at 37°C for 30 min and unadhered cells were washed out with HBSS(-). Fura 2/AM, in a final concentration of 10 μM of the acetoxymethylester form, was loaded in the presence of 0.03% (v/v) Pluronic F127 at 37°C for 30 min. The concentration of extracellular Fura 2/AM was reduced to 2 M, and then allowed to incubate at 37°C for 30 min again. After cells were washed twice

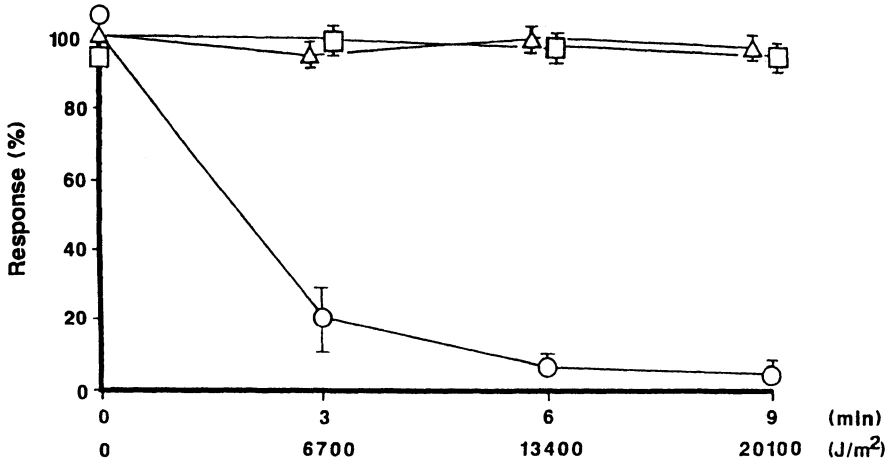


Figure 1. Effects of UV-B irradiation on MLR and platelet functions. Platelet concentrates were irradiated, and in vitro platelet functions (Δ HSR, ❑ aggregation response to ADP + collagen) and MLR (O) were measured. Mean ± SD, n=4.

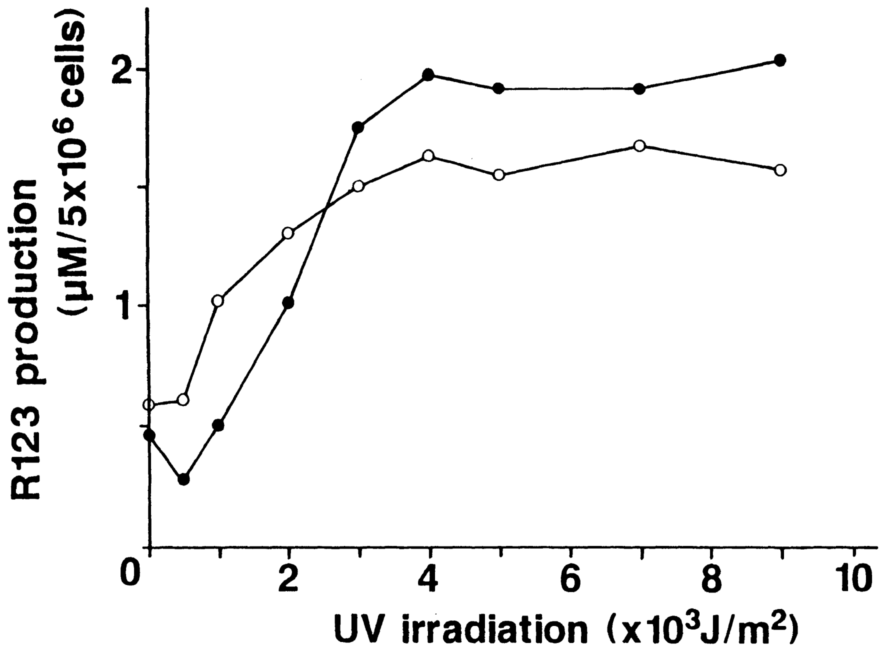


Figure 2. Production of fluorescent rhodamine 123 in lymphocytes (●) and monocytes (O) by UV-B irradiation.

with HBSS(-), changes in $[Ca^{2+}]i$ were monitored by fluorescence mycroscopy coupled to video-imaging analyzing computer system (CASALS, Olympus Optical CO. Ltd., Tokyo). To obtain irradiation-dependent changes, cells were irradiated by UV-B, and successively $[Ca^{2+}]i$ was measured with turning off the UV source. After $[Ca^{2+}]i$ measurement was completed, UV-B irradiation was restarted. In the presence of 20 mM EGTA, the same experiment was carried out.

Lipid peroxide assays and measurements of α-tocopherol and sulfhydryl group – Mononuclear cells at $1\text{-}5\times10^7$ cells/ml in PBS(-) were irradiated by UV-B, and the following assays were performed. Lipid peroxidation was estimated by measuring thiobarbituric acid reacting substanc (TBARS). After the irradiation, cells were centrifuged at 200×g for 5 min at 4°C. TBARS was assayed in the supernatant and the washed cells. The addition of α-tocopherol analog, 2,2,5,7,8,-pentamethyl-6-hydroxychroman (50 μM, Wako Pure Chemical, Tokyo) was examined. Phosphatidylcholine hydroperoxide was measured by a chemiluminescence-high performance liquid chromatography system [16]. The degree of peroxidation was expressed by the ratio of integrated chemiluminescence counts to phosphatidylcholine absorbance at 210 nm. α-Tocopherol in cells was measured with a high-performance liquid chromatography by an electrochemical detection. Total sulfhydryl group was assayed after whole cells were solubilized in the presence of 5% of sodium dodecyl sulfate. The observed value represents total thiols including glutathione and protein thiols when membrane and proteins are solubilized. Protein concentration was determined by Lowry method using bovine serum albumin as standard.

Results

UV-B irradiated platelet functions – Figure 1 shows the results of in vitro functions of UV-B irradiated platelets as measured by HSR and aggregability. Contrary to the complete inhibition of MLR, platelet functions were well preserved at any UV-B dose tested.

Detections of free radicals in UV-B irradiated cells – Purified lymphocytes and monocytes were irradiated with UV-B. First, the DHR 123 method was caried out. The fluorescent intensity of both cells increased as shown in Figure 2, indicating the generation of hydrogen peroxide. When the cytochrome c reduction method was applied, the sign of the generation of superoxide was also obtained (data not shown).

Effects of exogenous free radicals and radical scavengers – Exogenously provided free radicals (superoxide and singlet oxygen) inhibited MLR completely as shown in Figure 3, and cap formation partially as shown in Figure 4. However, inhibitory effects of gamma-ray irradiation on MLR and cap formation were not observed or, very small. Four radical scavengers were examined whether they could prevent the inhibition of MLR (Figure 5) and cap formation (Figure 6). Three of four scavengers (SOD, catalase, and peroxidase) remain extracellularly, and DMSO is permeable to cell membrane. All scavengers

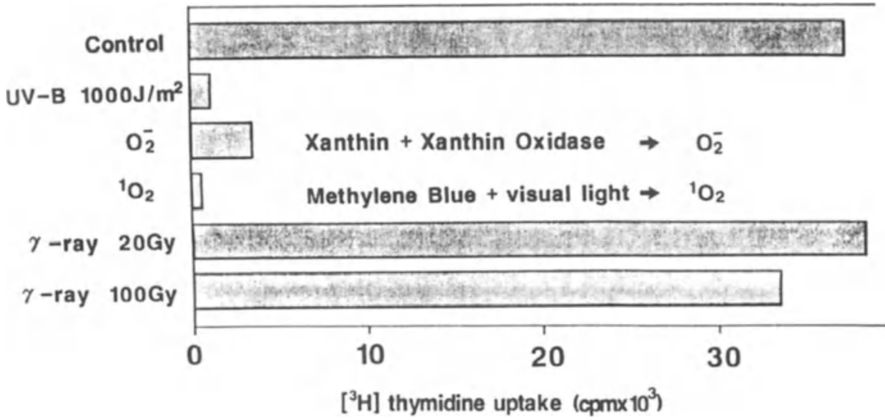


Figure 3. Effects of UV-B, gamma-ray irradiation, and exogenous free radicals on MLR. Superoxide (O_2^-) and singlet oxygen ($^1O_2$) were produced exogenously as described in Materials and Methods.

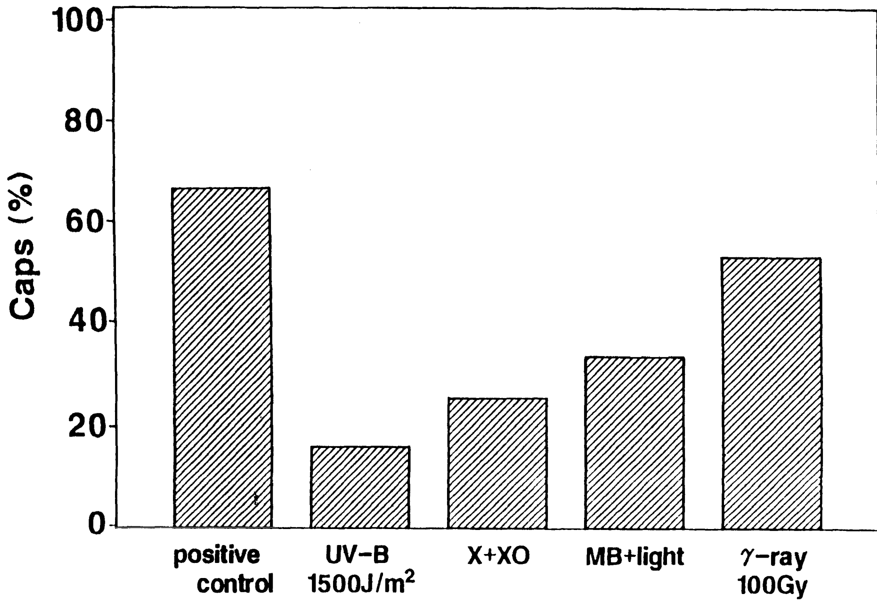


Figure 4. Effects of UV-B, gamma-ray irradiation, and exogenous free radicals on monoclonal anti-HLA-DR antibody induced cap formation. X: xanthine, XO: xanthine oxidase, and MB: methylene blue.

tested could not prevent the UV-B induced inhibition of MLR and cap formation.

[Ca^{2+}]i mobilization induced by UV-B irradiation – Changes in [Ca^{2+}]i were monitored during the irradiation of UV-B as shown in Figure 7a. The UV source was turned off during [Ca^{2+}]i assay, and the irradiation was restarted immediately after the assay was completed. The same experiment was carried out in the presence of 20 mM EGTA (Figure 7b). UV-B irradiation increased [Ca^{2+}]i during the irradiation, and this tendency was continued after the irradiation was stopped. EGTA blocked the increase in [Ca^{2+}]i by UV-B irradiation completely.

We then used the two specific calcium channel blockers, verapamil and nifedipine, to examine the role of calcium channels in UV-B irradiation inducible [Ca^{2+}]i mobilization. As shown in Figure 8, all calcium channel blockers were non-effective.

Lipid peroxidation and changes in endogenous antioxidants – Lipid peroxidation due to UV-B irradiation was evaluated by measuring the formation of TBARS (Figure 9) and phosphatidylcholine hydroperoxide (Table 1). In agreement with other reports [17,18], TBARS was increased as shown. This TBARS liberation was significantly inhibited by the addition of α-tocopherol analog. TBARS was also accumulated in cells by UV-B irradiation (data not shown). Although the specificity of TBARS assay to lipid peroxidation is questioned [19], we demonstrated the generation of phosphatidylcholine hydroperoxide *per se* at lower doses than that at which TBARS liberation was significant. In parallel experiments, endogenous α-tocopherol and total sulfhydryl group were decreased as shown in Table 1 and Figure 10, respectively.

Table 1. Formation of phosphatidylcholine hydroperoxide and consumption of α-tocopherol in cells due to UV-irradiation.

Dose J/m^2	**PCOOH/PC ratio of integrated count***	**α-tocopherol μg/mg of protein**
0	0.345± 0.13	112 ± 0.1
3800	0.442± 0.04	109 ± 9.4
7500	0.984± 0.12	100 ± 5.5
90000	2.260± 0.60	42.0± 7.1

* Integrated chemiluminescence count of phosphatidylcholine hydroperoxide (PCOOH) and integrated UV absorbance of phosphatidylcholin (PC) were obtained, and the ratio of PCOOH/PC was calculated. N=5, mean ± SE.

Discussion

In the present study, platelet concentrates can be irradiated with UV-B such that MLR are abolished while no detriment to in vitro platelet function was observed as measured by HSR and aggregation response to the synergistic stimulation of adenosine diphosphate and collagen, even if the dose of UV-B irradiation was

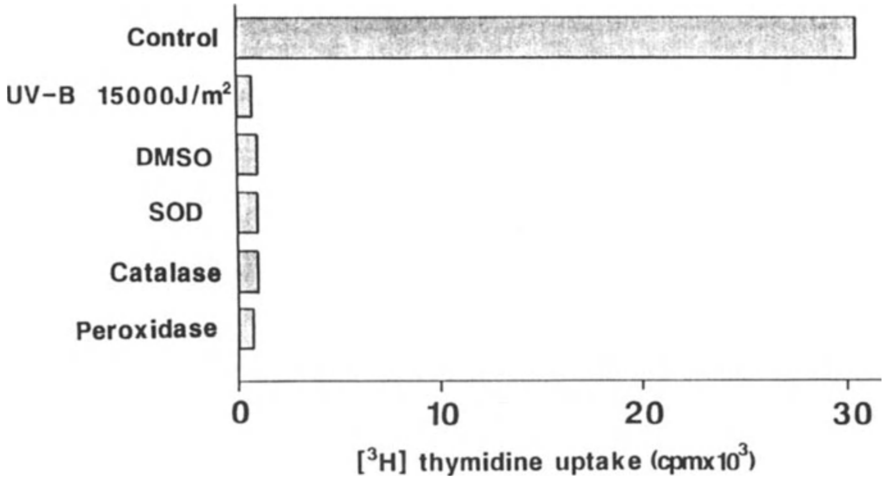


Figure 5. Effects of free radical scavengers on UV-B induced inhibition of MLR. Mononuclear cells were irradiated with UV-B in the presence of scavenger and examined in MLR.

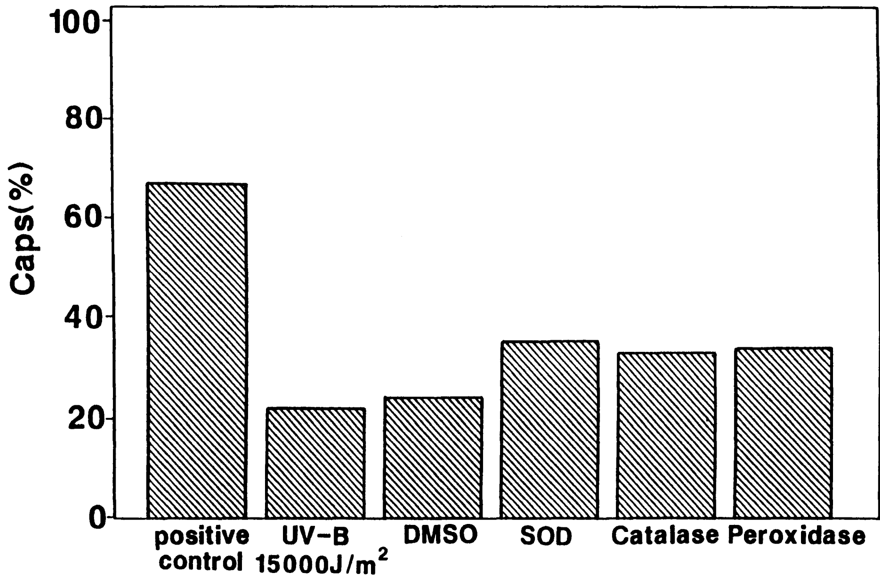


Figure 6. Effect of free radical scavengers on UV-B induced inhibition of monoclonal anti-HLA-DR antibody induced cap formation. Mononuclear cells were irradiated in the presence of scavengers and cap formation assay was carried out.

20,000 J/m^2 (Figure 1). The experiments studying in vivo platelet functions after UV-B irradiation conclude that platelet concentrates can be safely irradiated by UV-B, with satisfactory recovery, survival, and functions [20].

In vitro UV-B irradiation of human mononuclear cells inhibits their accessory cell funtions [2,21]. UV-B irradiation profoundly affects cellular constituents responsible for antigen presentation. However, the exact mechanism by which UV-B light mediates its effects has remained unclear. In the present study, we hypothesize that UV-B irradiation induces free radical reactions in mononuclear cells including monocytes, and then free radicals degrade membrane-associated and cytosolic constituents such as class II HLA, adhesion molecules, receptors, and other proteins (Figure 11). UV-B, from 280 nm to 320 nm, can be absorbed by several kinds of endogenous photosensitizers such as NADH [22] and tryptophan, and possibly generate active oxygen radicals in the presence of molecular oxygen. Singlet oxygen and superoxide are the candidates generated by UV-B irradiation. Singlet oxygen probably attacks cellular constituents directly. However, the detection of singlet oxygen is very difficult due to its very short life-time. On the other hand, superoxide can not attack directly because of its poor reactivity. Preferably, superoxide is converted to hydrogen peroxide by disproportion with proton. We have demonstrated that hydrogen peroxide is generated by the treatment of cells with UV-B irradiation by the DHR 123 method. As the assay can detect hydrogen peroxide diffused into the extracellular medium, more hydrogen peroxide seems to be generated inside the cells. Hydrogen peroxide as the product of superoxide will be further converted to metal complex radicals by the reaction with transmission metal elements such as iron, and then may degrade cellular constituents.

The generation of free radicals by UV-B irradiation is supported by the evidence that lipid peroxide was formed in UV-B irradiated cells. TBARS was detected in the medium (Figure 9). Furthermore, phosphatidylcholine hydroperoxide was also demonstrated in cells (Table 1). Concomitantly with lipid peroxide formation, endogenous antioxidants such as α-tocopherol and sulfhydryl group (Table 1 and Figure 10) were decreased. These antioxidants are thought to be consumed by the reaction with free radicals.

The significances of free radical reactions in inactivating leukocytes was examined by the treatment of cells with exogenously provided free radicals. All free radicals tested inhibited MLR and anti-HLA-DR antibody induced cap formation (Figures 3 and 4). Next, several radical scavengers were tested in preventing the inhibition of MLR and cap formation (Figures 5 and 6). DMSO is an effective scavenger for hydroxy radical, SOD for singlet oxygen, and catalase and peroxidase for hydrogen peroxide. Except for DMSO which is permeable to the cell membrane, scavengers remain in the extracellular medium. The results were that all scavengers were non-effective. The possible explanation is that the free radical reactions occurred inside the cells and are essential for MLR inhibition. In the case of the experiment using DMSO, the finding suggests that hydroxy radical is not generated in irradiation of UV-B. Another possibility is

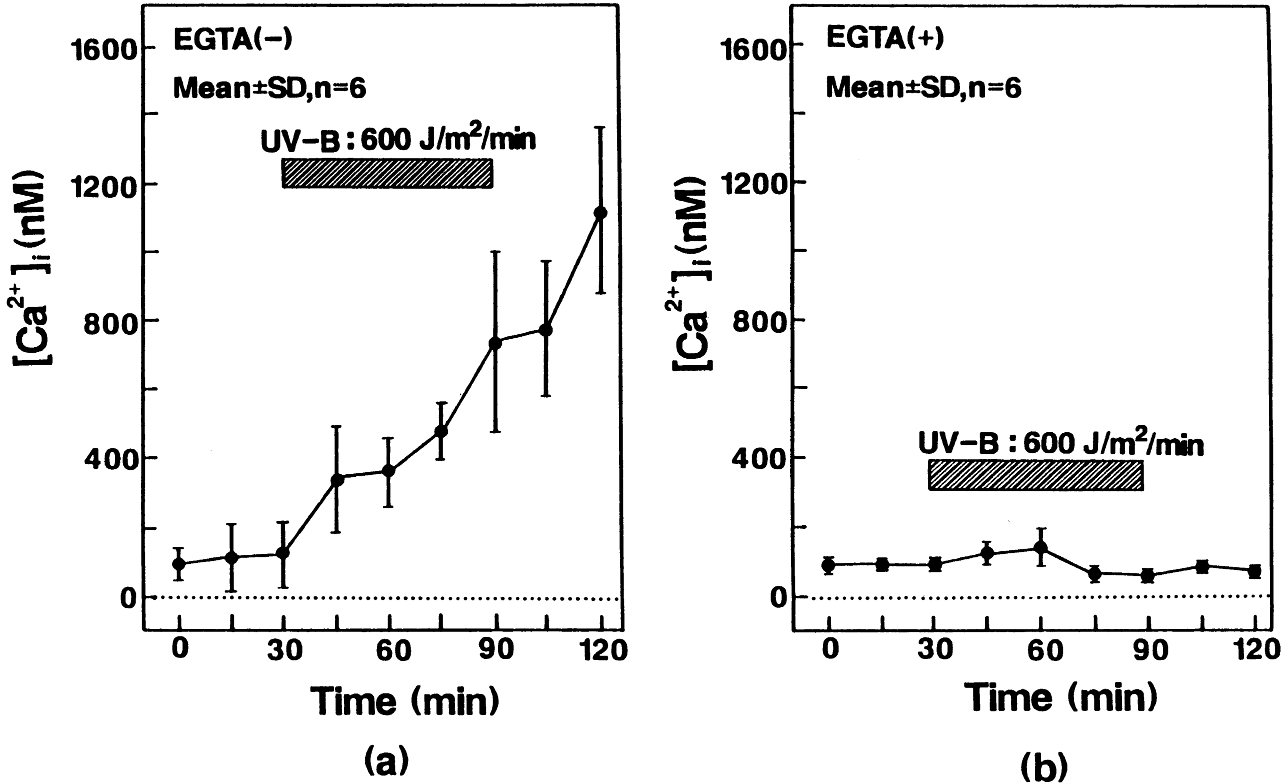


Figure 7. Increase in intracellular free Ca^{2+} level of monocytes by UV-B irradiation. The experiments were carried out in the absence (a) and presence (b) of 20 mM EGTA.

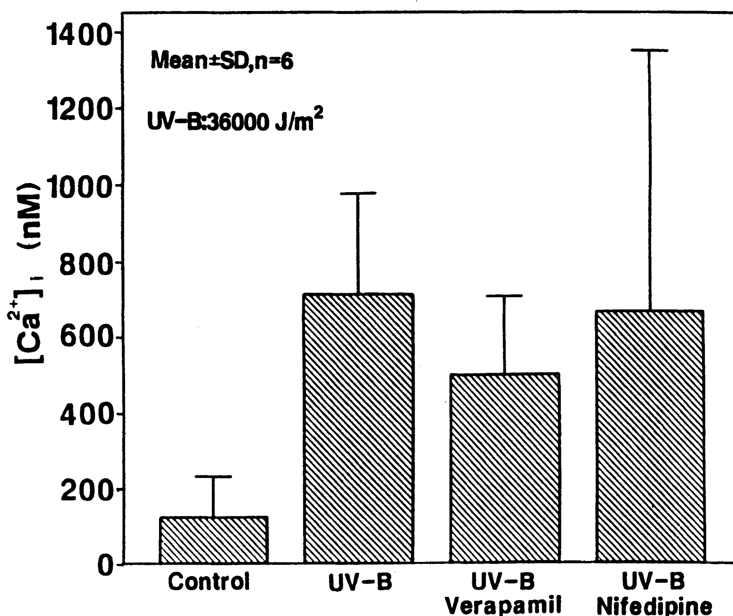


Figure 8. Effect of calcium channel blockers on UV-B induced augmentation in $[Ca^{2+}]i$. Monocytes were irradiated in the presence of a blocker. Mean ± SD, n=6.

that hydroxy radical does not work in inactivating leukocytes, even if hydroxy radical was generated by the irradiation of UV-B.

Gamma-ray irradiation is shown to damage cellular functions [23] and to prevent the occurrence of transfusion-associated graft-versus-host disease (GvHD) [24]. MLR is inhibited if the responder cell is irradiated by gamma-ray [25]. However, if the stimulator cell is treated in the model of alloimmunization, MLR can not inhibited as shown in the present study and reviewed by others [20]. We show that cap formation was scarcely affected by gamma-ray irradiation, but completely inhibited by UV-B. Although no $[Ca^{2+}]i$ augmentation is induced by gamma-ray irradiation [11], UV-B irradiation did induce. Considering that the major free radical generated by gamma-ray radiation is hydroxy radical, these findings suggest that superoxide and/or singlet oxygen is conceivably involved in leukocyte inactivation, but contribution of hydroxy radical is little.

$[Ca^{2+}]i$ plays an essential role in the regulation of various cellular functions. Polymerization and depolymerization of cytoskeleton proteins, which are responsible for the mobility of membrane-associated receptors and ligand inducible cap formation [26,27], are profoundly affected by $[Ca^{2+}]i$ mobilization. Using fluorescence microscopy coupled to digital video imaging, we demonstrated that UV-B irradiation increases $[Ca^{2+}]i$ influx (Figure 7a). Although the detailed mechanism is still unclear, the inhibition of cap formation by UV-B irradiation is likely to relate to the abnormal $[Ca^{2+}]i$ mobilization.

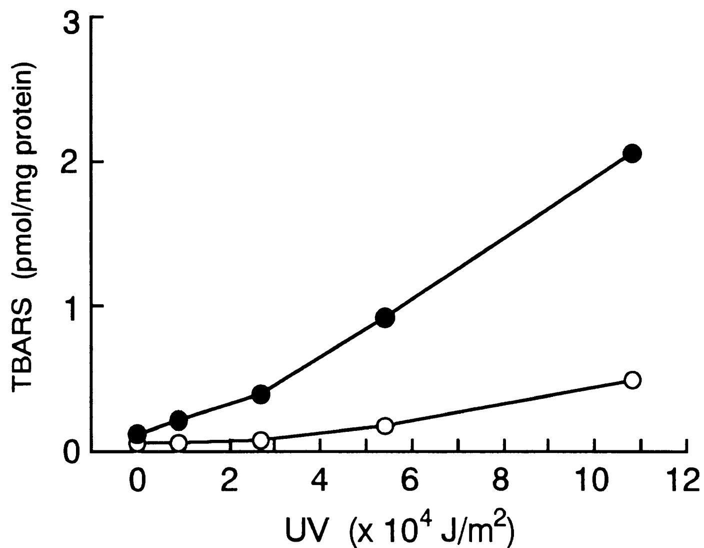


Figure 9. TBARS liberation from UV-B irradiated cells. Mononuclear cells were irradiated and centrifuged. TBARS in the supernatant was measured in the absence (●) and presence (○) of α-tocopherol analog. n=3.

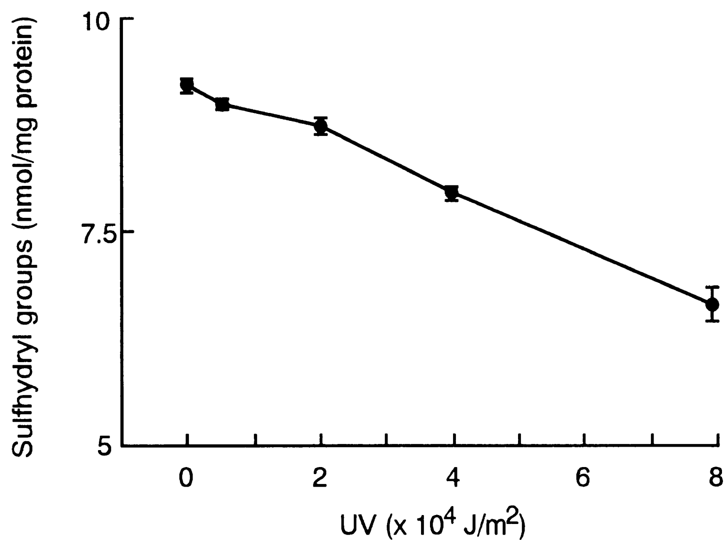


Figure 10. Changes in total sulfhydryl group in UV-B irradiated cells. After the irradiation, mononuclear cells were solubilized and the sulfhydryl group assay was carried out. Mean ± SE, n=10.

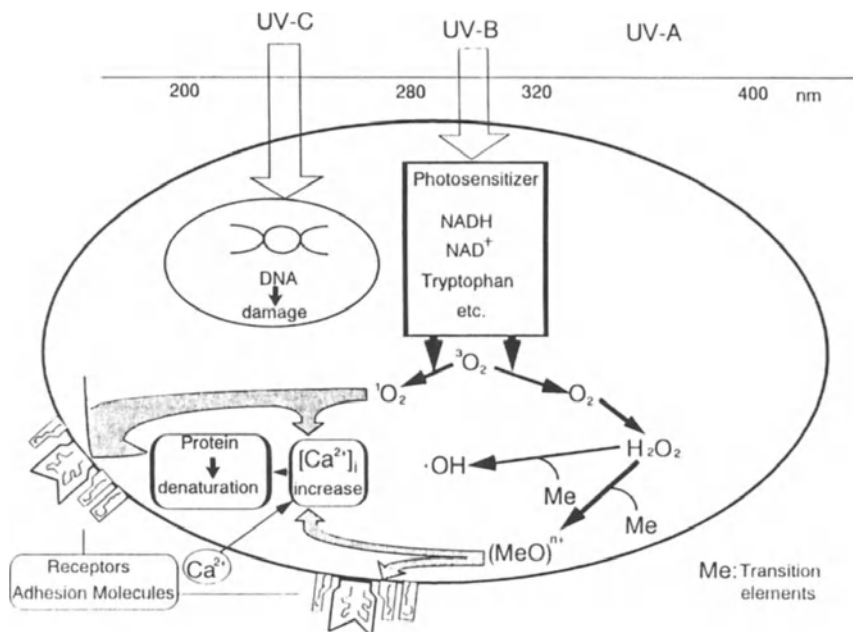


Figure 11. Proposed mechanism of leukocyte inactivation by UV-B induced free radical reactions.

Experiments were then performed to clarify the mechanism by which $[Ca^{2+}]i$ was increased. If extracellular calcium was buffered by the addition of EGTA, no calcium mobilization occurred (Figure 7b). This indicates that the augmentation in $[Ca^{2+}]i$ is due to the abnormal influx. Next, calcium channel blockers were used to examine the contribution of calcium channels (Figure 8). UV-B induced Ca^{2+} immobilization was not inhibited by any blocker. Recently, other investigators have found that UV-inducible Ca^{2+} influx is mediated through Ca^{2+} channels in lymphocytes by using Ca^{2+} channel blockers such as nifedipine [11]. The reason for the discrepancy between their results and ours is not clear; one possibility is that we irradiated cells at higher intensity and tried to observe the changes in $[Ca^{2+}]i$ during the irradiation, on the other hand, they detected the augmentation in $[Ca^{2+}]i$ 2-3 h after the irradiation at 200-500J/m^2, but could not within 1 h. At higher irradiation intensity, not only calcium channel but also other elements sensitive to UV-B irradiation might be disrupted.

In summary, this study provides evidences that free radical reactions are generated due to the irradiation of UV-B onto mononuclear cells, in parallel to the formation of lipid peroxide, decreases in endogenous anti-oxidants, and abnormal $[Ca^{2+}]i$ mobilization. In addition, exogenously provided free radicals (superoxide and singlet oxygen) can induce the inhibition of MLR and cap formation, which are characteristic phenomena to UV-B irradiation. These results suggest that free radicals are involved in inactivating leukocytes by UV-B irradiation.

References

1. Yankee RA, Grumet FC, Roquentine GN. Platelet transfusion therapy. The selection of compatible platelet donors for refractory patients by lymphocyte HLA typing. N Engl J Med 1969;281:1208-12.
2. Andreu G, Dewailly J, Leberre C, et al. Prevention of HLA immunization with leukocyte-poor packed red cells and platelet concentrates obtained by filtration. Blood 1988;72:964-9.
3. Slichter SJ, Deeg HJ, Kennedy M. Prevention of platelet alloimmunization in dogs with systemic cyclosporin and by UV-irradiation of cyclosporin-loading of donor platelets. Blood 1987;69:414-8.
4. Andreu G, Boccaccio C, Lecrubier C, et al. Ultraviolet irradiation of platelet concentrates: Feasibility in transfusion practice. Transfusion 1990:30:401-6.
5. Pamphilon DH, Alnagdy AA, Wallington TB. Immunology by ultraviolet light: Clinical studies and biological effects. Immunol Today 1991;12:119-23.
6. Slichter SJ. UV irradiation. Effects on the immune system and on platelet function, viability, and alloimmunization. In: Smit Sibinga CTh, Kater L (eds). Advances in haemapheresis. Kluwer Academic Publishers, Dordrecht 1992:207-28.
7. Aberer WG, Schuler G, Stingl G, Hönigsmann H, Wolff K. Ultraviolet light depletes surface markers of Langerhans cells. J Invest Dermatol 1981;76:202-10.
8. Krutmann J, Khan IU, Wallis RS, et al. Cell membrane is a major locus for ultraviolet B-induced alterations in accessory cells. J Clin Invest 1991;85:1529-36.
9. Jakway JP, Shevach EM. Stimulation of T-cell activation by UV-treated, antigen-pulsed macrophages: Evidence for a requirement for antigen processing and interleukin-1 secretion. Cell immunol 1983;80:151-62.
10. Lange-Wantzin G, Rothlein R, Kahn J, Faanes RB. Effect of UV irradiation on expression of membrane IL-1 by rat macrophages. J Immunol 1987;138:3803-7.
11. Spielberg H, June CH, Blair OC, Nystrom-Rosander C, Cereb N, Deeg HJ. UV irradiation of lymphocytes triggers an increase in intracellular Ca^{2+} and prevents lectin stimulated Ca^{2+} mobilization: Evidence for UV- and Nifedipine-sensitive Ca^{2+} channels. Exp Hematol 1991;19:742-8.
12. Hertl M, Kaplan DR, Fayen JD, Panuska JR, Ellner JJ, Elmets CA. The accessory function of B lymphocytes is resistant to the adverse effects of UV radiation. Eur J Immunol 1991;21:291-7.
13. Takahashi T, Hirsh A, Erbe E, Williams RJ. Mechanism of cryoprotection by extracellular polymeric solutions. Biophys J 1988;54:509-18.
14. Sekiguchi S, Takahashi TA, Yamamoto S, et al. A new type of blood component collector: Plasma separation using gravity without any electrical devices. Vox Sang 1990;58:182-7.
15. Babior BM, Kipnes RS, Curnutt JJ. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. J Clin Invest 1973;52: 741-4.
16. Miyazawa T, Yasuda K, Gujimoto K, Kaneda T. Presence of phosphatidylcholine hydroperoxide in human plasma. J Biochem 1988;103:744-6.
17. Punnonen K, A P, Ahotupa M. Effects of ultraviolet A and B irradiation on lipid peroxidation and activity of the antioxidant enzymes in keratinocytes in culture. Photodermal Photoimmunol Photomed 1991;7:173-7.
18. Thomas PD, Poznasky MJ. A modified tetramethylbenzidine method for measuring lipid hydroperoxides. Anal Biochem 1990;188:228-32.
19. Andreu G, Boccaccio C, Klaren J, et al. The role of UV radiation in the prevention of human leukocyte antigen alloimmunization. Transf Med Rev 1992;3:212-24.
20. Deeg HJ. Ultraviolet irradiation in transplantation biology: Manipulation of immunity and immunogenicity. Transplantation 1988;45:845-51.

21. Peak JG, Peak MJ, MacCoss M. DNA breakage caused by 334-nm ultraviolet light is enhanced by naturally occurring nucleic acid components and nucleotide coenzymes. Photochem Photobiol 1984;39:713-6.
22. Anderson RE, Standefer JC, Scaletti JV. Radiosensitivity of defined populations of lymphocytes. Cell Immunol 1977;33:45-61.
23. Linden JV, Pisciotto PT. Transfusion-associated graft-versus-host disease and blood irradiation. Transf Med Rev 1992;2:116-23.
24. Valerius NH, Johansen KS, Nielson OS, Platz P, Rosenkvist J, Sørensen H. Effect of in vitro X-irradiation on lymphocyte and granulocyte function. Scand J Haematol 1981;27:9-18.
25. Gabbiani G, Chaponnier C, Zumbe A, Vassalli P. Actin and tubulin co-cap with surface immunoglobulines in mouse B lymphocytes. Nature 1977;269:697-8.
26. Nelson WJ, ColaCo CA, Lazarides E. Involvement of spectrin in cell-surface receptor capping in lymphocytes. Proc Natl Acad Sci USA 1983;80:1626-30.

ALLOIMMUNIZATION AND PLATELET TRANSFUSION REFRACTORINESS

A. Brand

Introduction

Since over 20 years platelet transfusions are established therapy allowing prolonged survival of patients with hematologic and hemato-oncologic diseases.

Soon after the introduction of platelet supportive care it was recognized that alloimmunization frequently occurred and was a major cause of platelet refractoriness [1]. The development of white cell depletion from red cell and platelet transfusions in the seventies and eighties, coinciding with more aggressive chemotherapy resulted in a significant reduction of alloimmunization (Table 1) [1-15]. However non-immunological refractoriness seems increasing. This may also be due to altered quality of platelet transfusions, subjected to filtration and prolonged storage procedures [16], transfused to and increasing population of severely sick patients [17]. For optimal management of platelet transfusion therapy it is necessary to consider which patients are at risk for the development of alloimmunization, the clinical condition of the patient and the quality of the platelets transfused (Table 2).

Immunological platelet refractoriness

Three defined polymorphic systems are expressed on platelets (Table 3). These are the HLA class I antigens, the ABO antigens and the platelet-specific (HPA) antigens. Besides, not-yet defined non-HLA antigens, possibly new HPA specificities, are present. Although ABO antigens can be responsible for a reduced post-transfusion platelet increment, the role of ABO antibodies in transfusion refractoriness is easily revealed by the practice of transfusion of ABO-compatible non-stored platelet transfusions in case of insufficient post-transfusion increments. Increasing problems however are arising distinguishing antibody-mediated refractoriness from clinical factors. Several in vitro tests for the detection of platelet antibodies are available these days, the results are often confusing and are a reason for further extension of serological assays.

Therefore it is important to realize which antigens and which immunological factors contribute to alloimmunization and, when present, allow the selection of compatible donors improving the results of platelet transfusion therapy.

Tabel 1. Comparison of HLA alloimmunization by standard and leukocyte-depleted platelet transfusions.

Investigations	**Number of patients/ disease**	**Alloantibodies to platelets**	**Refractoriness diagnosis**
Grumet and Yankee [1] (1970)	7/AA		100%
Tejada et al. [2] (1973)	9/ANLL	100%	
Schiffer et al. [3] (1976)	60/ANLL	50%	
Howard and Perkins [4] (1978)	63/ANLL	50%	
Dutcher [5] (1981)	106/ANLL	42%	
Holohan et al [6] (1981)	108/cancer*	30%	
Eernisse and Brand [7](1981)	28/AA/ANLL 68/AA/ANLL**	72% 28%	93% 24%
Ford et al. [8] (1982)	23/ANLL		74%
Pegels et al. [9] (1982)	47/ANLL	73%	
McFarland et al. [10] (1982)	10/ANLL	90%	47%
Murphy et al. [11] (1986)	31/AL 19/AL**	48% 16%	23% 5%
Klingeman et al. [12] (1987)	264/AA	56%	
Brand et al. [13] (1988)	264/AA/ANLL**	18%	6%
Sniecinski et al. [14] (1988)	20/AA/ANLL 20/AA/ANLL**	50% 15%	50% 15%
Andreu et al. [15] (1988)	35 34**	31% 12%	46% 12%
A: N=811 B: N=405		51% 19%	57% 10%

A: No of patients with standard platelet transfusions
B: No of patients with leukocyte-depleted platelet transfusions

AA = aplastic anemia; AL = acute leukemia; ANLL = acute myeloid leukemia

* Single-donor random platelet transfusions
** Leukocyte-depleted platelet transfusions

Table 2. Platelet refractoriness.

Immunological	(Secondary) HLA-immunization ABO-antibodies Platelet-specific antibodies Innocent bystander destruction
Sick patients	Bone marrow transplantation Recombinant growth factors Drugs/infectious complications
Quality of platelets	Filtration Storage condition Preservation solutions

Table 3. Antigens expressed on platelets with relevance for transfusion therapy.

HLA-A and B antigens
ABO and (M, Ii, P, Le)
HPA antigens
Non-HLA platelet reactive Ag
(Auto-Ag, adhered ICX)

Platelet antibodies

Indirect platelet immunofluorescence and ELISA with platelet antigens allow the detection of HLA and non-HLA antibodies present on platelets. These can be further distinguished by monoclonal antibody immobilization of platelet antigens (MAIPA) using monoclonal antibodies against HLA and platelet specific determinants. However antibodies against non-polymorphic determinants such as auto-antigens, drug-associated antigens are detected by these techniques as well. Recently Heal et al. [18] described that recipient antibodies against the plasma proteins albumin, fibrinogen, C2 and C4 are detected on and can be eluted from platelets. Although such antibodies are indicative for broad immunization and related to the number of blood transfusions received and even may effect the life-span of transfused platelets, they offer no strategies for the definition and treatment of alloimmunization. In fact HLA antibodies are still the major cause of immunological platelet transfusion refractoriness, despite white cell depletion of blood transfusions. Of the 68 patients who sequentially developed immunological refractoriness during the period 1979-1986, sera were screened by the lymphocytotoxicity test (LCT) and a platelet immunofluorescence test (PIFT) without paraformaldehyde [13]. When antibodies were detected in both assays the PIFT was carried out with HLA-identical donors. The majority of the patients, as shown in Table 4 (N=56) showed HLA antibodies demonstrated by LCT and PIFT-concordance. Ten patients had HLA and non-HLA platelet reactive antibodies against HLA identical platelets and two

patients showed platelet reactive antibodies only. One of these latter could be specified by panel studies as anti-Zw^a. Recently Godeau et al. [19] evaluated 50 patients prospectively by regular screening in LCT, PIFT with and without paraformaldehyde (PFA) and MAIPA. They found HLA antibodies in 26%, platelet specific antibodies in 4% without identification and broad reactive platelet alloantibodies also in 4%, the latter without consequences for post-transfusion platelet increment. Although other investigators found a higher incidence of platelet-antibodies [20,21], antibodies against non-polymorphic systems and autoantibodies were not excluded in these publications.

Table 4. Platelet transfusion therapy: Pattern of antibodies in 68 immunological refractory patients evaluated with LCT and PIFT.

N of patients	Antibody pattern
56	HLA (LCT and PIFT-concordance)
10	HLA + non-HLA
	PIFT antibodies
2	PIFT*-antibodies

*One of these identified as anti-Zw^a.

The conclusion from all investigations after the role of platelet antibodies is that they often do not affect platelet survival and are often not directed against specific polymorphic antigens allowing optimal platelet donor selection.

HLA antibodies

HLA alloimmunization can be managed by treating the patient with HLA matched platelet transfusions, but increases costs of supportive care. It is important to minimize alloimmunization and to define risk factors for development (Table 5).

Table 5. Risk factors for HLA-immunization.

Amount of white cells transfused
Previous pregnancies
Immunosuppressive drugs (diagnosis)
DR-sharing between donor and patient
Immune-response loci

The amount of white cells transfused

The most important factor inducing HLA antibodies is the amount of leukocytes present in the blood components transfused. Although leukocyte depletion below 10^7 per transfusate significantly reduces HLA immunization, there is still a quantitative effect in this low range. (Van Marwijk-Kooy [22] compared patients who received $<5\times10^6$ leukocytes per platelet transfusion with patients who received $>5\times10^6$-5×10^7 leukocytes in a transfusate. He found in the first category 10 out of 17 patients (59%) who developed HLA antibodies and in the second 3 out of 36 (8%) patients. A study in prospective renal transplant patients [23] also showed variable immunization with leukocyte dosages between $<5\times10^6$ and 1.5×10^7. Although the reliability of the leukocyte counting in these low ranges can be questioned, the immunizing dose evoking primary immunization may well be between these low units.

Secondary booster

Human and animal studies have shown that for primary immunization donor antigen presenting cells (APC) are required and that by depletion the number of leukocytes of blood transfusions below at least 10^7 not enough APC's are left for primary immunization [24,25]. In classical immunology we assume that in case of a secondary booster the antigen is seen by the memory B cells and the stimulation is APC independent.

In a prospective randomized study conducted in four Dutch centers we found that females with previous pregnancies showed the same incidence of HLA antibodies (± 40%) after single donor leukocyte depleted platelet transfusions compared to standard single donor transfusions [26]. The degree of white cell depletion below 10^7 was similar to the amount of white cells that almost completely abolished primary immunization [13]. It is important to get insight in the process of secondary immunization in case of previous pregnancies to develop strategies for immunomodulation by either further reduction or inactivation (UV-B) of APCs or in case HLA class I antigens themselves are the inducers, by additional immune suppression.

DR-sharing between blood donor and recipient

Unfortunately we have no platelet transfusion experiments illustrating this subject, which is related to the immuno-suppressive effect of blood transfusions.

Prospective renal and cardiac transplant patients receive pretransplant blood transfusions to suppress the allograft response against the subsequent graft. Lagaay et al. [27] showed that such suppression of the alloimmune response was only elicited when the pretransplant blood donor and the recipient shared one or two HLA-DR antigens. Blood transfusions mismatched for both DR-antigens did not suppress alloreactivity. This suppression was illustrated in vitro by the lack of HLA antibody formation and the down-modulation of cytotoxic precur-

sor cells against the lymphocytes of the blood transfusion donor [28]. HLA-DR mismatched red cell transfusions resulted in broad HLA antibody formation in the recipients and maintenance or increase of cytotoxic precursor cell frequencies. Whether this phenomenon can be applied for modulation of immunity after platelet transfusions remains to be awaited.

Diagnosis and immunosuppressive drugs

Few publications have addressed the question whether the ability of alloantibody formation was related to the immune competence of the host in relation to diagnosis. For patients with AML, receiving the most aggressive chemotherapy and suffering the most severe infectious complications, it is well established that they are competent to mount an alloimmune response. Patients with aplastic anemia and acute lymphocytic leukemia with receiving high dose prednison during their treatment course are generally considered as high and low responders respectively. The timing of prednison treatment in relation to the first transfusion may be important and account for the difference. From elective donor specific blood transfusions preparing for family kidney transplantation it is known that concomitant immuno-suppressive therapy attacking the antigenically stimulated helper T cells, reduces the immunization incidence from 30 to below 10%. This approach can be considered to be evaluated when patients with aplastic anemia and myelodysplastic syndromes start transfusion therapy before treatment of the disease is started.

Immune response loci

For platelet specific antigens in which the polymorphism is due to one single amino acid substitution, HLA response loci have been established; of which the strongest association is found between HPA-1^a (Zw^a) and the DR52a antigen.

For HLA class I antigens providing multiple antigenic epitopes on one specificity the hypothesis is extremely difficult to study because multiple peptides are presumed to define the extreme polymorphism of the HLA class I antigens. Nevertheless non-responders and high responders exist, and data are offered [29] indicating that HLA-DR6 positive patients show enhanced allograft rejection and broad HLA antibody formation when triggered with a non-HLA-DR6 bearing graft.

Unfortunately complicated statistical methods applying multivariate analysis are required to define immune response loci as risk factors for alloimmunization due to platelet transfusions. But if patients at risk can be prospectively identified then treatment with immuno-modifying regimens to further reduce alloimmunization can be considered.

Conclusions

HLA immunization is still the main immunological factor causing platelet refractoriness. Leukocyte depletion seems not to result in complete prevention in all patients. High risk patients for alloimmunization must be identified and additional measurements to abolish alloimmunization in these patients must be developed.

Acknowledgements

Mrs. J. Paardekooper for her secretarial help.

References

1. Grumet FC, Yankee RA. Long-term platelet support of patients with aplastic anaemia. Ann Intern Med 1970;73:1-7.
2. Tejada F, Bias WB, Santos GW, Zieve PD. Immunologic response of patients with acute leukemia to platelet transfusions. Blood 1973;42:405-12.
3. Schiffer LA, Lichenfeld JL, Wiernik PH, et al. antibody response in patients with acute nonlymphocyte leukemia. Cancer 1976;37:2177-82.
4. Howard JE, Perkins HA. The natural history of alloimmunization to platelets. Transfusion 1978;18:496-503.
5. Dutcher JP, Schiffer CA, Aisner J, Wiernik PH. Alloimmunization following platelet transfusion: the absence of a dose response relationship. Blood 1981;57:395-8.
6. Holohan TV, Terasaki PI, Deisseroth AB. Suppression of transfusion-related alloimmunization in intensively treated cancer patients. Blood 1981;58:122-8.
7. Eernisse JG, Brand A. Prevention of platelet refractoriness due to HLA antibodies by administration of leukocyte-poor blood components. Exp Hematol 1981;9:77-83.
8. Ford JM, Brown LM, Cullen MH, et al. Combined granulocyte and platelet transfusions – development of alloimmunization as reflected by decreasing cell recovery values. Transfusion 1982;22:498-504.
9. Pegels JG, Bruynes ECE, Korthals Altes HR, et al. Immune unresponsiveness to platelets. Vox Sang 1982;42:211-6.
10. McFarland JG, Slichter SJ, Appelbaum F. Alloimmune versus consumptive platelet refractoriness in AML. Blood 1982;60(suppl 1):18a.
11. Murphy MF, Metcalf P, Thomas H, et al. Use of leukocyte-poor blood products and HLA matched donors to prevent alloimmunization by platelet transfusions. Brit J Haematol 1988;62:529-34.
12. Klingeman HG, Self S, Banaji M, et al. Refractoriness to random donor platelet transfusions in patients with aplastic anaemia: Multivariate analysis of data from 264 cases. Brit J Haematol 1987;66:115-21.
13. Brand A, Claas FMJ, Voogt PJ, et al. Alloimmunization after leukocyte depleted multiple random donor platelet transfusions. Vox Sang 1988;54:160-6.
14. Sniecinski I, O'Donnell MR, Nowicki B, Hill LR. Prevention of refractoriness and HLA-immunization using filtered blood products. Blood 1988;5:1402-7.
15. Andreu G, Dewailly J, Leberre C, et al. Prevention of HLA immunization with leukocyte-poor packed red cells and platelet concentrates obtained by filtration. Blood 1988;72:964-9.
16. Novotny VMJ, van Doorn R, Rozier Y, D'Amaro J, Harvey MS, Brand A. Transfusion results of filtered and subsequently stored random platelet suspensions prepared from buffy coats. Vox Sang 1992;63:23-30.

17. Bishop JF, Matthews JP, McGrath K, Yuen K, Wolf MM, Szer J. Factors influencing 20-hour increments after platelet transfusion. Transfusion 1991;31:392-6.
18. Heal JM, Cowles J, Masel D, Rowe JM, Blumberg N. Antibodies to plasmaproteins: An association with platelet transfusion refractoriness. Brit J Haematol 1992;80:83-90.
19. Godeau B, Fromont P, Seror T, Duedari N, Bierling P. Platelet alloimmunization after multiple transfusions: A prospective study of 50 patients. Brit J Haematol 1992; 81:395-400.
20. Murphy MI, Waters AJ. Immunological aspects of platelet transfusions. Brit J Haematol 1985;60:409-14.
21. McGrath K, Wolf M, Bishop J, et al. Transient platelet and HLA antibody formation in multitransfused patients with malignancy. Brit J Haematol 1988;68:345-60.
22. Van Marwijk-Kooy M, van Prooyen HC, Moes M, Bosma-Stants I, Akkerman JW. Use of leukocyte-depleted platelet concentrates for the prevention of refractoriness and primary HLA-alloimmunization: A prospective randomized trial. Blood 1991;77: 201-5.
23. Fisher M, Chapman JR, Ting A, Morris PJ. Alloimmunization to HLA antigens following transfusion with leukocyte-poor and purified platelet suspensions. Vox Sang 1985;49:331-5.
24. Claas FMJ, Smeenk RJ, Schmidt R, van Steenbrugge GJ, Eernisse JG. Alloimmunization against the MHC antigens after platelet transfusions is due to contaminating leukocytes in the platelet suspension. Exp Hematol 1981;9:84-9.
25. Bergstresser PR, Sullivan S, Streilein JW, Tigelaar RE. Origin and function of Thy-1 dendritic epidermal cells in mice. J Invest Dermatol 1985;85:90-26.
26. Sintnicolaas K, van Prooyen HC, van Marwijk-Kooy M, van Dijk BA, van Putten WLJ, Brand A. Leukocyte depletion of random single donor platelet transfusion for the prevention of secondary alloimmunization in patients with previous pregnancies. Brit J Haematol 1991;79:674(abstract).
27. Lagaay EM, Henneman PH, Ruigrok M, et al. Effect of one HLA-DR antigen matched and completely HLA-DR mismatched blood transfusions on survival of heart and kidney allografts. N Engl J Med 1989;321:701-5.
28. Van Twuyer E, Hooyaert RJD, ten Berge IJM. Pretransplantation blood transfusion revisited. N Engl J Med 1991;325:1210-3.
29. Hendriks GFJ, Schreuder GMTh, Claas FHJ, et al. HLA-DRw6 and renal allograft rejection. Brit Med J 1983;286:85-7.

GRAFT-VERSUS-HOST DISEASE

J. Jansen, Q. Chang, L.P. Akard

History

The development of nuclear arms at the end of World War II was a major stimulus for research into radiation damage to the hematopoietic system and into the treatment of such damage through bone marrow transplantation [1]. A large body of research with mice, dogs, and monkeys established a "bone marrow syndrome" with death through the complications of pancytopenia after a total body dose of 5-7 Gy (500-700 rad). Such animals could be salvaged by the infusion of syngeneic bone marrow [2] or allogeneic bone marrow [3,4]. These studies were trailblazers for bone marrow transplantation as therapeutic modality in man.

Soon after these first reports, delayed complications of allogeneic marrow (+ spleen) cell infusions into irradiated animals were reported. Barnes and Loutit were the first to demonstrate that most animals receiving allogeneic spleen cells died between 30 and 100 days after transplant [5].

This "secondary disease" was further defined by Van Bekkum's group at Rijswijk [4] and was explained by a reaction of the allogeneic cells (graft) against recipient cells (host), i.e. graft-versus-host disease (GvHD). Such reactions were similar to the "runting disease" in newborn mice transplanted with allogeneic spleen cells [6], and included severe diarrhea, weight loss, retarded growth and development, skin lesions, focal liver necrosis and lymphatic hypoplasia.

Billingham formulated the classic postulates for the development of GvHD [7]. These essential requirements were:

1. the graft must contain immunocompetent cells;
2. the host must possess transplantation antigens lacking in the graft donor, enabling the donor cells to recognize "foreign" antigens in the host; and
3. the host must be incapable of mounting an effective immunological reaction against the graft.

These requirements are still the basis for our understanding of the pathophysiology, prevention and treatment of GvHD.

Clinical features of GvHD

GvHD after allogeneic bone marrow transplantation in man is characterized by the attack at three organ systems: skin, gut, and liver [8]. The skin abnormalities can vary from mild maculopapular rash (often starting at palms of the hands, soles of the feet and behind the ears), through erythroderma to complete bullous epidermolysis. Gastrointestinal abnormalities, mostly involving jejunum and ileum, cause diarrhea (varying from mild to massive), abdominal cramps and even ileus. Liver GvHD involves cholestatic changes due to impairment of bile ducts [8,9]. A fourth target organ is the lymphohematopoietic system of the host, leading to pancytopenia and lymphocyte depletion. Since after allogeneic BMT, the hematopoietic system of the host has been eradicated or at least extremely suppressed by the preparative regimen, the circulating leukocytes are of donor origin; thus pancytopenia is not a common phenomenon [8]. In GvHD outside the BMT arena, however, pancytopenia is almost invariably present. GvHD induced by blood transfusion or liver transplants, is accompanied by severe pancytopenia and almost completely aplastic bone marrow [10,11].

The clinical features of GvHD in man are very similar to those observed in experimental studies with rodents, dogs, or primates [4,12].

Occurrence of GvHD in clinical medicine

The most frequent setting of GvHD is still in allogeneic bone marrow transplantation. Even when donor and recipient are HLA fully matched siblings, clinically detectable (acute) GvHD occurs in 10-80% of cases [13,14]. The incidence and severity of GvHD are related to donor/recipient sex match, allosensitization of the donor, recipient and donor age, and regimen of GvHD prophylaxis [13-15]. Thus, older male patients who receive a non-T-cell depleted marrow graft from a multiparous female donor, would be at a much higher risk of severe GvHD than a young child receiving a T-cell depleted graft from a young sibling. Survival after transplant is closely related with the severity of GvHD. In patients undergoing allogeneic BMT for severe aplastic anemia, the occurrence of severe (grade ≥ II) GvHD is associated with a much poorer survival [15]. For patients receiving bone marrow grafts from related HLA mismatched or from unrelated HLA matched donors, the incidence of acute GvHD is even higher and can reach 100% [16,17].

In solid organ transplants, one may expect GvHD to occur in situations where large numbers of immunocompetent lymphocytes are transplanted with the donor organs. Small bowel transplants first come to mind, and liver transplants are also at risk [11]. The proliferation and immunologic activity of donor lymphocytes in such transplants have been demonstrated [18].

Transfusions associated with GvHD were recognized as a significant problem in the 1980's [19,20]. Table 1 lists the different categories of conditions under which GvHD can occur because all three requirements of Billingham [7] have been fulfilled. The first category includes immunologically incompetent fetuses/

neonates/infants. The immunological disturbances can be either because of prematurity or because of congenital T-cell defects. Inadvertent transfusion of immunocompetent donor lymphocytes with the intended transfusion of red cells or platelets can lead to fatal GvHD in such patients [20-22].

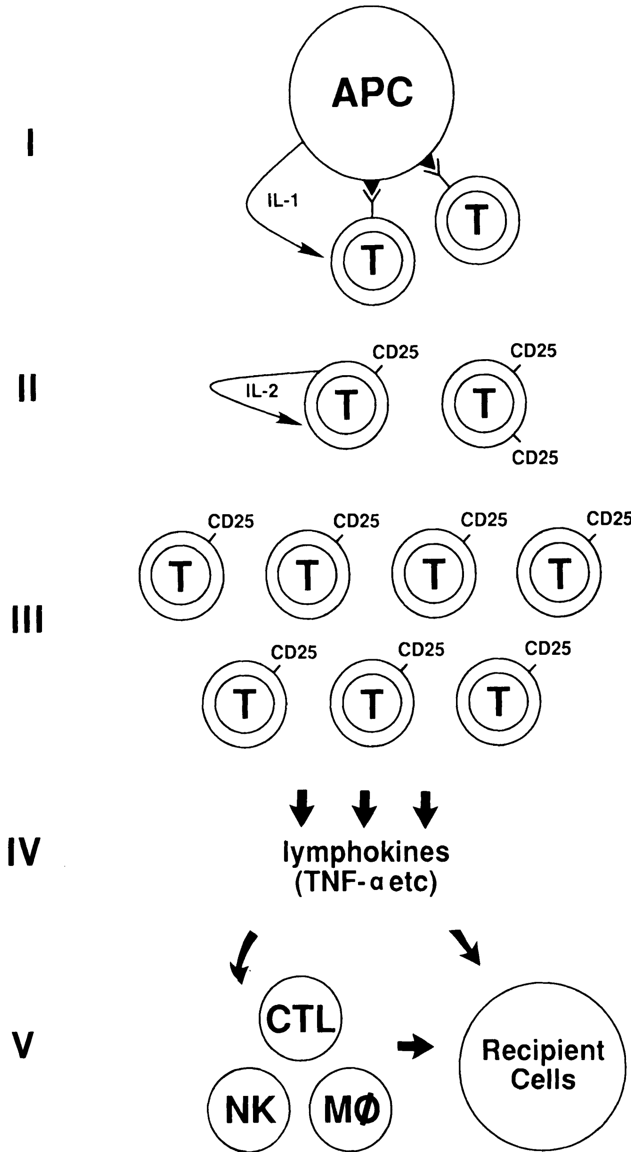
The second category are (pediatric or adult) patients with malignancies, who are immunocompromized either because of their disease (lymphoid malignancies), or because of chemotherapy. An increasing number of such cases have been reported, in particular in patients with Hodgkin's disease [20,23] and acute leukemia [20,24].

Table 1. Categories of patients at risk for the development of transfusion-induced graft-versus-host disease.

Immuno-incompetent recipients	
– congenital	– severe combined immunodeficiency (SCID) – thymic hypoplasia (DiGeorge) – Wiskott-Aldrich syndrome – Ataxia - telangiectasia
– premature infants and in-utero transfusions	
– acquired	– overwhelming viral/fungal infections – malignancies: Hodgkin's, acute leukemia – chemotherapy: acute leukemia, lymphomas, autologous BMT – allogeneic BMT recipients
Immunocompetent recipients	
– homozygous donor transfusions	
– family transfusions	

Table 2. Possible approaches to the prevention of graft-versus-host disease.

– Removal of donor T-cells	– complete (pan-T) (BMT) – selective (CD8) (BMT) – counterflow centrifugation (BMT) – leukocyte depletion (transfusions)
– Inactivation of donor T-cells	– irradiation (transfusions) – ultraviolet B (BMT, transfusions)
– Blocking of T-cell activation	– cyclosporin – FK506 – corticosteroids – methotrexate
– Blocking of cytokines	– IL-1 receptor antagonists – anti-IL-2 receptor antibodies – pentoxifylline



(modified from Ferrara and Deeg, 1991)

Figure 1. Afferent (I-III) and efferent phases (IV-V) of graft-versus-host disease.

I. The recipient antigen-presenting cells (APC) present alloantigens (peptide-HLA complexes) to resting donor T-cell. IL-1 provides an additional activation signal.
II. Activated donor T-cells produce IL-2 and start expressing IL-2 receptors (CD25).
III. Clonal expansion and differentiation occur.
IV. Lymphokines are released.
V. These lymphokines directly, or indirectly through various cell populations, damage recipient target cells.

CTL = cytotoxic T-cells; NK = natural killer cells; M0 = macrophages; CD25 = IL-2 receptor

The third category concerns immunocompetent patients who receive blood transfusion from HLA homozygous donors with whom they share a haplotype [20,25,26]. Infusion of fresh blood with viable lymphocytes, i.e. immunocompetent cells, now suddenly fulfils the Billingham requirements. The immunocompetent donor cells cannot be rejected by the recipient who does not recognize them as foreign. The recipient cells, however, do express HLA antigens lacking from the donor cells.

The risk of transfusion-induced GvHD depends on the severity of the immunocompromized state of the recipient, and on the dose of immunocompetent donor cells administered [20,27]. From animal experiments a dose of 10^7 viable donor T-lymphocytes per kilogram body weight of the recipient has been claimed to be capable of inducing GvHD [19]. In BMT, smaller numbers (10^5-10^6/kg) of donor T-lymphocytes have been shown to be capable of inducing GvHD [28,29], but it is unclear whether such small numbers will be capable of causing GvHD outside the BMT setting [20].

Immunopathophysiology

The pathophysiology of GvHD is subdivided into an afferent phase and an efferent phase (Figure 1) [30]. In the afferent phase, antigen presenting cells (APC) from the recipient digest large recipient proteins into smaller fragments and display these antigenic peptides bound to HLA molecules on their cell surface. Mature donor T-cells recognize the peptide-HLA complexes, because either the HLA molecules or the peptides are foreign. $CD4^+$ T-cells generally react to complexes including class II HLA antigens and $CD8^+$ T-cells to those with class I HLA antigens [30,31]. The antigen presenting cells release interleukin-1 to activate naive T-cells [32]. Several additional receptors between donor and recipient cells interact to intensify the cellular communication.

Individual donor T-cells become activated during this process of antigen presentation, as shown by activation of protein kinase C and tyrosine kinases [33]. Interleukin-2 and its receptors are expressed, leading to proliferation through autocrine and paracrine effects. Clonal expansion follows, and functional differentiation leads to the production of cytokines essential for the various effector cell functions.

The efferent phase of GvHD is very diverse and includes cellular and humoral mechanisms. Whether cytotoxic T-cells can cause damage directly or only through soluble mediators, such as tumour necrosis factor (TNF-α), is still under discussion [30,34-36]. TNF can be released by T-cells, macrophages and large granular lymphocytes and can result in cell destruction. Which cell types are involved in the effects of GvHD in humans is unclear. Possibly, the situation is similar to GvHD in mice, where natural killer cells and large granular lymphocytes are very important [37].

Although the GvHD reaction usually starts with the presentation of alloantigens, GvHD has also been reported in syngeneic BMT and even after autologous BMT [29,38]. This "syngeneic GvHD" appears to be mediated by autoreactive

T-cells directed at HLA class II antigens [39]. It has been suggested that these autoreactive T-cells develop in the thymic medulla of patients heavily treated with chemotherapy and radiation therapy in an environment deficient of HLA class II proteins. Thus, these autoreactive T-cells would escape clonal deletion within the thymus. In patients where other regulatory protection mechanisms are lacking, due to radiation and/or treatment with cyclosporin, the autoreactive T-cells could induce a limited form of GvHD [39].

Prevention and treatment of GvHD

Attempts to prevent the occurrence of GvHD can be directed at different stages of the afferent and efferent phases of the GvHD process. In the afferent phase, many prophylaxis protocols are aimed at preventing activation of donor T-cells; this can be accomplished by removing or inactivating the T-cells.

Elimination of donor T-cells has become a very attractive approach to prevent acute GvHD [40-42]. Many different techniques, including E-rosette separation with or without preceding soybean agglutination [43], counterflow centrifugation [44], and monoclonal antibodies with complement [41], with toxins [45] or with magnetic beads [46], can result in depletion of 95-99.9% of the total number of T-cells from the marrow graft. All these procedures can result in substantial reductions in the incidence and severity of GvHD. Unfortunately, T-cell depleted marrow grafts are associated with increased risks of graft rejection and relapse of malignancy [47]. The discussion of these complications is beyond the scope of this review. Outside the BMT setting, the removal of donor T-cells may be the simplest mehtod of preventing GvHD. Leukocyte depletion of transfusion products can be easily accomplished, and the latest generation of leukocyte filters should be capable of removing at least 99% of the leukocytes from red cell and platelet products [48,49]. Such a reduction should be sufficient to prevent transfusion-induced GvHD. The main drawback is that these filters are not full proof and occasionally much poorer depletion of leukocytes is obtained.

Selective depletion of T-cell subsets is a more recent approach to preserve the powerful anti-GvHD effect of T-cell depletion while, hopefully, avoiding the pitfalls of graft failure and relapse. In mice, elegant studies have demonstrated the involvement in GvHD of different T-cell subsets depending on the histocompatibility differences between donor and recipient [31,50]. Thus, donor $CD8^+$ cells appear to be associated with GvHD in case of an MHC class I antigen difference between donor and recipient, whereas $CD4^+$ cells are involved in case of a class II difference [31]. In case of both class I and class II differences, both $CD4^+$ and $CD8^+$ donor T-cells can induce GvHD. Mismatch for minor MHC antigens appeared to involve $CD8^+$ donor cells [31]. If these associations would be similar in humans, $CD8^+$ donor T-cells would be involved in the GvHD process in HLA identical sibling (=minor HLA antigens mismatched) and HLA-A or -B mismatched transplants, whereas $CD4^+$ donor T-cells would be involved in HLA-DR mismatched transplants. Some, indirect, evidence for the involvement of $CD8^+$ cells in GvHD in HLA-identical sibling transplants, exists.

Gratama et al. reported a more rapid reconstitution of donor $CD8^+$ cells in transplant recipients developing significant GvHD than in recipients showing only mild GvHD or none at all [51]. The same group also reported that donors inducing moderate to severe GvHD in their recipients, had a larger fraction of $CD8^+$ cells in their peripheral blood [52].

These observations would fit very well into a hypothesis that donor $CD8^+$ lymphocytes are primarily involved in the development of GvHD in HLA matched sibling transplants. A combination of $CD8^+$ depletion of the bone marrow graft and cyclosporin administration to the recipient has been studied by a number of groups [53,54]. This approach was shown to be effective in preventing GvHD in the HLA matched sibling transplants, without a clear increase in the risks of graft failure and relapse [54]. However, in matched unrelated bone marrow transplants, this combination appears insufficient to prevent moderate to severe acute GvHD (Jansen et al., unpublished data).

Alternative approaches to the functional elimination of donor T-cells exist. Radiation can effectively prevent proliferation of T-lymphocytes and doses of 5 Gy can abrogate the response to allogeneic cells; 15 Gy can decrease the response of lymphocytes to mitogens by 90% [55,56]. To virtually eliminate the risk of transfusion-induced GvHD, irradiation is by far the most convenient and most easily standardized approach, although pitfalls such as homogeneity of dose still exist. Because of this problem, a dose of 25 Gy probably should be preferred over 15 Gy in order to completely ablate the immunological reactivity of donor T-cells [57]. Obviously, for bone marrow grafts this technique cannot be used, since the hematopoietic stem cells are even more susceptible to radiation than lymphocytes [58].

A technique that holds promise for the elimination of GvHD without destroying the hematopoietic stem cells, involves the use of UV-B light [59,60]. In mice, exposure of donor bone marrow/spleen cells to 10-12.5 mJ/cm UV-B resulted in excellent bone marrow engraftment, but without the occurrence of GvHD [60]. Similarly, in a dog model, transfusion-induced GvHD was prevented by exposure of the transfusion product to UV-B [61].

Chemical ways to block the activation of donor T-cells in allogeneic BMT (and host T-cells in solid organ transplants) include cyclosporin and FK506. Cyclosporin blocks the synthesis of interleukin-2, and prevents the second stage of T-cell activation (Figure 1) [62]. This drug is one of the most commonly used agents to prevent GvHD in human BMT, but has only moderate activity and extensive toxicity. Renal toxicity, interactions with other drugs commonly used in BMT, and the possibility of hemolytic-uremic syndrome [63,64] all are major drawbacks for the use of cyclosporin. FK506 is a macrolide that has shown much promise in a rat model both to prevent and to treat GvHD [65,66]. Thus, the drug was capable of reversing the clinical and histological manifestations of GvHD, and did so more frequently and more completely than cyclosporin [65]. FK506 is about to enter clinical trials for the prevention of GvHD in human bone marrow transplantation. Although FK506 is also nephrotoxic, it is hoped that it will be at least as effective as cyclosporin, but less toxic [67].

A combination of immunosuppressive drugs may be superior to single agents. If synergism can be obtained, a superior effect could occur at the expense of fewer side effects. Just like in the use of antibiotics and cytotoxic drugs, one should aim for a combination of agents with different mechanisms of action and different dose-limiting toxicities. One such combination is cyclosporin and methotrexate which was shown to be superior in preventing human GvHD than either drug alone [68].

An alternative approach to the prevention of GvHD involves attempts to block the cytokines that have been produced by donor or host cells involved in the GvHD cascade. Important cytokines are IL-1 and IL-2 in the afferent phase and TNF-α and interferon-γ in the efferent phase (Figure 1) [30]. Interleukin-1 was found to be capable of exacerbating GvHD [69]. The in-vivo administration of Il-1 receptor antagonists, has been shown in mice to reduce the mortality from GvHD [70]. No detrimental effect on hematopoietic engraftment was seen. Similarly, anti IL-2 receptor antibodies have been successfully used in the treatment of human GvHD [71].

Pentoxifylline is a dimethylxanthine mainly used for the treatment of arterial vascular disease [72]. In addition, however, this drug is a potent inhibitor of TNF-α [73]. Recently in a non-randomized study pentoxifylline was found to reduce the toxicities of allogeneic bone marrow transplantation, including mucositis and also GvHD [74]. Randomized studies of this TNF-α blocking agent for the prevention of GvHD are under way at several institutions.

Undoubtedly, the next decade will see a rapid increase in the number of approaches to the prevention and treatment of GvHD. Almost certainly, no single method will prove to be perfect. More likely, combinations of methods will be used to "tailor" the amount of GvHD to accomplish its positive effects, but to avoid its negative complications.

Acknowledgement

The authors gratefully acknowledge the assistance of Mrs. Charlotte Franklin in the preparation of the manuscript.

References

1. Santos GW. History of bone marrow transplantation. Clin Haematol 1983;12:611-41.
2. Lorenz E, Uphoff DE, Reid TR, Shelton E. Modification of acute irradiation injury in mice and guinea pigs by bone marrow infections. J Nat Cancer Inst 1951;12:197-201.
3. Lorenz E, Congdon CC. Modification of lethal irradiation injury in mice by injection of homologous and heterologous bone marrow. J Nat Cancer Inst 1954;14:955-61.
4. Van Bekkum DW, de Vries MJ. Radiation chimaeras. Logos Press Limited, New York, 1967.
5. Barnes DWH, Loutit JF. Treatment of murine leukaemia with x-rays and homologous bone marrow. II. Brit J Haematol 1957;3:241-52.
6. Billingham RE, Brent L. A simple method for inducing tolerance of skin homografts in mice. Transplantation Bulletin 1957;4:67-71.

7. Billingham RE. The biology of graft-versus-host reactions. Harvey Lectures 1966-67;62:21-78.
8. Thomas ED, Storb R, Clift RA, et al. Bone-marrow transplantations. N Engl J Med 1975;292:832-43,895-902.
9. Sale G. Shulman HM (eds). The pathology of bone marrow transplantation. Masson Publishing USA, Inc., Seattle, 1984.
10. Brubaker DB. Human posttransfusion graft-versus-host disease. Vox Sang 1983; 45:401-20.
11. Burdick JF, Vogelsang GB, Smith WJ, et al. Severe graft-versus-host disease in a liver-transplant recipient. Medical Intelligence 1988;318:689-91.
12. Deeg HJ, Storb R, Shulman H. Engraftment of DLA-nonidentical unrelated canine marrow graft after high-dose fractionated total body irradiation. Transplantation 1982;33:443-6.
13. Vogelsang GB, Wagner JE. Graft-versus-host disease. Hemat/Oncol Clin Nrth Amer 1990;4:625-39.
14. Gale RP, Bortin MM, van Bekkum DW, et al. Risk factors for acute graft-versus-host disease. Brit J Haematol 1987;67:397-406.
15. Neudorf S, Filipovich A, Ramsay N, Kersey K. Prevention and treatment of acute graft-versus-host disease. Sem Hematol 1984;21:91-100.
16. Beatty PG, Clift RA, Mickelson EM, et al. Marrow transplantation from related donors other than HLA-identical siblings. N Engl J Med 1985;313:765-71.
17. Ash RC, Casper JT, Chitambar CR, et al. Successful allogeneic transplantation of T-cell-depleted bone marrow from closely HLA-matched unrelated donors. N Engl J Med 1990;322:485-94.
18. Starzl TE, Demetris AJ, van Thiel D. Liver transplantation. N Engl J Med 1989;321:1014-22,1092-9.
19. Von Fliedner V, Higby DJ, Kim U. Graft-versus-host reaction following blood product transfusion. Am J Med 1982;72:951-61.
20. Anderson KC, Weinstein HJ. Transfusion-associated graft-versus-host disease. N Engl J Med 1990;323:315-21.
21. Park BH, Good RA, Gate J, Burke B. Fatal graft-versus-host reaction following transfusion of allogeneic blood and plasma in infants with combined immunodeficiency disease. Transplant Proc 1974;6:385-7.
22. Parkman R, Mosier D, Umansky I, Cochran W, Carpenter CB, Rosen FS. Graft-versus-host disease after intrauterine and exchange transfusions for hemolytic disease of the newborn. N Eng J Med 1974;290:359-63.
23. Dinsmore RE, Straus DJ, Pollack MS, et al. Fatal graft-versus-host disease following blood transfusion in Hodgkin's disease documented by HLA typing. Blood 1980;55: 831-4.
24. Lowenthal RM, Grossman L, Goldman JM, et al. Granulocyte transfusions in treatment of infections in patients with acute leukemia and aplastic anaemia. Lancet 1975;*i*:353-8.
25. Thaler M, Shamiss A, Orgad S, et al. The role of blood from HLA-homozygous donors in fatal transfusion-associated graft-versus-host disease after open-heart surgery. N Engl J Med 1989;321:25-8.
26. Otsuka S, Kunieda K, Hirose M, et al. Fatal erythroderma (suspected graft-versus-host disease) after cholecystectomy: Retrospective analysis. Transfusion 1989;29: 544-8.
27. Holland PV. Prevention of transfusion-associated graft-versus-host disease. Arch Pathol Lab Med 1989;113:285-91.
28. Reisner Y, Kapoor N, Kirkpatrick D, et al. Transplantation for acute leukaemia with HLA-A and B nonidentical parental marrow cells from fractionated with soybean agglutinin and sheep red blood cells. Lancet 1981;*ii*:327-31.

29. De Witte T, Hoogenhout J, de Pauw B, et al. Depletion of donor lymphocytes by counterflow centrifugation successfully prevents acute graft-versus-host disease in matched allogeneic bone marrow transplantation. Blood 1986;67:1302-8.
30. Ferrara J, Deeg HJ. Graft-versus-host disease. N Engl J Med 1991;324:667-74.
31. Korngold R, Sprent J. Lethal graft-versus-host disease across minor histocompatibility barriers in mice. In: Nathan DG (ed). Clinics in haematology 1983;681-93.
32. Unanue ER, Allen PM. The basis for the immunoregulatory role of macrophages and other accessory cells. Science 1987;236:551-7.
33. Samelson LE, Patel MD, Weissmann AM, Harford JB, Klausner RD. Antigen activation of murine T-cells induces tyrosine phosphorylation of a polypeptide associated with the T-cells antigen receptor. Cell 1986;46:1083-90.
34. Ghayur T, Seemeyer TA, Kongshavn PAL, Gartner JG, Lapp WS. Graft-versus-host reactions in the beige mouse: An investigation of the role of host and donor natural killer cells in the pathogenesis of graft-versus-host disease. Transplantation 1987;44: 261-7.
35. Symington FW, Storb R. Mechanisms of tissue damage in human acute graft-versus-host disease. J Immunol Immunopharmacol 1989;9:214-9.
36. Piguet PF. Tumor necrosis factor and graft-versus-host disease. In: Burakoff SJ, Deeg HJ, Verrara J, Atkinson K (eds). Graft-vs-host disease: Immunology, pathophysiology and treatment. Marcel Dekker, New York 1990:225-76.
37. Ferrara JL, Guillen FJ, van Dijken PJ, Marion A, Murphy GF, Burakoff SJ. Evidence that large granular lymphocytes of donor origin mediate acute graft-versus-host disease. Transplantation 1989;47:50-4.
38. Rappeport J, Mihm M, Reinherz E, Lopansri S, Parkman R. Acute graft-versus-host disease in recipients of bone-marrow transplantation from identical twin donors. Lancet 1979;*ii*:717-20.
39. Hess AD, Horwitz L, Beschorner WE, Santos GW. Development of graft-versus-host disease-like syndrome in cyclosporine-treated rats after syngeneic bone marrow transplantation. I. Development of cytotoxic T lymphocytes with apparent polyclonal anti-Ia specificity, including autoreactivity. J Exp Med 1985;161:718-30.
40. Maraninchi D, Blaise, Rio B, et al. Impact of T-cell depletion on outcome of allogeneic bone-marrow transplantation for standard-risk leukaemias. Lancet 1987;*i*:175-8.
41. Mitsuyasu RT, Champlin RE, Gale RP, et al. Treatment of donor bone marrow with monoclonal anti-T-cell antibody and complement for the prevention of graft-versus-host disease. Ann Intern Med 1986;105:20-6.
42. Waldmann H, Hale G, Cividalli G, et al. Elimination of graft-versus-host disease by in-vitro depletion of alloreactive lymphocytes with a monoclonal rat anti-human lymphocyte antibody (CAMPATH-1). Lancet 1984;*i*:483-5.
43. Reisner Y, Kapoor N, Kirkpatrick D, et al. Transplantation of severe combined immunodeficiency with HLA-A,B,D,DR incompatible parental marrow cells fractionated by soybean agglutinin and sheep red blood cells. Blood 1983;61:341-8.
44. Schattenberg A, de Witte T, Preijers F, et al. Allogeneic bone marrow transplantation for leukemia with marrow grafts depleted of lymphocytes by counterflow centrifugation. Blood 1990;75:1356-63.
45. Filipovich AH, Vallera D, McGlave P, et al. T-cells depletion with anti-CD5 immunotoxin in histocompatible bone marrow transplantation: The correlation between residual CD5 negative T-cells and subsequent graft-versus-host disease. Transplantation 1990;9:401-15.
46. Jansen J, Hanks S, English D, Karaffa R, Akard L. Immunomagnetic depletion of $CD8^+$ lymphocytes from marrow grafts. In: Worthington-Write DA, Gee A, Gross S (eds). Advances in bone marrow purging and processing. Wiley-Liss, Inc, New York; 405-10.
47. Butturini A, Gale RP. T-cells depletion in bone marrow transplantation for leukemia: Current results and future directions. Bone Marrow Transpl 1988;3:185-92.

48. Sirchia G, Parravicini A, Rebulla P, Fattori L, Milani S. Evaluation of three procedures for the preparation of leukocyte-poor and leukocyte-free red blood cells for transfusion. Vox Sang 1980;38:197-204.
49. Lane TA, Anderson KC, Goodnough LT, et al. Leukocyte reduction in blood component therapy. Ann Intern Med 1992;117:151-62.
50. Korngold R, Sprent J. T-cells subsets and graft-versus-host disease. An overview. Transplantation 1987;44:335-9.
51. Gratama JW, Naipal A, Oljan, et al. T-lymphocyte repopulation and differentiation after bone marrow transplantation. II. Early shifts in the ratio between T4^{+} and T8^{+} T-lymphocytes correlated with the occurrence of acute graft-versus-host disease. Blood 1984;63:1416-23.
52. Gratama JW, de Groot-Swings G, Slats J, Zwaan FE, Jansen J. Correlation between the fraction size of the peripheral blood T8 T-lymphocyte subset of marrow donors and the development of acute graft-versus-host disease. Exp Hematol 1984;12:51-2.
53. Champlin R, Ho W, Gajewski J, et al. Selective depletion of CD8^{+} lymphocytes for prevention of graft-versus-host disease after allogeneic bone marrow transplantation. Blood 1990;76:418-23.
54. Champlin R, Jansen J, Ho W, et al. Retention of graft-versus-leukemia using selective depletion of CD8-positive T lymphocytes for prevention of graft-versus-host disease following bone marrow transplantation for chronic myelogenous leukemia. Transplant Proc 1991;23:1695-6.
55. Sprent J, Anderson RE, Miller JF. Radiosensitivity of T and B lymphocytes. II. Effect of irradiation on response of T-cells to alloantigens. Eur J Immunol 1974;4: 204-10.
56. Valerius NH, Johansen KS, Nielson OS, Platz P, Rosenkvist J, Sørenson H. Effect of in vitro x-irradiation on lymphocyte and granulocyte function. Scand J Haematol 1981;27:9-18.
57. Rosen NR, Weidner JG, Boldt HD, Rosen DS. Prevention of transfusion associated graft-versus-host disease. Selection of adequate dose of gamma radiation. Transfusion 1993;33:125-7.
58. Zwaan FE. Haemopoietic cells in human peripheral blood. (Thesis). J.H. Pasman, 's-Gravenhage 1980.
59. Deeg HJ, Bazar L, Sigaroudinia M, Cottler-Fox M. Ultraviolet B light inactivates bone marrow T lymphocytes but spares hematopoietic precursor cells. Blood 1989; 73:369-71.
60. Cohn ML, Cahill RA, Deeg HJ. Hematopoietic reconstitution and prevention of graft-versus-host disease with UVB-irradiated haploidentical murine spleen and marrow cells. Blood 1991;78:3317-22.
61. Deeg HJ, Graham TC, Gerhard-Miller L, Appelbaum FR, Schuening F, Storb R. Prevention of transfusion-induced graft-versus-host disease in dogs by ultraviolet irradiation. Blood 1989;74:2592-5.
62. Kahan BD, Cyclosporine. N Eng J Med 1989;321:1725-38.
63. Atkinson K, Biggs JC, Hayes J, et al. Cyclosporin A associated nephrotoxicity in the first 100 days after allogeneic bone marrow transplantation: Three distinct syndromes. Brit J Haematol 1983;54:59-67.
64. Leitner C, Sinzinger H, Pohanka E, Schwarz M, Kretschmer G, Syré. Occurrence of hemolytic uremic syndrome under cyclosporine treatment: Accident or possible side effect mediated by a lack of prostacyclin-stimulating plasma factor. Transplant Proc 1983;15(suppl 1):571-3.
65. Markus PM, Cai X, Ming W, Demetris AJ, Fung JJ, Starzl TE. FK506 reverses acute graft-versus-host disease after allogeneic bone marrow transplant in rats. Surgery 1991;8:357-64.

66. Markus PT, Cai X, Ming W, Demetris AJ, Fung JJ, Starzl TE. Prevention of graft-versus-host disease following allogeneic bone marrow transplantation in rats using FK506. Transplantation 1991;52:590-4.
67. Fung JJ, Slessiani M, Abu-Elmagd K, et al. Adverse effects associated with the use of FK506. Transplant Proc 1991;15:3105-8.
68. Storb R, Deeg HJ, Pepe M, et al. Methotrexate and cyclosporine versus cyclosporine alone for prophylaxis of graft-versus-host disease in patients given HLA-identical marrow grafts for leukemia: Long-term follow-up of a controlled trial. Blood 1989; 73:1729-34.
69. Atkinson K, Matias C, Guifere A, et al. In vivo administration of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF, interleukin-1 (IL-1), and IL-4 alone and in combination, after allogeneic murine hematopoietic stem cell transplantation. Blood 1991;77:1376-82.
70. McCarthy PL, Abhyankar S, Neben S, et al. Inhibition of interleukin-1 by an interleukin-1 receptor antagonist prevents graft-versus-host disease. Blood 1991;78:1915-8.
71. Hervé P, Wijdenes J, Bergerat JP, et al. Treatment of corticosteroid resistant acute graft-versus-host disease by in vivo administration of anti-interleukin-2 receptor monoclonal antibody (B-B10). Blood 1990;75:1017-23.
72. Fahr A, Langer R, Ziegoleit S. Influence of pentoxifylline administered in vivo on synthesis of prostacyclin in human varicos veins. Biomed Bioclin ACTA 1988;14: 29-36.
73. Han J, Thompson P, Beutler B. Dexamethasone and pentoxifylline inhibit endotoxin-induced cachectin/tumor necrosis factor synthesis at separate points in the signaling pathway. J Exp Med 1990;172:391-8.
74. Bianco JA, Appelbaum FR, Nemunaitis J, et al. Phase I-II trial of pentoxifylline for the prevention of transplant-related toxicities following bone marrow transplantation. Blood 1991;78:1205-11.

ANTI-IDIOTYPIC REGULATION OF THE ALLOIMMUNE RESPONSE IN PATIENTS TRANSFUSED WITH PLATELET CONCENTRATES[1]

J.W. Semple,[2,3] E. Atlas,[2] J. Freedman[2], V. Blanchette[4], M.D. Kazatchkine[5]

Introduction

Platelet transfusions have increased markedly in recent years with a resulting awareness of problems associated with transfusion therapy. A major problem in patients who receive multiple platelet transfusions has been the development of alloimmunization and a consequent state of clinical refractoriness to random donor platelet transfusions. Patients develop antiplatelet antibodies which shorten the survival of the transfused platelets; anti-HLA antibodies are most common, but platelet-specific antibodies can also occur. The incidence of alloimmunization to HLA in patients with acute leukemia is approximately 30-40% [1-5].

It has become evident that in approximately 50% of patients who develop anti-HLA antibodies, the antibodies may disappear, or become less reactive, despite continued exposure to platelet and red cell transfusions [6,7]. On the other hand, some transfusion recipients never become alloimmunized despite long-term antigenic stimulation.

One of the mechanisms by which the peripheral antibody repertoire is regulated is via idiotypic interactions [8-10]. One possible explanation of the above phenomenon in transfusion recipients is that anti-HLA reactivity in alloimmunized patients is under the regulation of anti-idiotypic antibodies [11-13]. This study provides evidence that the disappearance of anti-HLA antibodies in multiply transfused patients with acute leukemia is related to appearance of anti-idiotypic antibodies.

1. Supported by a grant from the Cutter Biological/Canadian Red Cross Society Research and Development Fund.
2. Division of Hematology, St. Michael's Hospital, Toronto, Ontario, Canada.
3. Departments of Pharmacology and Medicine, University of Toronto, Canada.
4. Hospital for Sick Children, Toronto, Ontario, Canada.
5. Hôpital Broussais, Paris, France.

Materials and methods

Patients and sera: Eighty-two patients (39 females and 43 males) were studied from a population of 134 newly diagnosed patients with acute leukemia receiving multiple platelet transfusions. Anti-HLA antibody reactivity in the patients sera was determined by three different methods (lymphocytotoxicity, flow cytometry and solid-phase red cell adherence), as described elsewhere [1,14,15]. None of the patients had detectible anti-HLA antibodies prior to transfusion therapy. Criteria for inclusion were either that the patients never made anti-HLA antibodies (N=39), or that they made anti-HLA which decreased over time (N=43, in 16 of whom antibody decreased to undetectable levels). Females were classified into those who had been previously pregnant (N=26) and those who were never pregnant (N=13). Twenty-two normal volunteers (7 females with no history of pregnancy and 15 males) were also studied. Serum samples were obtained prior to transfusion, every two weeks during remission-induction, and then monthly. Two hundred and thirty-six sera from the 82 patients were investigated in the current study. As a source of reference anti-HLA antibodies, ten sera, known to contain strong polyspecific anti-HLA, from patients repeatedly transfused with platelet concentrates, were pooled and $F(ab')_2$ fragments were prepared by the method of Hardy [16]. Details of the methods used in this study are described elsewhere [17].

Results and discussion

Using an ELISA technique [17], patient sera were tested for reactivity with reference $F(ab')_2$ fragments of anti-HLA antibodies obtained from multiply transfused patients with high anti-HLA titers. Overall, the sera of 55/82 patients contained anti-idiotypic antibodies reactive with the V region of anti-HLA $F(ab')_2$ fragments. None of the sera tested from 22 healthy volunteers showed reactivity against anti-HLA $F(ab')_2$ fragments. In addition, none of the patients' or normal sera showed reactivity against $F(ab')_2$ fragments derived from normal donor sera. Of 52 patients not formally included in this study because of high, persistent anti-HLA titers, 27 were tested for serum reactivity against the $F(ab')_2$ fragments and all were found to be negative. IVIg was also tested for its reactivity against either the reference anti-HLA or normal serum $F(ab')_2$ fragments. Enhanced reactivity to both types of fragments was seen at low dilutions (OD_{405} of 1/20 to 1/80 dilutions ranged from 0.35 ± 0.08 to 0.28 ± 0.04 respectively) of the IVIg.

Patients' sera which contained anti-idiotypic antibodies were tested for their ability to neutralize or inhibit the binding of the reference anti-HLA serum to platelet membranes in an ELISA [17]. Overall, the sera of 20/55 patients who made anti-idiotypic antibodies inhibited anti-HLA binding to platelet membranes. The class of the inhibitory anti-idiotypes was determined by pre-absorbing the sera on protein G-Sepharose beads. In all cases, the inhibitory effect of

the sera was reversed by protein G-Sepharose absorption indicating the anti-idiotypic antibodies were IgG.

Sixteen patients in the study made anti-HLA antibodies whose reactivity disappeared completely over time despite continued platelet transfusions. In order to determine whether this disappearance correlated to the appearance of anti-idiotypic antibodies, "internal" inhibitions were performed with the sera of these patients i.e. the sera lacking any detectable anti-HLA antibody were used to neutralize the patient's own previously detectable anti-HLA and then tested for $F(ab')_2$ reactivity in an ELISA [17]. We observed that 7/12 patients developed anti-idiotypic antibodies over time which could specifically inhibit platelet membrane binding of the anti-HLA they had previously made. Table 1 shows the percent inhibitions of each of these patient's sera. Of interest was the fact that 6 of these patients had been previously pregnant. Table 2 is a summary of the above results.

Table 1. Internal inhibitions of anti-HLA+ serum reactivity by patient's anti-HLA– serum.*

Patient no.	Percent internal inhibition
1	29 ± 10
2	31 ± 8
3	40 ± 12
4	42 ± 5
5	73 ± 14
6	53 ± 11
7	20 ± 5
Control**	4 ± 2

* Sera from 7/16 patients at the time they became anti-HLA negative contained IgG anti-idiotypes which were able to inhibit their previously Ab1 positive serum from binding platelet membranes. The results presented are the maximum percent inhibition ± standard error of the mean (SEM). Maximum inhibition was found at anti-HLA– serum: anti-HLA + serum ratios of 4:1 for all patients except patient 4 (2:1).
** Average percent inhibition ± SEM of control sera mixed with patients Ab1 positive sera.

One of the major problems encountered with platelet transfusion therapy is the development of anti-HLA antibodies which can lead to the refractory state. In approximately 50% of patients who develop anti-HLA antibodies, the antibodies may either disappear or become less reactive, despite continued exposure to platelet and red cell transfusions [6,7]. Some transfusion recipients however, never become alloimmunized despite long-term antigenic stimulation. This study investigated the possibility that failure to make anti-HLA, or reduction in anti-HLA despite continued platelet transfusions was due to the appearance of anti-HLA specific anti-idiotypic antibodies.

Anti-idiotypic antibodies are known to regulate the antibody repertoire through variable region interactions. These interactions may have beneficial effects in a variety of immune-mediated processes by down-regulating the immune response [9,10]. For example, anti-idiotypic antibodies have been shown to be present in the sera of patients receiving blood transfusions before renal transplantation [11-13,18], as well as in the sera of women alloimmunized by pregnancies [19,20].

Sixty seven percent of patients receiving platelet transfusion whose anti-HLA response changed over time developed anti-HLA specific anti-idiotypic antibodies. In addition, the demonstration of anti-HLA specific anti-idiotypic antibodies in patients who did not have detectable anti-HLA at any time during the study supports the possibility that these patients may have been previously stimulated to produce anti-idiotypic antibodies, either by alloimmunization and/or antigenic (anti-HLA) mimicry.

The development of the anti-idiotypic antibodies corresponded to a decrease or absence of anti-HLA, since anti-idiotypic antibodies could not be detected in the sera of patients with high, persistent anti-HLA reactivities [17]. It should be noted, however, that a lack of detectible anti-idiotypic antibodies in these patients may be due to competitive inhibitory influences by the anti-HLA antibodies.

Anti-idiotypic antibodies have been demonstrated in IVIg preparations [21-26]. When IVIg was tested in the ELISA against $F(ab')_2$ fragments derived from either the reference anti-HLA or normal sera, higher reactivities were recorded than seen with normal donor sera. This is consistent with a previous report showing that anti-idiotypic antibodies specific for anti-HLA are present in IVIg [27]. We also observed that enriched γ-globulins from a variety of species sera (rat, human, mouse) generally have higher reactivities than untreated sera against $F(ab')_2$ fragments in the ELISA [17].

Anti-idiotypic antibodies can be classified as either paratope-related (antigen binding site) or paratope-non-related (non-site-associated) [9,10]. Both types are believed to be involved in immune regulation at the B cell (via surface immunoglobulin) and possibly T cell (via the T cell receptor) levels [9,10]. In 36% of the patients whose sera contained anti-idiotypic antibodies, we found inhibition of binding of the reference anti-HLA sera to platelet membranes. This suggests that these antibodies have paratope-related specificities. This anti-idiotypic activity was contained in the IgG fraction of the serum, since pre-absorption of the serum by protein G-Sephadex, which binds all human IgG subclasses, completely reversed the inhibitory affect.

There was a consistent trend for a higher proportion of previously pregnant women to have paratope-related anti-idiotypes than observed in the never-pregnant patients. Alloimmunization to paternal HLA can occur as early as 8 weeks gestation [19] which may induce an anti-idiotypic response [19,20]. Furthermore, Claas et al. [28] have demonstrated that platelet transfusions in previously alloimmunized mice can induce a strong anamnestic alloimmune response. It may be that prior alloimmunization from pregnancy, together with subsequent

re-exposure to HLA via the platelet transfusions, results in hyperimmunization of the patient so as to generate anti-HLA idiotypes which preferentially induce inhibitory anti-idiotypes. The development of anti-idiotypic antibodies correlated with a loss in anti-HLA reactivity. These anti-idiotypic antibodies could specifically inhibit the binding of their own previously anti-HLA positive sera to platelet membranes. The present results suggest that in many patients who become alloimmunized by repeated platelet transfusions, the development of anti-idiotypes correlates with a decrease in anti-HLA alloantibodies. These data also support the possibility that anti-idiotypic antibodies may down-regulate alloimmunization in multiply transfused patients and suggests that anti-idiotypes may have potential therapeutic benefits in manipulations of the alloimmune response. We are currently developing a SCID mouse model of platelet alloimmunization in which SCID mice are injected with naive or previously alloimmunized human lymphocytes and various concentrations of human heterologous platelets. This murine model will allow us to examine several parameters regarding transfusion-induced alloimmunization including the role of contaminating leukocytes in the platelet concentrates and what factors (e.g. anti-idiotypic antibodies, cytokines) might specifically down-regulate the alloimmune response.

In summary, a majority of leukemic patients who had decreased or undetectable levels of alloimmunization (anti-HLA antibodies), despite multiple platelet transfusions, developed anti-HLA specific anti-idiotypes which could inhibit anti-HLA reactivity. Importantly, the loss of anti-HLA antibodies might be correlated with the appearance of anti-HLA specific anti-idiotypic antibodies and could potentially result in a better survival of transfused platelets.

Table 2. Summary of results.

	Controls	**Total**	**Never made Ab1[1)]**		**Decreasing Ab1 titer**	
			Preg+[2)]	**Preg–[3)]**	**Preg+**	**Preg–**
N	22	82	6	33 (26)[4)]	20	23 (17)
Anti-id+	0/22 (0%)	55/82 (67%)	6/6 (100%)	21/23 (64%)	13/20 (65%)	15/23 (65%)
Inhibition of F(ab')2	0/22 (0%)	20/55 (36%)	3/6 (50%)	6/21 (29%)	6/13 (46%)	5/15 (33%)
Internal inhibition[5)]	N/D[6)]	7/16 (44%)	N/D	N/D	6/12 (50%)	1/4 (25%)

1) Patients found to be negative for anti-HLA at all times
2) Female patients previously pregnant.
3) Males and never-pregnant females, no significant difference in results was seen.
4) Number of males in the group.
5) Only those patients whose anti-HLA titer decreased to zero.
6) Not determined.

References

1. Blanchette V, Freedman J, Adams M, MacMillan J, Wang E. Extreme leukodepletion as an intervention to prevent platelet alloimmunization in new leukemics: Results of a randomized study. Blood 1991;78:467a(abstract).
2. Howard JE, Perkins HA. The natural history of alloimmunization to platelets. Transfusion 1978;18:496-503.
3. Schiffer CA, Lichtenfeld JL, Wiernik PH. Antibody response in patients with acute nonlymphocytic leukemia. Cancer 1976;37:2177-82.
4. Holohan TV, Terasaki PI, Deisseroth AB. Suppression of transfusion-related alloimmunization in intensively treated cancer patients. Blood 1981;58:122-8.
5. Dutcher JP, Schiffer CA, Aisner J, Wiernik PH. Long-term follow-up of patients with leukemia receiving platelet transfusions: Identification of a large group of patients who do not become alloimmunized. Blood 1981;58:1007-11.
6. Bowen TJ, Berman-Wong E, Dawson D. Comparison of three crossmatch techniques to immunofluorescence test, and enzyme-linked immunoabsorbent assay. Curr Stud Hematol Blood Transf 1986;52:47-54.
7. McGrath K, Wolfe M, Bishop J. Transient platelet and HLA antibody formation in multitransfused patients with malignancy. Brit J Haematol 1988;68:345-50.
8. Paul WE, Bona C. Regulatory idiotopes and immune networks: a hypothesis. Immunol Today 1982;3:230-4.
9. Kearney JF. Idiotypic networks. In: Paul WE (ed). Fundamental Immunology. Raven Press, New York 1989:663-78.
10. Greally JM. The physiology of anti-idiotypic interactions: From clonal to paratopic selection. Clin Immunol Immunopath 1991;60:1-12.
11. Reed E, Hardy M, Latte C. Antiidiotypic antibodies and their relevance to transplantation. Transplant Proc 1985;17:735-8.
12. Barkley SC, Sakai RS, Ettenger RB, Fine RN, Jordan SC. Determination of anti-idiotypic antibodies to anti-HLA IgG following blood transfusion. Transplantation 1987;44:30-4.
13. Phelan DL, Rodey GE, Anderson CB. The development and specificity of antiidiotypic antibodies in renal transplant recipients receiving single-donor blood transfusions. Transplantation 1989;48:57-60.
14. Freedman J, Blanchette V, Hornstein A, et al. Utility of antiplatelet antibody detection methods in alloimmunized leukemics. Blood 1991;78:35a(abstract).
15. Freedman J, Hornstein A. Simple method for differentiating between HLA and platelet-specific antibodies by flow cytometry. Amer J Hematol 1991;38:314-20.
16. Hardy RR. Purification and characterization of monoclonal antibodies. In: Weir M (ed). Handbook of Experimental Immunology. Vol 1. Blackwell Scientific Pub, Oxford 1986:13.1-13.13.
17. Atlas E, Freedman J, Blanchette V, Kazatchkine M, Semple J. Down-regulation of the anti-HLA alloimmune response by variable region-reactive (antiidiotypic) antibodies in leukemic patients transfused with platelet concentrates. Blood 1993;81:(in press).
18. Burlingham WJ, Pan MH, Mason B, Ceman S, Sollenger HW. Induction of antiidiotypic antibodies to donor HLA-A2 following blood transfusions in a highly sensitized HLA-A2 recipient. Transplantation 1988;45:1066-71.
19. Reed E, Beer AE, Hutcherson H, King DW, Suciu-Foca N. The alloantibody response of pregnant women and its suppression by soluble HLA antigens and anti-idiotypic antibodies. J Reprod Immunol 1991;20:115-28.
20. Suciu-Foca N, Reed E, Rohowsky C, King P, King DW. Anti-idiotypic antibodies in anti-HLA receptors induced in pregnancy. Proc Natl Acad Sci USA 1983;80:830-4.
21. Rossi F, Sultan Y, Kazatchkine MD. Anti-idiotypes against autoantibodies and alloantibodies to VIII:C (anti-haemophilic factor) are present in therapeutic polyspecific normal immunoglobulines. Clin Exp Immunol 1988;74:311-6.

22. Rossi F, Kazatchkine MD. Antiidiotypes against autoantibodies in pooled normal human polyspecific Ig. J Immunol 1989;143:4104-9.
23. Rossi F, Dietrich G, Kazatchkine MD. Anti-idiotypes against autoantibodies in normal immunoglobulins: Evidence for network regulation of human autoimmune responses. Immunol Rev 1989;110:135-49.
24. Berchtold P, Dale GL, Tani P, McMillan R. Inhibition of autoantibody binding to platelet glycoprotein IIb/IIIa by anti-idiotypic antibodies in intravenous gammaglobulin. Blood 1989;74:2414-7.
25. Dietrich G, Kaveri S-V, Kazatchkine MD. Modulation of autoimmunity by intravenous immune globulin through interaction with the function of the immune/idiotype network. Clin Immunol Immunopath 1992;62:S73-S81.
26. Nydegger UE. Intravenous immunoglobulin in combination with other prophylactic and therapeutic measures. Transfusion 1992;32:72-82.
27. Glotz D, Sansonetti N, Francois A, Menyo-Cologne V, Druet P. In vitro inhibition of anti-HLA antibodies (Ab) by human polyclonal immunoglobulins (IVIg). J Am Soc Nephrology 1990;1(4):748(abstract).
28. Claas FHJ, Smeenk RJT, Schmidt R. Allo-immunization against the MHC antigens after platelet transfusion is due to contaminating leukocytes in the platelet suspensions. J Exp Hematol 1981;9:84-9.

DISCUSSION

T.H. The, L.R. Overby

F.H.J. Claas (Leiden, NL): Dr. Meryman, you showed that when there is incompetent APC, you can just restore the function by adding IL-2. Can you also do the opposite; not to deplete the blood from APCs but just add anti-IL-2 antibodies so the blood will not immunize anymore.

H.T. Meryman (Rockville, MD, USA): No, we have not done those experiments. A decade or so ago, as I recall, Terry Strom reported inducing allograft tolerance in rodents with an antibody against the IL-2 receptor[1,2] and I would presume it would also inhibit alloimmunization from transfusions. In our experiments the exogenous IL-2 is not making an incompetent APC into a competent one. It is merely supplying the IL-2 that would normally be secreted by the T-cell itself had the second signal been provided. But your suggestion is a good one.

T.H. The (Groningen, NL): The question was about the appropriateness of IL-2 addition to the responding cells. I wonder whether this effect does require the upregulation of the IL-2 receptors.

H.T. Meryman: Much of this work, of course, has been done by Jenkins and Schwartz at the NIH[2]. As they and we understand it, the acquisition of the MHC peptide signal is sufficient to induce a number of intracellular changes in the CD4 cell and the expression of the IL-2 receptor. This primes the CD4 cell to take the next step which will be the secretion of IL-2 which, among other functions, is necessary for its own proliferation. That is what, in this model, the accessory signal is presumed to do.

1. Strom TB, Gaulton GN, Kelley VE, et al. Treatment with anti-interleukin-2 receptor monoclonal antibody. In: Meryman HT (ed). Transplantation: approaches to graft rejection. Alan R. Liss Publ. New Yor 1986:227-38.
2. Jenkins MK, Schwartz RH. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. J Exp Med 1987;165:302-19.

H.J. Heiniger (Bern, CH): Dr. Jansen, how high are the TNF levels in these GvHD patients and how high are they when you give them pentoxiphylin?

J. Jansen (Indianapolis, IN, USA): To be honest, I think the only one who has measured the levels was Jack Singer, who did the study, but I do not recall the actual levels. They have looked at levels before and after treatment of TNF, published probably in 1991 in Blood.[1]

E. Racadot (Besançon, F): I can answer to the question on TNF levels in acute GvHD patients. We work in this field and we found only high levels in 25% of patients. But even in patients with low levels of TNF, we can have good results with an anti-TNF monoclonal antibody. We think that TNF maybe high in the skin or the liver and perhaps low in the serum.

A. Brand (Leiden, NL): Dr. Semple, you showed that 6 out of 6 pregnant women produce antibodies against HLA antibodies. Apparently, they are not protective for immunization in terms of antibody formation. Do you know how many of these 6 had HLA-antibodies as well?

J.W. Semple (Toronto, C): I believe 3 out of 6 of them had anti-idiotypes that were in fact inhibitory; whether they were protective I cannot answer. We could correlate refractoriness or lack of refractoriness with the production of anti-idiotypes.

A. Brand: Did you look of IVIg?

J.W. Semple: Yes, in terms of the inhibitory anti-idiotypes if you preabsorb the serum with protein G, which binds all human IgG subclasses; in fact you can reverse the anti-idiotype binding in all cases. So they are all IgGs. We have not found any IgMs, although they have been reported in pregnant women.

M. Cottler-Fox (Bethesda, MD, USA): Dr. Brand do you have any information or data on how many white cells might be needed for a recall HLA antibody formation in someone, who perhaps from pregnancy had previously HLA antibodies but does not originally, when you test them just prior to bone marrow transplant. When they are present two or three weeks later, presumably they could not have been formed de nove immediately poststransplant, so it must be a recall.

A. Brand: The minimal number of leukocytes necessary for recall are not known, but 10^7 is sufficient to induce antibody response. Initially, I thought

1. Bianco JA, Appelbaum FR, Nemunaitis J, et al. Phase I-II trial of pentoxifylline for the prevention of transplant-related toxicities following bone marrow transplantation. Blood 1991;78:1205-11.

white cells are not of any importance for recall; all you need is just antigenic stimulus in the form of class I on platelets or even red cells. I recently changed my opinion, because there are new preliminary data that if you deplete below 10^5 or 10^4, maybe you can also abolish the recall. I do not know whether Frans Claas can comment on animal studies related to this subject, because those studies showed that a recall in mice can be induced by class I antigens only. I am not aware that any leukocyte levels have been tested with respect to recall immunization in animals.

F.H.J. Claas: We did not do that systematically. But I can say, that in mice, for the platelet suspensions to have a boosting effect, the calculated amount of leukocytes should be less than 1000 in that suspension.

P. Rebulla (Milan, I): Dr. Jansen, is there a difference in the clinical expression of post-transfusion graft-versus-host disease in the immunocompetent versus immunodepressed patients? Could there be reactions which are not detected at the clinical level?

J. Jansen: Yes, I think obviously in the immunocompetent patient, where there is no chemotherapy given, it is mostly well recognized. It is probably pretty clear in open-heart surgery, when somebody develops pancytopenia, diarrhoea and a skin rash. I believe in particular in leukemia treatment, it is heavily underrecognized. I have always been convinced that part of the extreme skin rashes, that initially were reported with patients on high dose Ara-C, were transfusion induced graft-versus-host disease. I believe that in hemato-oncology patients receiving high dose chemotherapy, the reaction probably goes heavily underreported and the patient ends up pancytopenic; so that is not going to give you any help either. Diarrhoea is pretty common with chemotherapy. Although I believe that the picture probably is more or less the same, I think it is often very difficult to diagnose in patients who have been on chemotherapy.

E.F. van Leeuwen (Utrecht, NL): Drs. Meryman, Brand or Jansen, you all advocate the UV irradiation of blood to prevent alloimmunization as well as graft-versus-host disease. Are there any contra's for this in vitro treatment? Dr. Meryman, you said that UV will not suppress, will not prevent immunosuppression by blood transfusion.

H.T. Meryman: The conclusion that I would draw from our studies is that UV-B irradiation will induce immuno-suppression at the same time and by the same mechanism that it prevents alloimmunization, that is, by inhibiting the expression of the accessory signal.

J. Jansen: I think in bone marrow transplantation, it has only been studied in the murine model. the study of Jochem Deeg showed that mice get good engraft-

ment without creating graft-versus-host disease[1]. As far as I am aware, nobody has used it in clinical bone marrow transplantation. A good model perhaps to use would be autologous bone marrow transplantation where we have a back-up to make sure, that the bone marrow really gets established.

J.Th.M. de Wolf (Groningen, NL): I like to comment on the paper of dr. Meryman concerning the immunogenicity of stored blood. We are using in Groningen for many years more or less stored blood products. Until January 1st, 1992 we studied 90 acute leukemia patients treated with chemotherapy and a mean of 3 to 4 aplastic periods. They were treated with a red blood cell concentrate stored for 3 weeks and then leukocyte depleted by filtration, and random platelet concentrates stored for 4 to 5 days. In those 90 previously not sensitized patients, we only had 2% HLA immunization and refractoriness. Looking at the mechanisms, we investigated amongst others the role of the adhesive proteins necessary for the interaction between antigen presenting cells and T-cells and we found close to 90% reduction in the expression of ICAM-1 adhesive proteins.

F.H.J. Claas: Dr. Semple, your first screening on anti-idiotypic antibodies was on Fab's just made from typing sera. Does that mean, that everybody is using the same kind of idiotypes and anti-idiotypes to regulate this immune response against an HLA antigen or do you need many typing sera to screen for a certain specificity?

J.W. Semple: We have screened all the specificities we can of all the anti-HLA sera. Most of the patients actually tend to broaden in their specificity, but some of them tend to be narrow. Most of the specificities are the A2 and B7, which are the most common in the population. So any high titred HLA Fab-2 should be sufficient to at least screen a large majority of the anti-idiotypes.

F.H.J. Claas: But that means that if one person makes an anti-A2 idiotype, this is exactly the same as an anti-A2 made by another person.

J.W. Semple: I do not necessarily think that is true. I think in a polyclonal response against A2 for example you are going to see a variety of public and private idiotypes, that are generated. But I think what this assay screens for is generally the public idiotypes that are shared amongst the individuals.

We have tested IVIg in our system. In fact, against either anti-HLA Fab-2s or normal serum Fab-2s there is generally a higher reactivity. We have not done any specific tests to look at all the specificities. It is a crude test just to look at Fab-2 reactivities.

1. Cohn ML, Cahill RA, Deeg HJ. Hematopoietic reconstitution and prevention of graft-versus-host disease with UV-B irradiated haploidentical murine spleen and marrow cells. Blood 1991;78:3317-22.

P.C. Das (Groningen, NL): Dr. Vellenga, of all the varieties of cytokines described, we know in clinical practice erythropoietin is very good for red cells and granulocytic factor is good for white cells. What, in the context of transfusion medicine, will be your view on the future – which of the cytokines is going to play a very important role in for example alloimmunization, graft-versus-host disease and so on?

E. Vellenga (Groningen, NL): I think that depends on the underlying disease. When you look for example to the problem of autologous bone marrow transplantation then there is an increased tendency to isolate peripheral blood stem cells, which can enhance the recovery from pancytopenia after myeloblative chemotherapy. In that situation you think of IL-3 in combination of G-CSF or GM-CSF. But looking to the problems of graft-versus-host disease then alternative approaches are available. Recently, it was demonstrated that the use of the IL-1 receptor antagonist reduced significantly the incidence of acute graft-versus-host disease in pigs suggesting that this cytokine may be promisive in this field. Finally the cytokines affect not only the functional activity of immature cells but also of mature cells. It is conceivable that in the future different hematopoietic growth factors may be used to optimalize the storage of different blood products which contain terminal differentiated cells.

T.H. The: Dr. Takahashi as far as I can recollect UV-B irradiation effects the capping capacity of certain cells, B-cells perhaps or monocytes. In that way they loose their capacity to cap on treatment after treatment with anti-HLA antibodies. How could I understand these findings in the perspective of the immunological functions of these treated cells.

T.A. Takahashi (Sapporo, J): Well, first antigen presenting cells and T-cells should bind by MHC and accessory molecules, but only to give the complete signal to molecules on the antigen presenting cells. It is believed that the molecules not only adhere to each other, but they then give the signal to the T-cells. I think that is important. It is very interesting that UV-B irradiation completely inhibits that capping formation. I always just believed that cap formation is very important to give a complete signal to the T-cells. I think nobody could exactly show the capping formation to be very important, since the assay system is quite difficult, as the calcium is going to enter into cells to prevent capping formation. We like to do the humoral assay where the antigen presenting cells, of which only capping formation is prohibited but the response is not effected. It is quite difficult to have such an assay system.

H.J. Heiniger: Dr. Semple, according to Kazatchkine in Paris these anti-idiotypic antibodies play a central role in the regulation of the immune system in general[1,2]. Also I believe he showed that autoimmune responses were repressed. Among others, the formation of factor VIII inhibitors. Did you find in your studies a relationship with age, are the titres of the anti-idiotypes increasing with older age?

J.W. Semple: I have not. I have not looked at the patients in terms of age.

H.J. Heiniger: I think this will be important, because of a therapeutic approach as you said with IVIg. One should look at that, whether it is true that autoimmune responses are suppressed throughout life. If so, then older persons should have a high titre.

J.W. Semple: I do not think it has even been clearly shown that there is an increase with age of anti-idiotypes. If they do increase, it is unclear whether they suppress autoimmunity.

L. Dadiotis (Athens,G): Dr. Semple, do the anti-idiotypic antibodies downregulate the production or simply bind and inactivate the alloantibodies produced?

J.W. Semple: That is the 64,000 dollar question. They probably do both. Theoretically they should inhibit the interaction of soluble anti-HLA molecules with the platelet membrane. But with respect to non-site associated anti-idiotypes, they have been shown very clearly to regulate B-cells and T-cells based on confirmational changes when they bind to the cell's receptors. So, I think both mechanisms come into play in the idiotype network.

P.F.W. Strengers (Amsterdam, NL): Dr. Jansen, you did not mention the therapeutical approach with IVIg, although the group of Seattle published studies showing that in the group which has received IVIg there was a lower incidence of graft versus host compared to the other group[3,4,5]. Could you comment on that?

1. Rossi F, Dietrich G, Kazatchkine MD. Anti-idiotypes against autoantibodies in normal immunoglobulines: Evidence for network regulation of human autoimmune responses. Immunol Rev 1989;110:135-49.
2. Sultan Y, Kazatchkine MD, Maisonneuve P, Nydegger UE. Anti-idiotypic suppression of autoantibodies to factor VIII by high-dose intravenous immunoglobulins. Lancet 1984;ii:765-8.
3. Winston DJ, Ho WG, Lin CH, et al. Intravenous immunoglobulin for prevention of interstitial pneumonia after bone marrow transplantation. Ann Intern Med 1987;106:12.
4. Sullivan KM, Kopechky K, Jocom J, et al. Antimicrobial and immunomodulatory effects of intravenous immunoglobulin in bone marrow transplantation. Blood 1988;72:410a.
5. Elfenbein G, Krischer J, Grahampole J, et al. Intravenous immunoglobulin for cytomegalovirus pneumonia. In: Imbach P (ed). Immunotherapy with intravenous immunoglobulins. Academic Press, London/San Diego 1991:219-28.

J. Jansen: I think it would first have to be confirmed. I have a little difficulty with the set-up that is often used in Seattle, in which you take 200 patients. You randomize them over 200 different studies and you assume that for 199 of those studies they are randomly distributed. So, I think there are so many things going on at the same time, that I am not convinced that it really worked. I think what may support the concept is, that in the last five years, when everybody has been using immunoglobulin in general we have seen less graft-versus-host disease than before. But I thank it is too early to really recommend that as a way of preventing graft-versus-host disease.

C.Th. Smit Sibinga (Groningen, NL): May I come back to the presentations of dr. Meryman and Brand and related to that the presentation of dr. Takahashi. Indeed, the information shown in the in vitro set-up on aging of white cells while stored, I assume at 4°C in the red cells, is very interesting. Is there any data available at this point in time on dose relationship of the time of storage to the remaining amount of functional white cells?

The second question relates to dr. de Wolf's comment. Indeed, we have a very remarkable effect on the clinical incidence of sensitization and refractoriness, having used for years filtered blood which was stored for at least two to three weeks. Following leukocyte depletion by filtration the average remaining amount of white cells varies somewhere between 10^6 and 10^8. Would it be likely that the cells remaining in these products are of a completely different immune type, affected by the storage. What do you think would that effect be?

H.T. Meryman: Our data do suggest that there would be a progressively decreasing probability of alloimmunization during blood storage. However, I think that this relationship would be extremely difficult to demonstrate clinically. The probability of alloimmunization seems to be influenced by so many factors that it would be difficult to get data that would fall on a nice straight line. Our experiments really cannot be interpreted beyond saying that after thirteen days of storage we were able to demonstrate an in vitro immune response by our mononuclear cell preparation, but whether there would be a clinical dose relationship at shorter periods of storage I cannot say.

C.Th. Smit Sibinga: No, but I think these observations are very valid as there is also older information published in Nature[1], based on primate experiments. Primates reacted differently to stored homologous blood as compared to fresh homologous blood, where the rate of sensitisation to stored blood was significantly less than that to fresh blood. It may mean that the whole story of the remaining amount of white cells in a filter unit of red cells may be put into a different perspective related to the storage time, the shelf-life of the red cells, instead of what we are thinking now for in general on transfusions of red cells. That brings me

1. Batchelor JR, Welsh KI, Burges H. Transplantation antigens per se are poor immunogens within a species. Nature 1978;273:54-7.

to the point of dr. Brand's presentation. We cannot store the white cells in platelet concentrate for the same period of time and under the same conditions as we can store the red cells.

So, there is a different phenomenon. The more likely the white cells in platelet transfusions are basically the trigger. What you said is, that in the secondary type of immune response the class I alone probably might be the trigger. Could it be so that class II antigens, which have gone of the white cells and became soluble might induce in the host an autotype of phenomenon through an adhesion on the host antigen presenting cells, where that particular host cell loaded with the class II antigens of the donor might trigger the phenomenon and give a mimicking effect to the class I alone, where in the fact it is the combination of both.

A. Brand: That is smart, very smart. Several publications have shown that at the first place, during storage the class I antigens are not decreasing, where class II antigens are decreasing already after a couple of days. What may be important especially in relation to the presentation of dr. Takahashi is: What is the medium of the storage. One can imagine and that was also a question to dr. Meryman, that if one stores the nucleated cells in whole blood, one will have a different storage medium in which definitely substances like free oxygen radicals are generated as compared to storage of nucleated cells in a red cell depleted medium. That may be one of the reasons. Additionally, class I is a soluble antigen present in the plasma, but I am not aware how class II is processed and degraded, is there any consumption of adhesion to be expected from soluble class II antigens?

J.W. Semple: These then must be isolated α and β chains though in the plasma.

F.H.J. Claas: Yes, sure. I think the theory of soluble HLA is a good idea and may be even so if you have a primed immune system. It is very likely that when you have already B-cells which are activated specific for a certain antigen, that via those B-cells for instance even the class I molecules can be picked up from the platelets and presented in an autologous APC system, so that the B-cells for the recipient start to present the allopeptides to its own immune system and that you get help from different types of helper cells compared to the primary immuneresponse. So you first start with T-helper cells recognizing the class II of the donor, but may be later on you might also have T-helper cells recognizing donor peptides on your own APC's. In kidney transplantation there is a lot of evidence for that type of regulation of the immune response. So the same might hold true in a secondary immune response to platelets.

H.T. Meryman: There is also the possibility in the platelet transfusion that the CD8 and the B-cells will recognize the class I on the platelets and then receive the necessary help from a totally independent and unrelated immunological

event such as a virus infection that is going to activate cells and provide that additional service signal.

C.Th. Smit Sibinga: So, actually we come to the concept of the immune bystander phenomenon related to autologous lymphocyte activation triggering the whole phenomenon. Then a last question to dr. Takahashi. Very interestingly you showed that the UV-light radical formation apparently on the membrane changes the structure and therefore, makes the cell less prone to be active as an antigen presenting cell. However, I wonder why not in the same time intracellular structures are affected because the major source of radicals is water and the cell is just a watery balloon with a membrane and its cytoplasmic structures inside. It is known for instance, that in cancer treatment the application of radicals has an effect on the mitosis of cancer cells, so apparently effects the DNA. Why is it, that you did not observe that?

T.A. Takahashi: The UV-B-irradiated cells also showed more morphological changes under an electronmicroscope following incubation. After 12 hours of incubation the microvilli have disappeared and we could see some holes in the membrane. So there is, indeed, a big morphological change inside and on the surface of the cells, not only the membrane.

C.Th. Smit Sibinga: But did you observe in vitro any proliferation capacity still left?

T.A. Takahashi: Yes, there is still some. The same morphological changes are observed in the cells irradiated by gamma-rays. So the same morphological changes are seen in the UV-irradiated and the gamma-irradiated cells, but the gamma-irradiated cells serve as the stimulator cells, where UV-radiated cells do not, reflecting the difference in proliferative capacity.

H.T. Meryman: In that regard, dr. Takahashi, what would be the role of free radical scavengers such as superoxide dismutase?

T.A. Takahashi: I can only assume that more superoxides are produced.

H.T. Meryman: I would like to add a comment regarding the effect of gamma versus UV-B irradiation. We looked also at gamma irradiation in our system and found that, at clinically acceptable levels, there is a reduction in the proliferation of the T-cells although not sufficient to be clinically interesting. The gamma-irradiated cells also were found to recover during the first hour following irradiation, although not if the cells were held for an hour in the cold, following which the damage became irreversible. In the case of UV-B irradiation, as I showed earlier, T cells retained the capacity to proliferate when IL-2 was provided, showing that the UV-B does not kill these cells.

M.K. Elias (Groningen, NL): Dr. Meryman, we now understand that a transfusion can either immunize or immuno-suppress according to the production of co-stimulatory signals or failures of production of these signals. We know from the history of platelet transfusion, that a certain percentage of patients never becomes alloimmunized whatsoever. Could this failure of alloimmunization in these patients be explained on the basis of the concept of anergy and if so what makes that in some patients these signals are produced and in others not.

H.T. Meryman: Anergy is a possible explanation for the absence of alloimmunization following transfusion but it is not necessarily a requirement. The experiments of Oh and McClure that I showed you demonstrate that it does not take very much to prevent alloimmunization by transfusions[1]. Since they prevented immunization by administering autologous platelets, a cellular immune process clearly was not involved. They were probably stimulating prostaglandin release or some other phenomenon of that sort which induced a alloimmunization. My conclusion is that alloimmunization by transfusions is a borderline event and a mild amount of immune suppression can prevent it. We also have the impression that if the first few transfusions do not alloimmunize, you are probably home free, but I am not prepared to argue that a cellular event such as anergy is essential.

1. Oh JH, McClure HM. Lymphocytotoxic antibodies induced by fresh blood, stored blood and platelets in rhesus monkeys. Transplantation Proc 1982;14:410-2.

II. PROCESSING ASPECTS

OPTIONS FOR PREVENTION OF T CELL ALLOACTIVATION BY BLOOD TRANSFUSIONS[1]

F.H.J. Claas, H.S. de Koster, E.L. Lagaaij, J.J. van Rood

Introduction

Blood transfusions may have opposite immunological effects. They can either lead to immunization of the recipient or induce immunological tolerance. The latter effect might be very helpful for clinical transplantation, whereas immunization is generally considered to be a negative effect for both patients in need for further blood transfusions and for patients waiting for an organ transplantation. The most easy parameter for immunization is the presence of circulating alloantibodies. In HLA-alloimmunization, it is known that most of these alloantibodies are of the IgG type, which is a clear indication that also T cell activation has taken place during the immunization process. A pivotal role in allorecognition is played by $CD4^+$ T cells, which recognize the foreign HLA class II molecules of the transfused cells. Once these $CD4^+$ T cells are properly triggered by alloantigen presenting cells in the first phase of the immune response, they will, either directly or indirectly via cytokines and other factors, initiate and facilitate the actual effector processes of the immune response leading to IgG antibody formation and maturation of cytotoxic T lymphocytes. Therefore one can expect that prevention of activation of these $CD4^+$ T cells may be an effective way to prevent alloimmunization. Induction of tolerance, is another possible strategy to prevent alloimmunization. For the induction of tolerance, we hypothesize that not prevention of activation but activation of certain (allopeptide specific) $CD4^+$ T cells might be essential.

Prevention of T cell activation

Already in the early fifties it was shown that blood transfusions can lead to the induction of leukoagglutinins [1], which later proved to be alloantibodies against the HLA antigens of the donor. Although such antibodies were very useful for the definition of the different HLA antigens, as were pregnancy sera [2], they

1. These studies were partially supported by the Dutch Kidney Foundation, the J.A. Cohen Institute for Radiopathology and Radiation Protection (IRS) and the "Praeventiefonds".

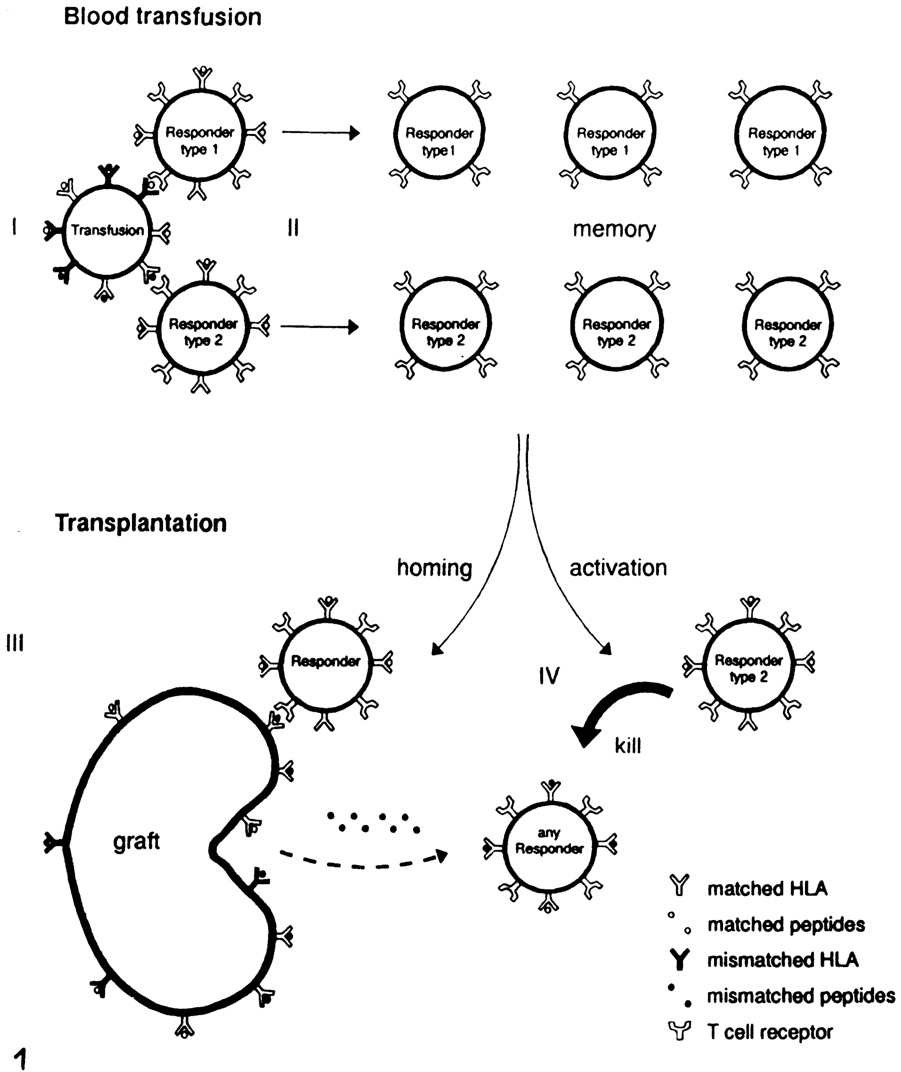


Figure 1. Active downregulation of graft rejection by a haploidentical blood transfusion.
Stage I: The blood transfusion results in priming of $CD4^{+}$ T cells directed against the mismatched HLA-DR molecules (type 1) and against matched HLA-DR molecules carrying allogeneic peptides (type 2).
Stage II: These sets of T cells remain present as memory cells.
Stage III: Introduction of the graft will activate all kind of allospecific T cells. Activated T cells start to express HLA-DR molecules and home to the graft.
Stage IV: Peptides shared between kidney and transfusion donor are presented by activated T cells to type 2 $CD4^{+}$ memory cells, resulting in elimination of any activated cell in the graft.

were not very helpful for patients in need for further transfusion therapy. The presence of such antibodies was associated with the occurrence of severe transfusion reactions and a poor survival of transfused platelets. Therefore several approaches were tried to prevent induction of alloantibodies after transfusion.

An essential role for mononuclear cells in the induction of alloantibodies by blood transfusions was shown by the fact that removal of contaminating leukocytes from platelet transfusates will prevent immunization against the HLA class I antigens of the platelet donor [3,4].

Probably, HLA class II positive leukocytes will activate $CD4^+$ helper T cells , which, on their turn, provide help to B-cells in order for these to develop into plasma cells producing antibodies against the HLA class I antigens of the donor. In the absence of leukocytes such activation of the B cells will not occur in naive recipients. It might well be that the situation is different in primed recipients as it can not be excluded that pure platelets are able to induce a secondary antibody response [3].

Another way to prevent antibody formation after platelet transfusion is by UV-irradiation of the leukocyte containing platelet suspensions [5,6]. UV-irradiation will prevent the secondary signals of the class II positive leukocytes, which are necessary to activate the $CD4^+$ helper T cells. This information is mainly coming from in vitro data in man and animals and in vivo data in mice. Clinical trials are performed at the moment in order to evaluate the effectiveness of UV-irradiated blood components in preventing antibody formation.

Another approach used to prevent alloantibody formation is the use of blood transfusions in combination with immunosuppressive drugs. This has mainly been done in renal transplant patients waiting for a graft and has indeed resulted in a lower percentage of antibody positive patients [7,8]. Of potential importance might also be the approach of injecting antibody coated blood cells, which, in a rat model, did prevent antibody formation and led to unresponsiveness against subsequent injection with uncoated blood cells [9].

Tolerization by blood transfusions

The potential tolerizing effect of blood transfusions was first observed in clinical renal transplantation. The success of the early transplantations was sometimes hampered by blood transfusion induced antibodies, that caused hyperacute rejection. In order to prevent immunization of the patients, less and less patients were transfused. However, instead of an improved graft survival the results became worse. After Opelz et al. [10] showed that pretransplant blood transfusions had a favourable effect on graft survival, patients received deliberate blood transfusions before transplantation.

The beneficial effect of blood transfusions has been confirmed in several studies, although recent reports show that the effect gradually diminishes [11,12] probably due to better patient care and more advanced immunosuppressive regimens. As was found for the immunizing effect of blood transfusions, leukocytes seem to be essential to obtain an immunosuppressive effect

of blood transfusions as well [13]. This immunosuppressive effect seems not to be restricted to organ transplantation but may also be involved in the recurrence of colorectal cancer in patients undergoing surgery. Patients receiving a perioperative transfusion have a significantly worse prognosis than patients undergoing cancer surgery without receiving a transfusion [14,15].

The need for partially HLA-matched blood transfusions

Both immunization and tolerization after blood transfusion have in common that they occur only in a proportion of the patients. Recent studies showed that sharing of at least one HLA-DR antigen between blood transfusion donor and recipient will lead to tolerization, whereas HLA-DR mismatched transfusions will lead to immunization [16]. Originally, these observations were made in relation to renal allograft survival. A prospective study in cardiac transplant recipients showed a similar effect on the number of rejection episodes after transplantation. Sharing of at least one HLA-DR antigen between transfusion donor and recipient will reduce the number of rejection episodes significantly. Later studies showed that one HLA-DR shared blood transfusion had less immunizing and in some cases tolerizing effects on several parameters of the immune response compared to HLA-DR mismatched transfusions (summarized in Table 1). An HLA-DR shared transfusion will lead to less antibody formation and there will be no increase in the donor specific MLR (mixed leukocyte reaction) and donor-specific CML (cell-mediated lympholysis), whereas HLA-DR mismatched transfusions do trigger antibody formation and T cell reactivity.

Limiting dilution studies performed to measure donor specific cytotoxic T cell precursor (CTLp) frequences in the presence of IL-2 showed that blood transfusion initially induces an increase of donor specific CTLp [17]. However at a later stage (after 4-5 weeks) donor specific CTLp disappear in the case of an HLA-DR (+ HLA-A and -B) shared blood transfusion. This was not found in the

Table 1. HLA compatibility between transfusion donor and recipient determines the immunological consequence of the transfusion.

	HLA-DR (haplotype) matched transfusion	**HLA-mismatched transfusion**
Renal transplant survival	↑	↓
Rejection episodes after heart transplantation	↑	↑
Antibodies	=	↑
MLR	=	↑
CML	=	↑
CTLp	↓↓	=↑

case of a mismatched blood transfusion [18]. These data suggest that sharing of at least one HLA-DR antigen and some of the HLA class I antigens between blood transfusion donor and recipient may lead to tolerization.

An additional requirement for the induction of the blood transfusion effect seems to be the presence of at least one HLA-DR mismatch between donor and recipient. This was demonstrated by studies of Lazda et al. [19], who showed a beneficial effect of haplo-identical blood transfusion only when there was HLA-DR disparity on the unshared haplotype.

A pivotal role of allogeneic peptides in the blood transfusion effect

From the foregoing one can conclude that to obtain a tolerance inducing effect of pretransplant blood transfusion, the following prerequisites are necessary: blood transfusion donor and recipient must share at least one HLA-DR antigen [16] or, a haplotype [18] and must be mismatched for the other one [19]. Because HLA-DR antigens play a central role and because both B cell responses (leukocyte antibodies) and T cell responses (cytotoxic T lymphocytes) are influenced by the blood transfusion, we suggest that a $CD4^+$ regulatory T cell is closely involved in the mechanism of the transfusion effect.

The difference between a one HLA-DR matched transfusion compared to a mismatched transfusion is that in the latter case $CD4^+$ T cells of the recipient are only confronted with allogeneic HLA-DR molecules whereas in the first case also self HLA-DR is present on the transfused cells.

Since the main function of HLA molecules is presentation of peptides [9] and empty HLA molecules are hardly expressed on the cell surface, these self HLA-DR molecules will be loaded by different kind of donor peptides, including peptides derived from mismatched HLA or other alloantigens.

Blood transfusion will lead to the activation of both $CD4^+$ T cells of the recipient which recognize the HLA alloantigens as such (type 1 alloreactive T cells, Figure 1) but also of allogeneic peptide specific self HLA-DR restricted $CD4^+$ T cells (type 2 alloreactive T cells, Figure 1). These sets of T cells remain as memory cells in the body, until a graft is introduced, which carries the same allogeneic peptides as the ones against which the T cells were initially primed (stage III, Figure 1). Recipient T cells are activated by the kidney donor alloantigens and start to express HLA class II antigens.

These T cells home to the graft and are able to pick up kidney donor peptides, including the ones shared by kidney donor and blood transfusion donor. In this way the activated T cells of the recipient are presenting the same allogeneic peptide on the same self HLA-DR molecules as the original blood transfusion donor and become targets for the reactivity of the type 2 $CD4^+$ cells (stage IV, Figure 1). This will lead to the elimination of all activated T cells that surround the graft (and that are able to pick up the particular allogeneic peptide) and thus in enhanced graft survival. A similar mechanism may also be involved in the tolerance to recurrent rectal cancers assuming shared peptides between allogeneic blood transfusion and malignant autologous cells.

Correlation between donor cell recirculation and tolerance

Another possible explanation for tolerance induction might be that the transfused cells act as deletional antigen presenting cells or veto cells. For this mechanism to occur, there is a need of a certain level of chimerism after blood transfusion, which should persist in order to keep the tolerant situation. Recent studies in the mouse showed indeed a correlation between persistence of transfused leukocytes in the recirculating pool of the recipient and the occurrence of tolerance [21] whether donor cells persists was correlated inversely with the level of natural killer cell (NK) activity in the host.

Although the specificity of NK cell recognition is not very clear yet, recent studies suggest that NK activity is based on the recognition of lacking self molecules [22]. This might be the reason why fully allogeneic cells cannot persist in case of normal NK activity, whereas semi-allogeneic cells, sharing a MHC haplotype, do. It remains to be established whether such a phenomenon is also involved in the observations described above.

Conclusions

Prevention of T cell alloactivation can either be based on a passive effect for instance, by giving transfusions lacking proper antigen presenting cells or an active process, in which transfusion can lead to immunological tolerance. Both approaches might be useful for prevention of antibody formation after blood transfusion, while the latter is preferred by immunologist involved in clinical transplantation. More research is needed, however, to clarify the mechanisms involved, in order to have a better insight in and more clinical benefit of the options described.

Acknowledgements

We wish to thank Ingrid Curiël for rapidly preparing the manuscript.

References

1. Dausset J. Leuco-agglutinins. IV. Leuco-agglutinins and blood transfusion. Vox Sang 1954;4:190-8.
2. Van Rood JJ, Eernisse JG, van Leeuwen A. Leucocyte antibodies in sera from pregnant women. Nature 1958;181:1735-6.
3. Claas FHJ, Smeenk RJT, Schmidt R, van Steenbrugge GJ, Eernisse JG. Alloimmunization against the MHC antigens after platelet transfusion is due to contaminating leukocytes in the platelet suspension. Exp Hematol 1981;9:84-9.
4. Eernisse JG, Brand A. Prevention of platelet refractoriness due to HLA antibodies by administration of leukocyte poor blood components. Exp Hematol 1981;9:77-83.
5. Deeg HJ. Transfusion with a tan: Prevention of alloimmunization by UV irradiation. Transfusion 1989;29:453-8.
6. Pamphillon DH, Corbin SA, Saunders J, Tandy NP. Applications of ultraviolet light in the preparation of platelet concentrates. Transfusion 1989;29:379-83.

7. Haisa M, et al. Donor-specific transfusion (DST) with intermittent administration of azathioprine induces suppressor T cells and MLR-inhibiting factors without sensitization. Transplant Proc 1989;21:1814-7.
8. Glass NR, Miller DT, Sollinger HW, Belzer FO. Comparative analysis of the DST and imuran-plus DST protocols for life donor renal transplantation. Transplantation 1983;36:636-41.
9. Susal C, Terness P, Opelz G. An experimental model for preventing alloimmunization against platelet transfusions by pretreatment with antibody-coated cells. Vox Sang 1990;59:209-15.
10. Opelz G, Sengar DPS, Mickey MR, Terasaki PI. Effect of blood transfusions on subsequent kidney transplants. Transplant Proc 1973;5:253-9.
11. Lundgren G, Albrechtsen D, Flatmark A, et al. HLA-matching and pretransplant blood transfusions in cadaveric renal transplantation, a changing picture with cyclosporin. Lancet 1986;ii:66-8.
12. Opelz G. Improved kidney graft survival in non-transfused recipients. Transplant Proc 1987;19:149-52.
13. Persijn GG, van Leeuwen A, Parlevliet J, et al. Two major factors influencing kidney graft survival in Eurotransplant: HLA-DR matching and blood transfusion(s). Transplant Proc 1981;13:150-4.
14. Voogt PJ, van de Velde CJH, Brand A, et al. Perioperative blood transfusions and cancer prognosis: Different effects of blood transfusion on prognosis of colon and breast cancer patients. Cancer 1987;59:836-43.
15. Wu H-S, Little AG. Perioperative blood transfusions and cancer recurrence. J Clin Oncol 1988;6:1348-54.
16. Lagaaij EL, Henneman JPH, Ruigrok M, et al. Effect of one HLA-DR antigen matched and completely HLA-DR mismatched blood transfusions on survival of heart and kidney allografts. N Engl J Med 1989;321:701-5.
17. Vandekerckhove BAE, van Bree S, Zhang LI, Datema G, Zantvoort F, Claas FHJ. Increase of donor-specific cytotoxic T lymphocyte precursors after transfusion. Transplantation 1989;48:672-5.
18. Van Twuyver E, Mooijaart RSD, ten Berge IJM, de Waal LP. Pretransplantation blood transfusion revisited. N Engl J Med 1991;325:1210-3.
19. Lazda VA, Pollak R, Mozes MF, Barber PL, Jonasson O. Evidence that HLA class II disparity is required for induction of renal allograft enhancement by donor-specific blood transfusions in man. Transplantation 1990;49:1084-7.
20. Neefjes JJ, Ploegh HL. Assembly and intracellular transport of MHC class II molecules. Immunol Today 1992;13:179-84.
21. Sheng-Tanner X, Miller RG. Correlation between lymphocyte-induced donor-specific tolerance and donor cell recirculation. J Exp Med 1992;176:407-13.
22. Moretta L, Ciccone E, Moretta A, Höglund P, Öhlén C, Kärre K. Allorecognition by NK cells: Nonself or no self? Immunol Today 1992;13:300-6.

USE OF MONOCLONAL ANTIBODIES FOR IMMUNE PURGING IN BONE MARROW TRANSPLANTATION

E. Racadot, P. Hervé

Introduction

Bone marrow transplantation (BMT) programs frequently include an ex vivo manipulation of the marrow cells either to remove T cells from allografts or to remove the remaining tumour cells from autografts. The specificity of immunologic purging methods is the use of monoclonal antibodies (mAbs).

The hybridoma technology, discovered by Köhler and Milstein [1] consists in the fusion of a normal immunological B cell committed to making antibodies with a malignant myeloma partner, thus producing a hybrid cell with the genetic information of both immortality and antibody synthesis. Each cell is thus empowered to produce unlimited amounts of a single or monoclonal antibody [2].

A defined mAb offers the major advantage of selectively recognizing cell targets, tumour cells or normal T lymphocytes, without affecting normal bone marrow progenitors [3,4]. However, since mAbs are not toxic by themselves, they must be used in conjunction with an additional agent such as heterologous complement, toxins or magnetic particles in order to cause specific elimination of target cells. Rabbit complement is the oldest and most commonly used adjunct for bone marrow purging with mAbs in autologous or allogeneic transplantation. For this reason we will focus on describing the protocol of bone marrow purging using complement-mediated cytolysis. The other available procedures using mAbs will also be mentioned.

Bone marrow purging methods

The three main techniques for bone marrow purging are complement-mediated cytolyses, immunotoxins and mAb-coated magnetic beads.

Monoclonal antibodies and complement-mediated cytolysis

In this manipulation, three partners are involved: the bone marrow which must be purified to limit non-specific toxicities and make the manipulation more reproducible, the monoclonal antibodies which must be carefully tested to determine their optimal dose and optimal association, and the complement which

must be carefully defined. Lastly, a precise protocol of immune depletion must be set up to obtain reproducible results.

I. Bone marrow purification

Several cell separators have been appraised with respect to their ability to concentrate the harvested whole bone marrow. Regardless of the machine used and the procedure followed (Ficoll Hypaque gradient or not), the mononuclear cell (MNC) suspension must not be contaminated by more than 10% granulocytes and 1% red blood cells [5,6,7]. After purification, MNCs are suspended in human albumin 4% and are ready for in vitro treatment.

II. Selection of rabbit complement

Rabbit complement must be well characterized to allow reproducible manipulations. Since 1984 we have produced our own complement in our institution. Baby rabbits (21-28 days) are bled by incision of the carotid artery and blood is collected in 20-ml sterile tubes. After centrifugation a sample of each tube is taken to look for the presence of heterophilic antibodies. All the positive sera are eliminated. The negative sera are pooled: 90 to 100 rabbits usually yield a 500 to 600 ml-batch. The quality control procedures which were performed on each batch are summarized in Table 1. All complement batches showed low endotoxin levels, negative viral testing, bacterial and fungal sterility. The optimal complement dilution is tested on each lot and may vary from dilution 1/2 to dilution 1/3.

Table 1. Characteristics of the baby rabbit complement (21-28 days old) produced in our institution.

- Lack of heterohemagglutinins
- Stable levels of Ca++ 3.06 ± 0.156 mmol/1
Mg++ 0.8 ± 0.05 mmol/l
- Reproducible hemolytic activity: 29.6 ± 7 units
- Lack of non-specific toxicity evaluated on peripheral blood lymphocytes, bone marrow mononuclear cells and CFU-GMs (GFU-GM recovery: 85% ± 9)
- Confirmed efficiency as evaluated on blood and bone marrow mononuclear cells after treatment with monoclonal antibodies (less than 1% residual T cells after treatment of blood MNCs with anti-T mAbs and two rounds of complement)
- After filtration on 0.22 μm filters, bacteriological controls are negative
- Limulus test negative
- Lack of viral particles (controls on monkey kidney cells, newborn mice, embryonic eggs)

III. Monoclonal antibody selection

– *Choice of mAb isotype:* Most antibodies are produced in mice or in rats and do not generally fix human complement. One exception is the well known CAMPATH-1, a rat IgM antibody which is able to fix human complement [8].

The other mAbs are able to fix rabbit complement if they are of IgG2, IgG4 or IgM isotype.

– *Choice of the antibody according to the target epitope:* It is necessary to choose antibodies which recognize one epitope on target cells: e.g. anti-T mAbs which eliminate normal T cells from a purged bone marrow in the case of allografts, anti-B or anti-T mAbs which eliminate residual leukemic cells from autografts. The choice is related with the leukemic phenotype: anti-CD19, anti-CD10, anti-CD20 [9], and anti-B activation antigens are the different possible anti-B mAbs [10], anti-CD2, anti-CD5, anti-CD7, and anti-CD25 can be chosen among anti-T mAbs [11]. In allogeneic BMT, the choice lies between pan-T or anti-T subset mAbs (i.e. CD8, CD6) [12].

– *Choice of the mAb with the highest cytotoxicity:* In order to choose one antibody among several antibodies belonging to the same cluster of differentiation (CD), an analysis of their cytotoxic capacities has to be performed, either by appreciating the rate of lysis of 51Cr labelled clonogenic cell lines [3,13], or by evaluating the viability of clonogenic cell lines or fresh leukemic cells with a Trypan Blue dye [4]. Figure 1 reports the results we obtained with this technique. We compared the cytotoxic activity of 5 different anti-CD10 mAbs:

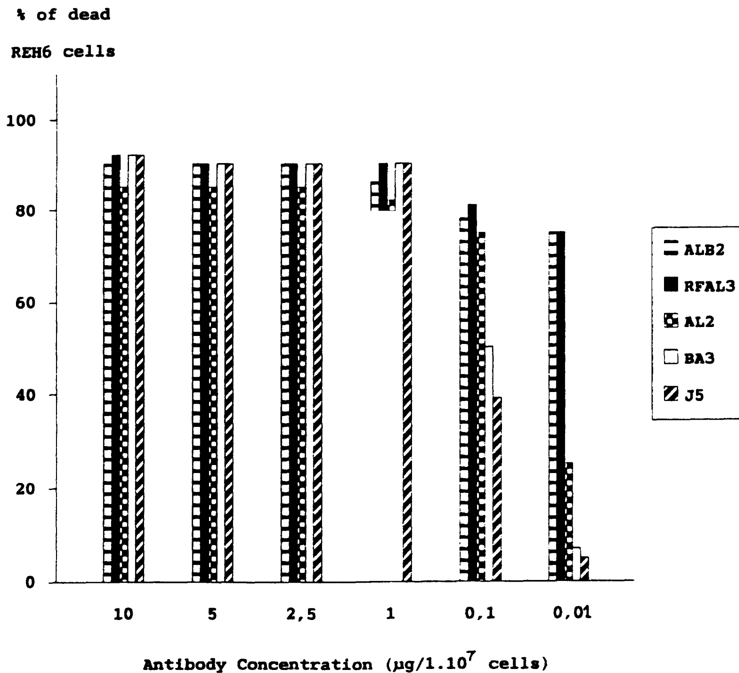


Figure 1. Comparative analysis of the cytotoxic activity of 5 anti-CD10 mAbs against REH6 cell line after one round of complement according to their concentration. Each value represents the mean of three tests (S.D. <10%).

J5/IgG2a [14] Ba3/IgG2b [15] ALB2/IgG2a [16], AL2/IgG2a (rat) [17] and RFAL3/IgM [18] on REH6 cells (REH6 cell line is a human B cell line expressing CD10). Except for AL2, a plateau of cytotoxicity was reached in a comparable way regardless of the antibody used. The highest cytotoxicity was obtained with concentrations of 0.5 to 1 ug per 1.10^7 cells. ALB2 retained its cytotoxic activity at very low concentrations, moreover it was available. We therefore chose this antibody for bone marrow purging.

– *mAb purity:* In order to be used for ex-vivo bone marrow purging, mAbs must meet the following pharmaceutical criteria: be sterile; contain no pyrogenic substance; contain less than 10 pg/ml of mouse DNA; and contain no mouse virus.

IV. Treatment modalities

Different parameters must be analyzed to optimize the manipulation: cell suspension concentration, number of complement rounds, use of an mAb cocktail.

– *Optimal cell suspension concentration (Figure 2):* We performed this analysis with the Trypan Blue exclusion test. Blood MNCs were incubated with an anti-T mAbs at progressive doses and then rabbit complement was added (dilution 1/2). A higher cell concentration was associated with a decrease in cytotoxicity levels. The cell concentration at which the best results were obtained was 2.10^7 cells/ml. The limiting factor seems to be the complement activity.

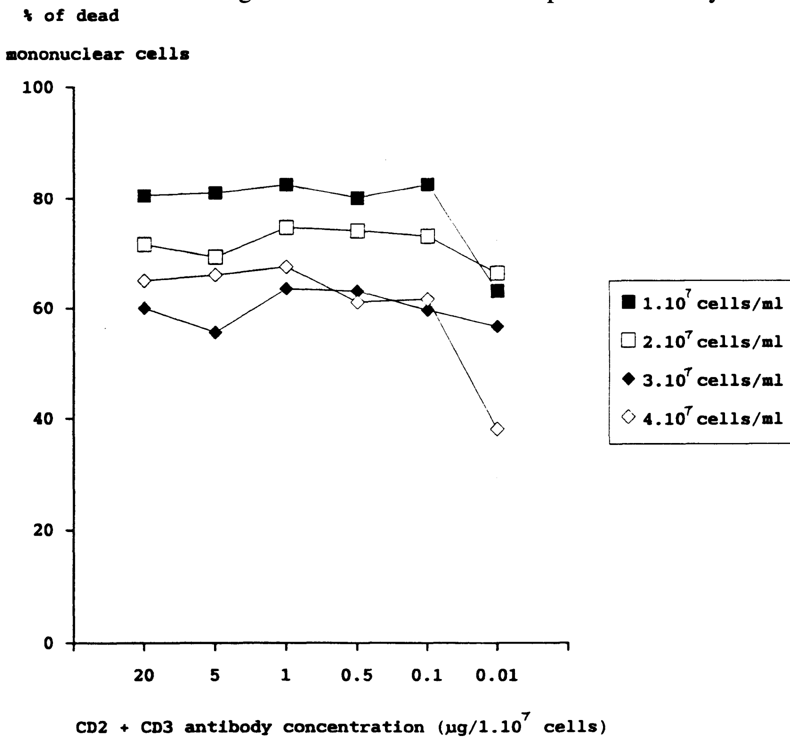


Figure 2. Effect of cell suspension concentration on the cytotoxic activity of pan-T mAbs against mononuclear cells after one round of complement. (Each point is the mean of three tests (S.D. <10%).

– *Optimal number of treatments:* We performed the treatment with mAbs and complement after contamination of a normal bone marrow with 5% REH6 clonogenic cells. This clonogenic assay appears to be a sensitive tool to evaluate the efficacy of bone marrow purging as is shown in Table 2. Two rounds of complement clearly resulted in a higher cytotoxicity level than did a single round (Figure 3)

Table 2. Quantitative assays for the detection of residual leukemic cells or residual T cells.

Methods	Log killing	Reference
Indirect IF + Ethidium bromide	2	19
Double staining combination TdT / c CD3 – CD13/TdT cμ/TdT	4	20,21
Flow cytometry	1.5-2	22
Hoechst dye technique	3-4	23
51 Cr release	2	3
Clonogenic assay	> 4	24
Limiting dilution assay	4	22,25,26
Gene rearrangements	1.5-2	27
Polymerase chain reaction	5	28

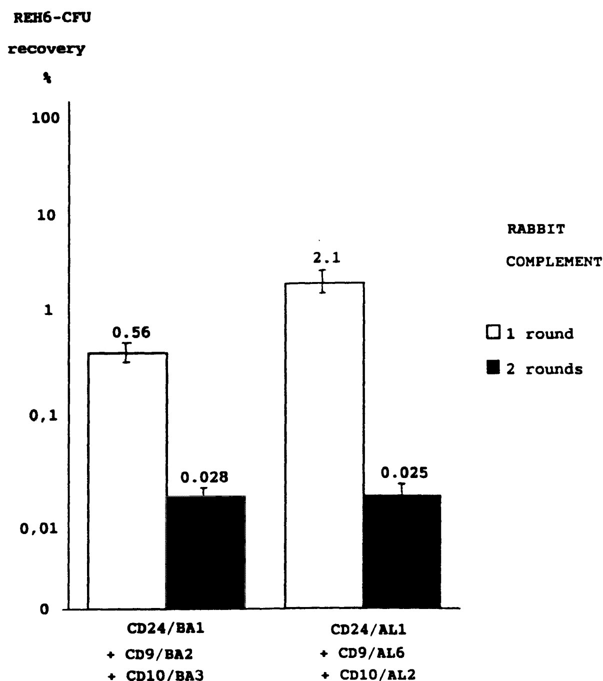


Figure 3. REH6-CFU recovery after cytolysis with an mAb cocktail and one or two rounds of complement. Each value represents the means of three tests (S.D. <10%).

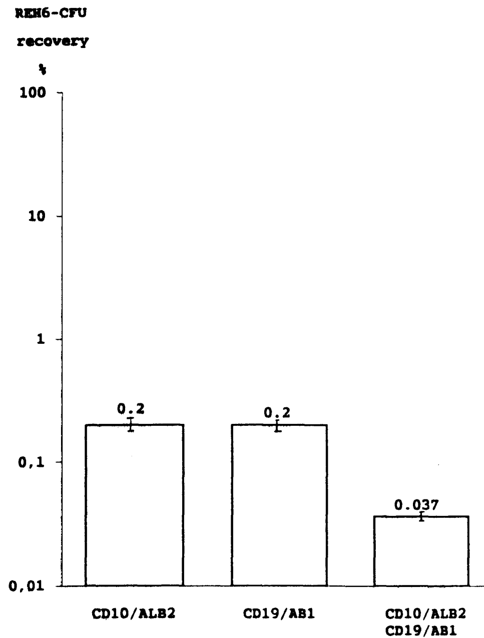


Figure 4. REH6-CFU recovery after cytolysis with one mAb or with an mAb cocktail and one round of complement. Each value represents the means of three tests (S.D. <10%).

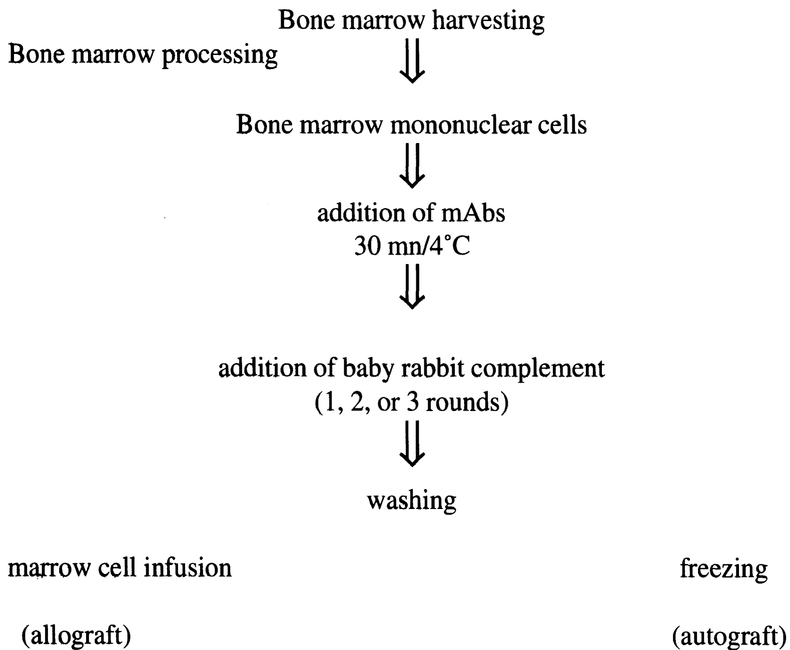


Figure 5. Bone marrow purging procedure using monoclonal antibodies and complement.

– *Optimal mAb cocktail (Figure 4):* The clonogenic assays we performed demonstrated the higher efficacy of an mAb cocktail recognizing different epitopes on the target cell surface as compared to a single antibody.

V. Feasibility of the manipulation

The feasibility of the in vitro treatment must be assessed by a study of CFU-GM and BFU-E bone marrow progenitors and by long-term cultures whenever a new mAb is used. For instance with a cocktail of anti-CD19 (AB1) + anti-CD10 (ALB2) mAbs plus two rounds of complement, we observed a CFU-GM recovery of 75 ± 15% [4].

The results of these preclinical studies led us to propose a bone marrow purging protocol which is described in Figure 5. The elimination of residual leukemic cells may require two or even three rounds of complement. A clonogenic assay using REH6 cells, bone marrow processing with anti-CD10/ALB2 mAbs + one round of complement resulted in 1-log depletion of leukemic cells whereas an mAb + two rounds of complement led to a 3-log depletion [4]. The same observation was made by Gribben [28]. The elimination of T cells from allografts may require only one round of complement in HLA-identical situations [7] but a more complete depletion may be required in mismatched allografts [29].

Monoclonal antibodies conjugated to toxins

Immunotoxins (IT) are hybrid molecules consisting of a monoclonal antibody covalently linked to a toxin [30]. But it is difficult to find a highly potent toxin devoid of high non-specific toxicity. Most single chain toxins, like pokeweed antiviral protein (PAP), saporin and gelonin have shown little toxicity against target cells when linked to mAbs. Similar results have been obtained with immunotoxins containing ricin A-chain alone. In this case it is necessary to add activators like NH4C1 and to respect strict pH conditions (7.5 to 7.8) for them to be active [30]. The intact ricin is the most powerful natural toxin. Its A-chain inactivates the 6 S ribosomes, and its B-chain binds to the galactose moieties found on the surface of all eukaryotic cells. The use of intact ricin offers the advantage of a simple, short ex-vivo incubation and does not require any additional biological activation [31]. However, the B-chain may be responsible for non-specific binding to normal cells. The blocking of the binding sites of the whole ricin makes it possible to avoid this non-specific binding while maintaining the unimpaired activity of both chains for increased intracellular toxicity. This "blocked-ricin" has been covalently linked to different mAbs thus resulting in ITs with increased cytotoxic activity [33].

Bone marrow purging with ITs usually requires a purification procedure, prior to the immune reaction. Mononuclear cells are then incubated with an IT for 2 h [31,32]. After incubation, washes are needed before infusion or freezing. When an IT is coupled to anti-T or anti-B mAbs no toxicity has been observed

on progenitors cells. But with anti-My9 blocked-ricin long incubations are toxic on the CFU-GM which express CD33 [34].

Evaluation of IT efficacy can be done with the tests described in Table 2. However, the viability tests performed at the end of the procedure (indirect IF, flow cytometry, Hoechst dye technique and 51Cr release) cannot be done because of the long delay necessary for target cell death: several hours, if not several days.

When compared with complement-mediated cytolysis the efficacy of IT seems similar: Moretti [25] reports a 3.7-log depletion of IL-2 precursors after treatment with anti-T IL-2 mAbs plus complement versus a 3-log depletion with a ricin A-chain anti-CD3 IT. In a clonogenic assay using the HL60 myeloid cell line, Roy [34] reports a depletion of 3.6-log for anti-My9 HL60 myeloid cell line + complement and 4.4-log for anti-My9 blocked-ricin after a 5-hour incubation. The main limitation for the use of these ITs is their current unavailability.

Monoclonal antibodies linked to magnetic beads

By coating magnetic beads with mAbs against different cell surface antigens, it is possible to deplete a bone marrow of the target cells expressing these antigens. Kemshead and his associates pioneered immunomagnetic purging in the treatment of neuroblastoma [35,36]. Currently, magnetizable beads are used for other malignancies like B lymphoma [37].

The immunomagnetic beads, Dynabeads M-450 (Dynal), have a diameter of 4.7 μm. They contain 22% Fe and they have surface of 69 μm^2/particle [38]. The mAbs, mainly of IgM isotype, may be directly coupled to M-450 beads. With IgMs of IgG isotype it seems more efficient to incubate the cells first with primary and then with magnetizable M-450 beads coated with a second antibody (sheep anti-mouse IgG antibody (SAM)).

A new type of beads: M-280 (Dynal) are now under study. They are smaller (diameter 2.8 μm) and contain less iron than M-450 beads. When they are coated with a polyclonal sheep anti-mouse antibody they have the same efficacy, whether used together with IgG or IgM antibodies. Thus bone marrow purging may be done with a cocktail of mAbs regardless of the Ig isotype [Kvalheim, unpublished]. The optimal conditions for marrow treatment using the magnetic procedure are well defined: the marrow inoculum must be purified prior to magnetic separation. Bone marrow MNCs are incubated with the chosen bone marrow purging for 30 min at 4°C. the cell suspension is washed twice in phosphate-buffered saline (PBS), resuspended to a final concentration of 1.10^6/ml and incubated with SAM beads at 4°C for 30 min under gentle rotation. The bead-coated cells are then collected in a magnetic field. The entire procedure is non-toxic for the progenitor cells. The mean recovery rate of normal CFU-GM is usually higher than 70% [37]. The techniques of immunobead purging and complement-mediated cytolysis yield similar results in terms of target cell depletion. In a culture assay with a mixture of 1% HL60 cells and normal marrow, Guyotat [39] found a lysis of more than 99% with an SAM cocktail plus two rounds of complement and with antibody-coated magnetic beads.

Another approach, described by Bieva et al. [40] uses specific SAMs directly coated with smaller-size super paramagnetic particles (iron content > 96%). These particles are inexpensive. The purging time is reduced, due to a simple manipulation, and a more than 3-log depletion can be expected after one purging cycle. Studies are being carried out to compare these beads with Dynal beads and with complement-mediated cytolysis.

The possibility of positive selection of human hematopoietic progenitor cells with M-450 Dynabeads coated with anti-CD34 mAbs must be mentioned [41]. The subsequent detachment of Dynabeads makes it possible to isolate pure CD34+ cells (purity higher than 90%) without encountering viability problems [42].

Clinical applications

Allogeneic Bone Marrow Transplantation

In HLA-identical allogeneic BMT, it is clearly established that T cell depletion decreases acute graft-versus-host disease (aGvHD). Table 3 reviews some of the methods which have been reported until now. In a recent analysis made by the International Bone Marrow Transplant Registry (IBMTR) [50], 371 T cell-depleted transplants were compared with 2 480 non-T cell-depleted transplants. It appears that T cell-depletion decreased not only aGvHD (RR=0.45;p< 0.0001). Two other complications may occur after allogeneic BMT: higher incidence of graft failure (RR=9.29; p< 0.0001) and leukemia relapse (RR=5.61; p< 0.0001 in CML, RR=1.94; p< 0.007 in AML and RR=1.83;p< 0.05 in ALL).

Table 3. A survey of the different mAbs used for T-cell depletion in HLA-identical allogeneic BMT.

Methods	References	
CD2 + C1	Mitsyasu	[43]
CD6 + C1	Bosserman	[44]
CD2 + CD3 + C1	Hervé	[45]
CD2 + CD5 + CD7 + C1	Racadot	[7]
CD2 + CD5 + CD7 + C1	Gegmo	[46]
CD4 + CD5 + CD8 + C1	Gegmo	[46]
CD6 + CD8 + C1 + C2	Prentice	[47]
Cocktail of 8 anti-T mAbs + C1 (CD2-CD5-2CD3-CD6-2CD28-CD4)	Martin	[48]
CAMPATH-1 + C1	Apperley	[49]
Chain-A ricin - CD5	Filipovich	[50]
IT - CD3	Martin	[51]

C1 = One round of complement; C2 = Two rounds of complement.

Another IBMTR study analyzed the results of T cell-depletion in patients who had received a BMT from related donors other than HLA-identical siblings [51]. 470 such patients were compared with 868 patients receiving HLA-identical sibling transplants: T cell-depletion increased graft failure and reduced aGvHD but did not improve leukemia-free survival rates.

To overcome these T-cell-depletion-related complications, new approaches must be developed. Among them we can quote the use of a fixed low number of blood T cells with a T cell-depleted marrow [54], the depletion of selected T cell subsets [44] or the prophylactic in vivo use of an mAb like anti LFA-1 administered before BMT [55]. Finally the reinforcement of pre-transplant conditioning regimens may reduce the risk of relapse.

Autologous Bone Marrow Transplantation

High-dose ablative therapy with rescue for bone marrow function by autologous bone marrow transplantation has become widely used in patients with hematologic and solid tumours [56-59]. A number of methods for selective removal of malignant residual cells have been reported. But until now the debate remains largely opén concerning the real impact of purging in malignant residual cells have been reported. But until now the debate remains largely open concerning the real impact of purging in malignant hemopathies. However a recent report from the European Bone Marrow Transplantation (EBMT) group documents convincing results in favour of chemical purging versus no purge in AML [60]. The development of sensitive techniques for the detection of residual disease, like bcl-2 proto-oncogene amplification by the polymerase chain reaction (PCR) [28] may make it possible to appreciate the effectiveness of malignant cell-depletion and the interest of bone marrow purging. However another question remains unanswered to date: does the malignant stem cell express the antigens that are expressed by more mature malignant cells? In other words, are the mAbs chosen for bone marrow purging able to kill the malignant stem cells?

Conclusions

Several immune purging techniques are currently available. The feasibility has been confirmed: hematopoietic reconstitution occurs within a delay equivalent to that observed with unmanipulated marrow. In allogeneic bone marrow transplantation the clinical efficacy of this approach has been demonstrated. However due to the occurrence of further complications, we must be careful when using such techniques. In autologous bone marrow transplantation thanks to the development of sensitive methods to assess the efficacy of purging, it will soon be possible to appreciate the effectiveness of depletion in vitro. As to its clinical efficacy, prospective randomized studies including many patients need to be carried out.

References

1. Kohler G, Milstein D. Continuous culture of fused cells secreting antibody of predefined specificity. Nature 1975;256:495-7.
2. Kohler G. Derivation and diversification of monoclonal antibodies. Science 1986; 233:1281-6.
3. Bast RC, Ritz J, Lipton JM, et al. Elimination of leukemic cells from human bone marrow using monoclonal antibody and complement. Cancer Res 1983;43:1389-94.
4. Racadot E, Herve P, Lamy B, Peters A. Preclinical studies of a panel of 12 monoclonal antibodies in view of bone marrow purging in acute lymphoblastic leukemia. Leukaemia Res 1987;11:987-94.
5. Gilmore MJML, Prentice HG, Blacklock HA, Janossy G, Hoffbrand AV. Technique for rapid isolation of bone marrow mononuclear cells using Ficoll-Metrizoate and the IBM 2991 blood cell processor. Brit J Haematol 1982;50:619-26.
6. Herve P, Andreu G. Bone marrow processing and ex vivo purging prior to allogeneic and autologous bone marrow transplantation. Plasma Ther Transf Technol 1986;7: 455-62.
7. Racadot E, Herve P, Beaujean F, et al. Prevention of graft versus host disease in HLA-matched bone marrow transplantation for malignant diseases; a multicentric study of 62 patients. J Clin Oncol 1987;5:426-35.
8. Waldmann H, Hale G, Cividalli G, et al. Elimination of graft-versus-host-disease by in vitro depletion of alloreactive lymphocytes with a monoclonal rat anti-human lymphocyte antibody (campath-1). Lancet 1984;i:483-5.
9. Foon KA, Schroff RW, Gale RP. Surface markers on leukemia and lymphoma cells: Recent advances. Blood 1982;60:1-19.
10. Foon KA, Todd RF, Immunologic classification of leukemia and lymphoma. Blood 1986;69:1-31.
11. Borowitz MJ, Dowell BL, Boyett JM, et al. Monoclonal antibody definition of T-cell acute leukemia: A pediatric oncology group study. Blood 1985;65:785-8.
12. Janossy G, Tidman N, Papa Georgiou ES, Kung PC, Goldstein G. Distribution of T lymphocyte subsets in the human bone marrow and thymus: An analysis with monoclonal antibodies. J Immunol 1981;126:1608-13.
13. Bast RC, de Fabritis P, Liptom J, et al. Elimination of malignant clonogenic cells from human bone marrow using multiple monoclonal antibodies and complement. Cancer Res 1985;45:499-503.
14. Ritz J, Pesando JM, Notis-McConatry J. A monoclonal antibody to human acute lymphoblastic leukemia antigen. Nature 1979;63:583-5.
15. Lebien TW, Boue DR, Bradley JC, Kersey JH. A monoclonal antibody to the common ALL antigen which does not mediate antigenic modulation in vitro. J Cell Biochem 1982;suppl 6:34-9.
16. Boucheix C, Perrot JY, Mirshahi M, et al. A new set of monoclonal antibodies against acute lymphoblastic leukemia. Leukaemia Res 1985;9:597-604.
17. Lebacq-Verheyden AM, Ravoet AM, Bazin H, Sutherland DR, Tidman N, Greaves MF. Rat AL2, AL3, AL4 and AL5 monoclonal antibodies bind to the common acute lymphoblastic leukemia antigen (CALLA gp100). Int J Cancer 1983;32:273-9.
18. Campana D, Janossy G. Leukemia diagnosis and testing of complement fixing antibodies for bone marrow purging in acute lymphoblastic leukemia. Blood 1986;68: 1264-71.
19. Granger S, Janossy G, Francis G, Blacklock H, Poulter LW, Hoffbrand AV. Elimination of T lymphocytes from human bone marrow with monoclonal T antibodies and cytolytic complement. Brit J Haematol 1982;50:367-74.
20. Campana D, Coustan-Smith E, Janossy G. The immunologic detection of minimal residual disease in acute leukemia. Blood 1990;76:163-71.

21. Gore SD, Kastan B, Goodman N, Civin CI. Detection of minimal residual T cell acute lymphoblastic leukemia by flow cytometry. J Immunol Methods 1990;132:275-86.
22. Martin PJ, Hansen JA. Quantitative assays for detection of residual T cells in depleted human marrow. Blood 1985;65:1134-40.
23. Favrot M, Herve P. Detection of minimal malignant cell infiltration in the bone marrow of patients with solid tumors, non Hodgkin lymphomas and leukaemias. Bone Marrow Transpl 1987;2:117-22.
24. Estrov Z, Grunberger T, Dube ID, Wang YP, Freedman MH. Detection of residual acute lymphoblastic leukemia cells in cultures of bone marrow obtained during remission. N Engl J Med 1986;315:538-42.
25. Moretti L, Corpuz S, Giuliodori L, et al. Quantitation of T cell depletion by limiting dilution analysis. Haematologica 1991;76:188-92.
26. Roy DC, Felix M, Cannady WG. Cannistra S, Ritz J. Comparative activities of rabbit complement of different ages using an in vitro marrow purging model. Leukaemia Res 1990;14:407-16.
27. Bregni M, Siena S, Neri A, et al. Minimal residual disease in acute lymphoblastic leukemia detected by immune selection and gene rearrangement analysis. J Clin Oncol 1989;7:338-43.
28. Gribben JG, Freedman AS, Neuberg D, et al. Immunologic purging of marrow assessed by PCR before autologous bone marrow transplantation for B cell lymphoma. N Engl J Med 1991;325:1525-33.
29. Herve P, Cahn JY, Tiberghien P, et al. Bone marrow transplantation from donors other than HLA identical siblings. What have we learned from previous experiences? Bone Marrow Transpl 1990;6(suppl 1):112-4.
30. Jansen FK, Blythman H, Carriere D, et al. Immunotoxins, hybrid molecules combining high specificity and potent cytotoxicity. Immunol Rev 1982;62:185-216.
31. Filipovich AH, Vallera DA, Youle RJ, Neville DM, Kersey JH. Ex-vivo T cell depletion with immunotoxins in allogenic bone marrow transplantation: The pilot clinical study for prevention of graft versus host disease. Transplant Proc 1985;17:442-4.
32. Press OW, Vitetta ES, Farr AG, Hansen JA, Martin PJ. Evaluation of ricin A-chain immunotoxins directed against human T-cells. Cellular Immunol 1986;102:10-20.
33. Lambert JM, McIntyre GM, Gouthier MN, et al. The galactose binding sites of the cytotoxic lectin ricin can be chemically blocked by modification with reactive ligands prepared by chemical modification of triantennary N-linked oligosaccharides. Biochemistry 1991;30:3234-45.
34. Roy DC, Griffin JD, Belvin M, Blattler WA, Lambert JM, Ritz J. Anti-My9-blocked-ricin = an immunotoxin for selective targeting of acute myeloid leukemia cells. Blood 1991;77:2404-12.
35. Treleaven JG, Gibson FM, Ugelstad, et al. Removal of neuroblastoma cells from bone marrow with monoclonal antibodies conjugated to magnetic microspheres. Lancet 1984;i:70-3.
36. Kemshead JT, Health L, Gibson FM, et al. Magnetic microspheres and monoclonal antibodies for the depletion of neuroblastoma cells from bone marrow. Experience, improvements and observations. Brit J Cancer 1986;54:771-8.
37. Kvalheim G, Fodstad O, Pihl A, et al. Elimination of B lymphoma cells from human bone marrow: Model experiments using monodisperse magnetic particles coated with primary monoclonal antibodies. Cancer Res 1987;47:846-51.
38. Ugelstad J, Mork PC, Mfutakamba HR, Soleimany E, Nordhuus I. Thermodynamics of swelling of polymer, oligomer and polymer oligomer particles. In: Poehlein GV, Ottervill RG, Goodwin JW (eds). Science and Technology of polymer colloids. NATO ASI Series 1983:51-99.

39. Guyotat D, Shi ZH, Campos L, et al. Pre-clinical evaluation of anti lacto-N-fucopentoase III (CD15) monoclonal antibodies for ex vivo bone marrow purging in acute myeloid leukemia. Bone Marrow Transpl 1990;6:385-90.
40. Bieva C, Vander Brugghen F, Stryckmans P. Malignant leukemic cell separation by iron colloid immunomagnetic absorption. Exp Hematol 1989;17:914-20.
41. Givin CI, Strauss LC, Fackler MJ, Tismann TM, Willey JM, Loken RL. Positive stem cell selection – Basic Science. In: Gross S, Gee A, Worthington-Write DA (eds). Advances in bone marrow purging and processing. Wiley-Liss, Inc, New York 1990:387-402.
42. Rasmussen AM, Smeland EB, Erikstein BK, Caignault L, Funderud S. A new method for the detachment of Dynabeads from positively selected B lymphocytes. J Immunol Methods 1992;146:195-202.
43. Mitsuyasu R, Champlin R, Gale T, et al. Treatment of donor bone marrow with monoclonal anti T cell antibody and complement for the prevention of graft versus host disease. Ann Intern Med 1986;105:20-6.
44. Bosserman LD, Murray C, Takvorian T, et al. Mechanism of graft failure in HLA-matched and HLA-mismatched bone marrow transplant recipients. Bone Marrow Transpl 1989;3:239-45.
45. Herve P, Cahn JY, Flesch M, et al. Successful GvHD prevention without graft failure in 32 HLA-identical allogenic bone marrow transplantations with marrow depletion of T cells by monoclonal antibodies and complement. Blood 1987;69:388-93.
46. Maraninchi D, Gluckman E, Blaise D, et al. Impact of T cell depletion on outcome of allogenic bone marrow transplantation for standard-risk leukaemias. Lancet 1987;ii: 175-8.
47. Prentice HG, Brenner MK, Grob, et al. HLA matched T cell depleted allogeneic BMT in the treatment of acute leukaemia in 1st CR. Bone Marrow Transpl 1986; 1(suppl 1):91.
48. Martin PJ, Hansen JA, Torok-Storb B, et al. Graft failure in patients receiving T cell-depleted HLA-identical allogeneic marrow transplants. Bone Marrow Transplant 1988;3;445-6.
49. Apperley J, Jones L, Hale G, et al. Bone marrow transplantation for patients with chronic myeloid leukemia: T-cell depletion with Campath-1 reduces the incidence of graft versus host disease but may increase the risk of leukaemic relapse. Bone Marrow Transpl 1986;1:53-66.
50. Filipovich AH, Vallera DA, Youle RJ, et al. Graft versus host disease prevention in allogeneic bone marrow transplantation from histocompatible siblings: A pilot study using immunotoxins for T cell depletion of donor bone marrow. Transplantation 1987;44:62-9.
51. Martin PJ, Hansen JA, Torok-Storb, et al. Effects of treating marrow with a CD3-specific immunotoxin for prevention of acute graft versus host disease. Bone Marrow Transpl 1988;3:437-44.
52. Marmont AM, Horowitz MM, Gale RP, et al. T-cell depletion of HLA-identical transplants in leukemia. Blood 1991;78:2120-30.
53. Ash RC, Horowitz MM, Gale RP, et al. Bone marrow transplantation from related donors other than HLA-identical siblings: Effect of T cell depletion. Bone Marrow Transpl 1991;7:443-52.
54. Verdonck LF, de Gast GC, van Heugten HG, Dekker AW. A fixed low number of T cells in HLA-identical allogeneic bone marrow transplantation. Blood 1990;75: 776-80.
55. Fischer A, Friedrich W, Fasth A. Reduction of graft failure by a monoclonal antibody (anti-LFA-a/CD11a) after HLA non identical bone marrow transplantation in children with immunodeficiencies, osteopetrosis and Fanconi's anemia: A European group for immunodeficiency/European group for bone marrow transplantation report. Blood 1991;77:249-56.

56. Santos GW. Marrow transplantation in acute non lymphocytic leukemia. Blood 1989; 74:901-8.
57. Weisdorf DJ, Haake R, Miller WJ, et al. Autologous bone marrow transplantation for progressive non-Hodgkin's lymphoma: Clinical impact of immunophenotype and in vitro purging. Bone Marrow Transpl 1991;8:135-42.
58. Kersey JH, Weisdorf D, Nesbit ME, et al. Comparison of autologous and allogeneic bone marrow transplantation for treatment of high risk refractory acute lymphoblastic leukemia. N Engl J Med 1987;317:461-8.
59. Blaise D, Gaspard MH, Michel G, et al. Allogeneic or autologous bone marrow transplantation for acute lymphoblastic leukemia in first complete remission. Bone Marrow Transpl 1990;5:7-12.
60. Gorin NC, Labopin M, Meloni G, et al. Autologous bone marrow transplantation for acute myeloblastic leukemia in Europe: Further evidence of the role of marrow purging by mafosfamide. Leukemia 1991;5:896-904.

REDUCTION OF THE IMMUNOGENIC CAPACITY OF BLOOD COMPONENTS FOR THE PREVENTION OF ALLOIMMUNIZATION TO WHITE CELLS

P. Rebulla, F. Bertolini, G. Sirchia

Introduction

In 1957 Brittingham and Chaplin published a study showing that non-hemolytic febrile transfusion reactions (NHFTR) are due to donor blood cells (WBC) and platelets [1]. In a subsequent study, Perkins et al. found a relation between the number of WBC transfused and the temperature rise in NHFTR [2]. Serological investigations in multitransfused patients included in these and other similar studies revealed that many reactive patients had WBC antibodies in the serum [3,4].

Further work carried out in more recent years allowed the identification of additional posttransfusion complications related to the transfusion of non WBC-depleted allogeneic blood, including refractoriness to random donor platelet transfusion [5-9], cytomegalovirus (CMV) transmission [10-12], increased cancer recurrence and postoperative infection rates [13-15]. In addition to the identification of these negative effects, it was recently found that it might be unwise to remove WBC from blood too soon after collection, since leukocytes, if present in the unit for some hours, could remove contaminating bacteria [16]. This issue has become particularly relevant since the recent reports of bacteremia and endotoxic shock associated with blood transfusion [17]. Although some aspects of this work are still in progress, the bulk of these observations indicate that the presence of WBC in blood components at the time of transfusion is undesirable. This prompted the development of methods for reducing the WBC content of red blood cells (RBC) and platelet concentrates (PC).

The whole issue of blood transfusion and WBC reduction has been extensively reviewed in recent publications [18-20]. This paper focuses on current technical aspects of WBC reduced blood components obtained by filtration, their quality control and the rationale for their clinical use. It does not consider UV irradiation, a recently developed approach to prevent some of the above complications, which is still in the investigational phase [21-25].

WBC reduction by filtration

Filtration is one of the most effective technologies routinely available for reducing the number of WBC that contaminate standard RBC and PC [19]. It has progressively replaced previous more cumbersome and less effective methods based on centrifugation, washing and sedimentation, which took advantage of the different specific density of red cells, white cells and platelets. Filtration can be performed during blood component preparation [26,27] or after storage [28-31]. Several manufacturers provide filters specifically designed for use in the laboratory or at the patient's bedside. In some cases the filter performance varies in relation to storage time, flow rate and temperature of RBC or PC.

With some currently available filters 3-$\log_{10}$ WBC depletion can be obtained in RBC [32-34]. This corresponds to decreasing the total WBC load of an average RBC unit from 3,000 to less than 3×10^6 WBC. Although removal of the buffy coat from RBC prior to filtration generally improves WBC depletion, this is not so with all filters [34]. In this regard, it seems that the platelets (which are removed if the buffy coat or the platelet rich plasma is removed from RBC) can play an important role in leukodepletion because they can form a bridge between WBC and the fibers of some filters [35]. Several current filters allow RBC average yields of more than 90% [34], well above the 80% minimum level required by the Standards of the American Association of Blood Banks [36]. However, if leukodepletion is performed in buffy coat free RBC, an additional loss of 10-15% RBC due to initial buffy coat removal must be considered [32,34]. Time of filtration under gravity (no pressure cuff applied to the unit) varies according to the filter characteristics, ranging in many cases between 10 and 20 minutes per RBC unit [34].

Recent work on filtered PC indicates that platelet quality is not significantly affected by filtration [37,38], and that prestorage WBC depletion has no effect in reducing the platelet storage lesion [39].

Choice of filter

Important characteristics of a WBC reduction filter include cost, absolute WBC removal, recovery of the primary blood component (RBC or platelets) and flow rate. The choice of the filter depends on several variables including the financial resources, the case mix of transfusion recipients and the availability of technicians and/or nurses trained to perform filtration in the laboratory or at the patient's bedside. As far as financial resources are concerned, it must be noted that the current cost of the more effective filters is not trivial, and in some countries this cost cannot be justified on the basis of health priorities. It is expected that the cost per procedure could decrease if scientific evidence is provided supporting a more generalized use of filters. In this regard, it should be evaluated what percentage of leuko-poor blood recipients would make a 100% production of WBC depleted blood components more convenient than production for selected cases or patient categories.

The case mix of transfusion recipients is another important issue to be considered in the choice of the filter and of the technique to be used. For example, the prevention of NHFTR in multiply transfused recipients such as the thalassemics can be done at low cost with a technique known as "spin, cool and filter" [31], which uses a low cost microaggregate blood filter at the patient's bedside. In this method RBC units are centrifuged at high speed in inverted position; during this centrifugation WBC concentrate in the buffy coat layer, which is then consolidated by a few hours of storage at +4°C. Aggregated WBC are finally trapped in a microaggregate filter inserted along the transfusion set. Although this technique shows limited effectiveness in removing WBC as compared to more recent filters [31,34], its ability in preventing NHFTR in thalassemics is not significantly inferior to that of more expensive devices and methods if nurses are properly trained for handling the units so as not to disturb the buffy coat layer formed during RBC centrifugation [40]. This bedside method allows regular transfusion support in certain settings where filtration in the laboratory is unfeasible or too expensive. This can be the case of single centers where several hundreds thalassemia patients receive each 2-3 units of WBC reduced RBC every 3-4 weeks throughout their entire life. In such settings, which are not uncommon in the Mediterranean area [41-44], a bedside technique is more convenient than filtering in the laboratory also because in the former case the cost of laboratory technicians required for WBC depletion is reduced or eliminated. The choice would be different for the treatment of newly diagnosed leukemic patients. Differently from the case of thalassemia patients, for whom a clear indication of WBC depleted RBC has been expressed [45], there is no consensus on routine leukodepletion for leukemic patients. In these patients the goal would be to prevent not only NHFTR, but also alloimmunization to WBC and refractoriness to random donor platelet transfusion, which occurs in 30-60% of leukemic recipients of standard blood components [46]. In this regard, Schiffer pointed out that only 10-15 out of 100 leukemics given from the beginning WBC depleted RBC and PC would ultimately benefit from this expensive treatment [46]. This is mainly due to previous alloimmunization, early deaths, absent or limited alloimmunization in a proportion of cases, and the unlikelihood that filtration, similarly to any other approach to decrease alloimmunization, could be 100% effective. Filtration in fact has been shown to prevent or delay primary alloimmunization [47], but not the secondary immune response to foreign blood cell antigens [48]. Therefore, at present there is no scientific evidence indicating that the routine treatment of all leukemics with WBC depleted RBC and PC is cost effective. In this regard, many centers follow local policies which depend on the case mix, the financial resources, the availability of potential bone marrow transplantation, etc. Having set this important premise, some studies have shown that primary alloimmunization to WBC is greatly reduced in leukemics receiving blood components containing less than 5×10^6 WBC per unit [49,50]. The validity of this threshold is debatable due to the rather insensitive and inaccurate methods of counting residual WBC that were available when these studies were done. It must also be considered that RBC filtered through some

modern filters and evaluated with improved methods of counting such as the Nageotte chamber [32] (see below) contain less than 1×10^6 WBC per unit [34]. As a consequence, and also in consideration of the great technical improvement achieved in the field of blood filtration in recent years, it would be desirable to repeat some of the clinical studies performed in the past, with the aim of collecting more accurate figures on the level of RBC contamination capable of preventing primary alloimmunization, to transmit some viruses, or to perform new studies on other rare but almost invariably fatal complications such as the transfusion-associated graft-versus-host disease (TA GvHD) [51-53]. Hopefully, the Trial to Reduce Alloimmunization to Platelets (TRAP) performed in the USA [46] will soon answer several of the pending questions.

The cost issue is certainly less relevant in case of patient categories who receive blood transfusion very infrequently and/or require it as a single event throughout their entire lifetime, particularly if this event occurs very early in live like in the neonatal period. However, despite the evidence that CMV is not transmitted by WBC depleted RBC and PC [54-56], there is no final consensus on the indication of WBC depleted blood components for infants [57,58]. Consensus is limited to the agreement that transfusion-associated CMV infection should be avoided in low-birth-weight infants [59].

Similarly, there is no current consensus on a possible indication of WBC depleted RBC for neoplastic surgical patients. This is a hot issue for several research groups currently reporting conflicting data [13,14,60-63]. Clearly, such an indication could contribute to significantly decreasing the cost of filters.

Quality control of filtered products

Postfiltration WBC contamination cannot be assessed with automated counters, which generally show a lower detection limit around 500 WBC per microliter. Manual microscopic counting techniques and different chambers (Bürker, Neubauer, Nageotte) have been used to this aim [64,32,34]. The volume of Bürker and Neubauer chambers is 0.9 microliters; that of the Nageotte chamber is 50 microliters. Since the sensitivity, accuracy and variability of postfiltration WBC counts depend on the number of WBC detected [65], larger chambers offer greater sensitivity. Compared to the Bürker and Neubauer chambers, the Nageotte chamber shows improved sensitivity, since its volume of 50 microliters allows the evaluation of 5 microliters of native sample if this is diluted 1:10 in a staining/diluent fluid. However, also with the Nageotte chamber the number of WBC that can be counted after filtration with several currently available filters is limited [32,34]. This prevents the sensitivity (lower detection limit) of such method to go below approximately 1 WBC per microliter [32,66], which corresponds to a total of 350,000 WBC in a 350 ml filtered unit. Although a modified Nageotte counting method with improved sensitivity has been reported [67], the performance of this method in different laboratories under routine conditions has not been evaluated. Another approach is based on flowcytometric detection of residual WBC stained with the DNA fluorophore propidium iodide

[68]. The volume of native sample that can be examined with this technique is larger than in the Nageotte chamber, but variability at counts below 1 WBC per microliter is still high [34,66]. In addition, this approach has the disadvantage of requiring expensive equipment. Since with the more advanced filters postfiltration WBC counts are frequently below 1-2 WBC per microliter [32,34], more sensitive methods for routine quality control (QC) are necessary.

Greatly improved sensitivity can be obtained with the polymerase chain reaction (PCR) technology [69]. In this method WBC from a large volume of filtered blood are concentrated by Ficoll-Hypaque gradient centrifugation in a small volume. The DNA material contained in WBC collected with this method is then amplified with the PCR. The method needs to be improved and standardized, also due to the variability of WBC yield after gradient centrifugation performed to collect WBC [70,71]. In addition, PCR is not suitable for routine purposes, and this approach requires the use of a large volume of the unit, which therefore is not usable for transfusion. However, it is possible that after technical refinements this method can find practical applications [72].

An important question related to QC is: How many units should be counted to be confident that the unit given to a certain recipient contains less than a defined number of WBC? Traditionally, QC in this area has been done by evaluating a certain (small) number of units sampled from a large production. The proportion of units checked for QC does not generally exceed 1-2 percent of those produced. Although this is based on the rational premise of reducing costs and increasing the feasibility of QC, there are several reasons why this approach should be changed with current WBC depleted blood components. First of all, many current filters show highly consistent and effective performances [34]. This might seem a good reason to check from time to time such performances. However, if a small number of units is sampled from a large production, and the filter performance shows little variability, the probability of detecting an abnormal result is small. Moreover, to increase the number of sampled units can be unpractical and too expensive. A possible alternative could be to differentiate QC in relation to the complication to be avoided. For example, a limited number of postfiltration WBC counts on each new filter lot could be enough if filtered RBC are used to prevent NHFTR, while accurate counts on all filtered units might be necessary if the aim is to prevent complications caused by lower WBC contamination.

As far as the expression of the results is concerned, confidence intervals (CI) of measured parameters are probably better than means, standard deviations, medians or ranges [73,74]. If one takes as an example the postfiltration WBC count, the user is interested in knowing how sure (or confident) one can be that the unit given to a certain patient contains less than a certain number of WBC. This number is the upper limit of the CI, and the level of confidence is expressed by a value that accompanies the CI: generally 90%, 95% or 99%. The 95% CI includes a lower and an upper limit set at the 2.5th and the 97.5th percentiles of the distribution respectively. Although being 95% confident in something can seem a great level of safety, this is not necessarily so. For example, if

a certain patient must receive a RBC unit containing no more than 5×10^6 WBC and the 95% CI of postfiltration WBC count obtained with a certain filter is $0.5\text{-}5\times10^6$, in 1 of 40 units the WBC count will be less than 0.5×10^6 (or below the 2.5th percentile) while in 1 of 40 units it will be more than 5×10^6 (or above the 97.5th percentile). If, for example, a contamination of more than 5×10^6 is associated with the transmission of a certain viral infection, and the patient is a leukemic receiving throughout his leukemic life 40-50 exposures to WBC depleted donor blood, a failure rate as low as 1 in 40 cases (i.e. 95% confidence) may be dangerous: it is in fact possible that a single failure is responsible for an important complication, such as CMV transmission, HLA alloimmunization, etc. With the present level of knowledge it is not easy to define accurate CI of residual WBC counts, mainly because accurate figures on the number of postfiltration WBC to calculate reliable distributions are scanty [32-34]. In addition, also relying on 95% confidence may not be enough in some cases. This reasoning has been presented to indicate that QC of WBC reduced blood components is difficult and further work is necessary at the theoretical/statistical level, at the bench level (to set up more sensitive and accurate counting methods) and at the biological/clinical level (to better define levels of WBC contamination capable of preventing different complications.

Cooperative efforts

A large number of data concerning WBC reduction by filtration have been collected during the last 20 years. This huge amount of information is scattered in the scientific and industrial communities. A cooperative effort of scientists and manufacturers sharing expertise and knowledge at this level is therefore the best premise to further improve leukodepletion and benefit transfusion recipients with safer products. Based on this reasoning, in 1990 the International Society of Blood Transfusion formed a working party named BEST, the acronym of Biomedical Excellence for Safer Transfusion. BEST includes representatives of the scientific and manufacturing community involved in transfusion medicine. BEST work is aimed at a rapid collection of a large number of data on issues related to the safety of blood transfusion through the organization of wet workshops. Two studies on postfiltration WBC counting methods have been carried out so far [66]. Future studies on routine use outside the group of methods evaluated within BEST have also been planned. In addition, a subcommittee has been formed in charge of identifying feasible methods to perform QC of platelet concentrates. Thorough discussion in the working party and timely circulation of the results outside BEST are expected to contribute to improving the quality of blood components and, in turn, the safety of blood transfusion.

References

1. Brittingham TE, Chaplin H. Febrile transfusion reactions caused by sensitivity to donor leukocytes and platelets. JAMA 1957;167:819-25.
2. Perkins HA, Payne R, Ferguson J, Wood M. Nonhemolytic febrile transfusion reactions. Quantitative effects of blood components with emphasis on isoantigenic incompatibility of leukocytes. Vox Sang 1966;11:578-600.
3. Décary F, Ferner P, Giavedoni L, et al. An investigation of nonhemolytic transfusion reactions. Vox Sang 1984;46:277-85.
4. Brubaker DB. Clinical significance of white cell antibodies in febrile nonhemolytic transfusion reactions. Transfusion 1990;30:733-7.
5. Herzig RH, Herzig GP, Bull MI, et al. Correction of poor platelet transfusion responses with leukocyte-poor HL-A-matched platelet concentrates. Blood 1975;46:743-50.
6. Murphy MF, Waters AH. Immunological aspects of platelet transfusion. Brit J Haematol 1985;60:409-14.
7. Slichter SJ. Mechanism and management of platelet refractoriness. In: Nance SJ (ed). Transfusion medicine in the 1990's. American Association of Blood Banks, Arlington, VA 1990:95-179.
8. Godeau B, Fromont P, Seror T, Duedari N, Bierling P. Platelet alloimmunization after multiple transfusions: A prospective study of 50 patients. Brit J Haematol 1992; 81:395-400.
9. Chow MP, Yung CH, Hu HY, Tzeng JL, Lin JS, Lin WM, Lin CK. HLA antibodies – the cause of platelet alloimmunization in Chinese. Am J Hematol 1992;39:15-9.
10. Rinaldo CR, Black PH, Hirsh MS. Interaction of cytomegalovirus with leukocytes from patients with mononucleosis due to cytomegalovirus. J Infect Dis 1977;136: 667-78.
11. Verdonk LF, Middeldorp JM, Kreeftenberg HAG, Hauw The T, Hekker A, de Gast GC. Primary cytomegalovirus infection and its prevention after autologous bone marrow transplantation. Transplantation 1985;39:455-7.
12. Tolpin MD, Stewart JA, Warren D, et al. Transfusion transmission of cytomegalovirus confirmed by restriction endonuclease analysis. J Pediatr 1985;107:953-6.
13. Blumberg N, Heal JM. Preoperative blood transfusion and solid tumor recurrence. Blood Rev 1987;1:219-29.
14. Blumberg N, Heal JM. Transfusion and host defenses against cancer recurrence and infection. Transfusion 1987;29:236-45.
15. Triulzi DJ, Vanek K, Ryan DH, Blumberg N. A clinical and immunologic study of blood transfusion and postoperative bacterial infection in spinal surgery. Transfusion 1992;32:517-24.
16. Högman CF, Gong J, Eriksson L, Hambraeus A, Johansson CS. White cells protect donor blood against bacterial contamination. Transfusion 1991;31:612-26.
17. *Yersinia enterocolitica* bacteremia and endotoxin shock associated with red blood cell transfusions – United States, 1991. JAMA 1991;265:2174-5.
18. Meryman HT, Hornblower M. The preparation of red cells depleted of leukocytes. Review and evaluation. Transfusion 1986;26:101-6.
19. Sirchia G, Parravicini A, Rebulla P. Leukocyte-depleted blood components. In: Cash JD (ed). Progress in Transfusion Medicine. Vol 1. New Science, Churchill Livingstone 1988;vol 3:87-109.
20. Chambers LA, Garcia LW. White blood cell content of transfusion components. Lab Med 1991;22:857-60.
21. Slichter SJ, Deeg HJ, Kennedy MS. Prevention of platelet alloimmunization in dogs with systemic cyclosporine and by UV-irradiation or cyclosporine-loading of donor platelets. Blood 1987;69:414-8.

22. Andreu G, Boccaccio C, Klaren J, et al. The role of UV irradiation in the prevention of human leukocyte antigen alloimmunization. Transf Med Rev 1992;6:212-24.
23. Deeg HJ. Modification of alloreaction after blood transfusion and marrow transplantation by ultraviolet light. Sem Hematol 1992;29:95-101.
24. Pamphilon DH, Blundell EL. Ultraviolet-B irradiation of platelet concentrates: A strategy to reduce transfusion recipient allosensitization. Sem Hematol 1992;29: 113-21.
25. Sherman L, Menitove J, Kagen LR, et al. Ultraviolet-B irradiation of platelets: A preliminary trial of efficacy. Transfusion 1992;32:402-7.
26. Lovric VA, Schuller M, Raftos J, Wisdom L. Filtered microaggregate-free erythrocyte concentrates with 35-day shelf life. Vox Sang 1981;41:6-10.
27. Davey RJ, Carmen RA, Simon TL, et al. Preparation of white cell-depleted red cells for 42-day storage using an integral in-line filter. Transfusion 1989;29:496-9.
28. Diepenhorst P, Engelfriet CP. Removal of leukocytes from whole blood and erythrocyte suspensions by filtration through cotton wool. V. Results after transfusion of 1820 units of filtered erythrocytes. Vox Sang 1975;29:15-22.
29. Wenz B, Gurtlinger KF, O'Toole A, Dugan EP. Preparation of granulocyte-poor red blood cells by microaggregate filtration. A simplified method to minimize febrile transfusion reactions. Vox Sang 1980;39:282-7.
30. Sirchia G, Parravicini A, Rebulla P, Bertolini F, Morelati F, Marconi M. Preparation of leukocyte-free platelets for transfusion by filtration through cotton wool. Vox Sang 1983;44:115-20.
31. Parravicini A, Rebulla P, Apuzzo J, Wenz B, Sirchia G. The preparation of leukocyte-poor red cells for transfusion by a simple cost-effective technique. Transfusion 1984;24:508-9.
32. Masse M, Andreu G, Angue M, et al. A multicenter study on the efficiency of white cell reduction by filtration of red cells. Transfusion 1992;32:792-7.
33. Pieterz RNI, Steneker I, Reesink HW, et al. Comparison of five different filters for the removal of leukocytes from red cell concentrates. Vox Sang 1992;62:76-81.
34. Rebulla P, Porretti L, Bertolini F, et al. White cell reduced red blood cells prepared by filtration: A critical evaluation of current filters and methods for counting residual white cells. Transfusion 1993;33:1-6.
35. Stencker I, van Luyn MJA, van Wachem PB, Biewenga J. Electronmicroscopic examination of white cell reduction by four white cell-reduction filters. Transfusion 1992;32:450-7.
36. Walker RH (ed). Technical Manual (10th ed). American Association of Blood Banks, Arlington, VA 1990.
37. Bertolini F, Rebulla P, Marangoni F, Sirchia G. Platelet concentrates stored in synthetic medium after filtration. Vox Sang 1992;62:82-6.
38. Holme S, Snyder E, Heaton A, Keegan T, Napychank P. In vitro and in vivo evaluation of cotton wool filtration of platelet concentrates obtained by automated and manual apheresis. Transfusion 1992;32:328-33.
39. Dzik WH, Cusack WF, Sherburne B, Kickler T. The effect of prestorage white cell reduction on the function and viability of stored platelet concentrates. Transfusion 1992;32:334-9.
40. Parravicini A, Rebulla P, Bertolini F, et al. Clinical evaluation of the spin, cool and filter technique for the preparation of leukocyte-poor red cells at the bedside. In: Sirchia G, Zanella A (eds). Thalassemia today. The Mediterranean experience. Centro Trasfusionale Ospedale Maggiore Policlinico di Milano Editore, Milano 1987:45-51.
41. Modell B. Thalassemia, a problem of the Mediterranean area. In: Sirchia G, Zanella A (eds). Therapy of thalassemia. The Mediterranean experience. Centro Trasfusionale Ospedale Maggiore Policlinico di Milano Editore, Milano 1985:3-8.

42. Schettini F. Organization of thalassemia services in the south of Italy and perspectives. In: Sirchia G, Zanella A (eds). Thalassemia today. The Mediterranean experience. Centro Trasfusionale Ospedale Maggiore Policlinico di Milano Editore, Milano 1987:243-5.
43. Carnelli V, Terzoli S, Boffa C, et al. Organization of services for thalassemia patients at the Center of Pediatric Hematology of the University of Milano. In: Sirchia G, Zanella A (eds). Thalassemia today. The Mediterranean experience. Centro Trasfusionale Ospedale Maggiore Policlinico di Milano Editore, Milano 1987:247-50.
44. Fattoum S, Guemira F, Abbes S. Thalassemia in Tunisia. In: Sirchia G, Zanella A (eds). Thalassemia today. The Mediterranean experience. Centro Trasfusionale Ospedale Maggiore Policlinico di Milano Editore, Milano 1987:251-5.
45. Cao A, Gabutti V, Masera G, Modell B, Sirchia G, Vullo C. A short guide to the management of thalassemia. In: Sirchia G, Zanella A (eds). Thalassemia today. The Mediterranean experience. Centro Trasfusionale Ospedale Maggiore Policlinico di Milano Editore, Milano 1987:635-70.
46. Schiffer CA. Prevention of alloimmunization against platelets. Blood 1991;77:1-4.
47. Sirchia G, Rebulla P, Mascaretti L, et al. The clinical importance of leukocyte depletion in regular erythrocyte transfusion. Vox Sang 1986;51(suppl 1):2-8.
48. Scornik JC, Ireland JE, Howard RJ, Fennell RS, Pfaff WW. Role of regular and leukocyte-free blood transfusions in the generation of broad sensitization. Transplantation 1984;38:594-8.
49. Andreu G, Dewailly J, Leberre C, et al. Prevention of HLA immunization with leukocyte-poor packed red cells and platelet concentrates obtained by filtration. Blood 1988;72:964-9.
50. Sniecinski I, O'Donnel MR, Nowicki B, Hill LR. Prevention of refractoriness and HLA-alloimmunization using filtered blood products. Blood 1988;71:1402-6.
51. Decoste SD, Boudreaux C, Dover JS. Transfusion-associated graft-vs-host disease in patients with malignancies. Report of two cases and review of the literature. Arch Dermatol 1990;126:1324-9.
52. Sanders MR, Graeber JE. Posttransfusion graft-versus-host disease in infancy. J Pediatr 1990;117:159-63.
53. Moroff G, Luban NLC. Prevention of transfusion-associated graft-versus-host disease. Transfusion 1992;32:102-3.
54. Gilbert GL, Hayes K, Hudson IL, James J, and the Neonatal Cytomegalovirus Infection Study Group. Prevention of transfusion-acquired cytomegalovirus infection in infants by blood filtration to remove leukocytes. Lancet 1989;i:1228-31.
55. Bowden RA, Slichter SJ, Sayers MH, Mori M, Cays MJ, Meyers JD. Use of leukocyte-depleted platelets and cytomegalovirus-seronegative red blood cells for prevention of primary cytomegalovirus infection after marrow transplant. Blood 1991;78: 246-50.
56. Eisenfeld L, Silver H, McLaughlin J, et al. Prevention of transfusion-associated cytomegalovirus infection in neonatal patients by the removal of white cells from blood. Transfusion 1992;32:205-9.
57. Preiksaitis JK. Cytomegalovirus-seronegative blood support for perinatal patients – CON. In: Kurz SR, Baldwin ML, Sirchia G (eds). Controversies in transfusion medicine: Immune complications and cytomegalovirus transmission. American Association of Blood Banks, Arlington, VA 1990:103-30.
58. Dock NL. Cytomegalovirus-seronegative blood support for perinatal patients – PRO. In: Kurz SR, Baldwin ML, Sirchia G (eds). Controversies in transfusion medicine: Immune complications and cytomegalovirus transmission. American Association of Blood Banks, Arlington, VA 1990:131-53.
59. Lamberson HV, Dock NL. Prevention of transfusion-transmitted cytomegalovirus infection. Transfusion 1992;32:196-8.

60. Nowak MM, Ponsky JL. Blood transfusion and disease-free survival in carcinoma of the breast. J Surg Oncol 1984;27:124-30.
61. Nathanson SD, Tilley BC, Schultz L, Smith RF. Perioperative allogeneic blood transfusions: Survival in patients with resected carcinomas of the colon and rectum. Arch Surg 1985;120:734-8.
62. Voogt PJ, van de Velde CJ, Brand A, et al. Perioperative blood transfusion and cancer prognosis: Different effects of blood transfusion on prognosis of colon and breast cancer patients. Cancer 1987;59:836-43.
63. Crowe JP, Gordon NH, Fry DE, Shuck JM, Hubay CA and participating investigators. Breast cancer survival and preoperative blood transfusion. Surgery 1989;106: 836-41.
64. Friedman LI, Sadoff BJ, Stromberg RR. White cell counting in red cells and platelets: How few can we count? Transfusion 1990;30:387-9.
65. Dumond LJ. Sampling errors and the precision associated with counting very low numbers of white cells in blood components. Transfusion 1991;31:428-32.
66. Vyas GN for the BEST Working Party of the ISBT. Evaluation of Nageotte chamber **and flow cytometry for WBC count in WBC-reduced RBC. Transfusion 1992; 32:(suppl.):765(S296).**
67. Masse M, Naegelen C, Pellegrini N, Segier JM, Marpaux N, Beaujean F. Validation of a simple method to count very low white cell concentrations in filtered red cells or platelets. Transfusion 1992;32:565-71.
68. Wenz B, Burns ER, Lee V, Miller WK. A rare event analysis model for quantifying white cells in white cell-depleted blood. Transfusion 1991;31:156-9.
69. Rawal BD, Schwadron R, Busch MP, Endow R, Vyas GN. Evaluation of leukocyte removal filters modelled by the use of HIV-infected cells and DNA amplification. Blood 1990;76:2159-61.
70. Boyum A, Lovhaug D, Tresland L, Nordlie EM. Separation of leukocytes: Improved cell purity by fine adjustments of gradient medium density and osmolarity. Scand J Immunol 1991;34:697-712.
71. Ford TC, Rickwood D. Improved isolation of mononuclear cells from stored blood. Clin Chim Acta 1992;206:249-52.
72. Rawal BC, Prati D, Dang C, Capelli C, Vyas GN. DNA enzyme immunoassay using PCR-amplified HLA-DQ alpha gene applied to evaluate high efficiency leukocyte removal filters (In preparation).
73. Garne MJ, Altman DG (eds). Statistics with confidence: Confidence intervals and statistical guidelines. London. Brit Med J 1989.
74. Confidence interval analysis (CIA): Microcomputer programme manual and disk. London. Brit Med J 1989.

THE EFFECT OF PLATELETS IN RED CELL CONCENTRATES ON THE LEUKOCYTE DEPLETION CAPACITY OF LEUKOCYTE DEPLETION FILTERS[1]

I. Steneker,[2] H.K. Prins,[3] J. Biewenga,[4] R.N.I. Pietersz[2]

Introduction

Leukocytes and platelets are known to adhere to a variety of materials [1]. Already in 1928, Fleming used cotton wool to remove leukocytes from small amounts of blood [2]. This was the basis for the preparation of leukocyte-poor red cell concentrates (RBC) for transfusion many years later [3,4]. In 1972 Diepenhorst et al. [3] described the first leukocyte depletion filter which was composed of a column filled with tightly packed cotton wool fibers, providing a network with equally distributed pores. The retention of both granulocytes and lymphocytes in this cotton-wool filter was independent of Ca^{2+} and Mg^{2+} and hematocrit and slightly temperature dependent.

The originally used cotton wool was soon replaced by cellulose-acetate fibers, because of convenience and better possibilities to control the quality of the fibers [5-7]. Comparison of the cellulose-acetate column filter with the cotton-wool column filter showed that the retention of leukocytes in the cellulose-acetate filter seemed higher than in the cotton wool filters [8].

The more recent availability of non-wovens has led to the development of flat-bed filters with coarse filter layers for the removal of gross blood clots and medium and fine filter layers for the removal of leukocytes [9-12]. These filters differ in 1) fiber material, 2) number of pre-filter layers, 3) number of fine layers, 4) fiber diameter, 5) distance between two adjacent fibers, 6) thickness of the filter bed ,and 7) available surface area. These filters were quite rapidly introduced for routine use. However, several questions regarding the mechanisms of leukocyte removal on these filters have not been solved. Such questions are: where and how are leukocytes captured in the filters?; do the cells become acti-

1. Support in part by the "Nederlands Produktielaboratorium voor Bloedtransfusieapparatuur en Infusievloeistoffen BV (NPBI)", Emmer-Compascuum, The Netherlands and by the Ministry of Economic Affairs of the Dutch Government, The Hague, The Netherlands.
2. Red Cross Blood Bank Amsterdam, The Netherlands.
3. Central Laboratory of the Netherlands Blood Transfusion Service (retired staff member).
4. Department of Cell Biology, Free University Amsterdam.

vated and/or do they release mediators?; do cell-cell interactions occur?; what is the effect of plasma proteins and the cellular composition of the blood cell concentrates on the filtration process?; does storage of RBC effect the results of filtration?; what is the effect of temperature on the filtration process? Such information will be needed for further developments and the correct application of the leukocyte depletion filters.

The questions were studied by histological methods, electronmicroscopic techniques and a standard filter loading test. Histological examination of various parts of the filters, embedded in paraffin or plastics, was used to evaluate where leukocytes are captured in the filters, following filtration of RBC [13,14]. Cells captured in the various parts of the filters were washed-out with saline/glucose (0.9% NaCl, 0.1% glucose) to establish whether leukocytes were irreversibly bound onto the filter material. Electronmicroscopical examination of various parts of the filters was used to study the interaction of leukocytes with the filter material as well as cell-cell interactions [15]. Moreover, detailed examination of the cell morphology, cell spreading and cell activation was possible with transmission-electronmicroscopy. The filters were loaded with three RBC consecutively, to study the filtration characteristics of the filters and to study which factors affected the filtration characteristics [16]. Leukocyte counting and differentiation was performed with a Bürker hemocytometer. The lower limit of this technique is 110 cells per µl [17].

The Cellselect™, a cellulose-acetate column filter [13] and the Cellselect Optima™, Pall RC 100™ and Sepacell R500 B2™ filter, all three polyester flatbed filters were investigated in the study [14,15].

Distribution of captured leukocytes

The function and morphology of platelets and leukocytes is well preserved at 20°C within 24 h after blood collection. In this study we always used RBC containing about 2.0×10^9 leukocytes and 100×10^9 platelets, prepared from whole blood within 24 h after collection (fresh RBC). Based on the functional and physical properties of these cells it could be expected that the retention of platelets and granulocytes in the filters differs from the retention of lymphocytes. This was confirmed by the histological and immunohistochemical studies on a column filter [13] and flat-bed filters [14].

Cellulose-acetate column filter

Following filtration on fresh RBC, platelets were almost exclusively captured in the top of the cellulose-acetate column filter on the surface of the fibers as aggregates or mixed clusters with granulocytes. Because fresh RBC hardly contain platelet aggregates or mixed aggregates of platelets and leukocytes, the formation of cell aggregates or clusters must have been induced by cell-fiber and cell-cell interactions during the filtration.

In our studies the concentration of leukocytes captured by the cellulose-acetate column filter gradually decreased from top to bottom, which confirmed the

previously found distribution of radiolabelled leukocytes in this column filter [17]. Granulocytes and monocytes were almost exclusively captured in the top part, whereas lymphocytes were captured in the middle and bottom part. The majority of granulocytes could not be washed-out from the filter bed with saline-glucose solution, whereas more than 95% of the leukocytes could be recovered following the wash-out procedure with saline-glucose [13]. Therefore, the depletion mechanism of lymphocytes and granulocytes must differ.

Polyester flat-bed filters

In the polyester flat-bed filters the distribution of captured granulocytes and monocytes also differed from that of lymphocytes. It was expected that the pre-filter, developed for the removal of storage-generated microaggregates, remained empty after filtration of fresh RBC. However, single granulocytes as well as mixed aggregates of granulocytes and platelets were found in the pre-filter layers (coarse) of all three flat-bed filters. Thus, the polyester fabric induced the formation of cell clusters by cell-cell or cell-fiber interaction. The amount of platelets and granulocytes captured in the pre-filter depended on the packing density of the fibers. This was most obvious in the Sepacell R500 filter, in which the concentration of captured leukocytes and platelets showed a positive correlation with the increase of dense packing of the 14 pre-filter layers. When the distance between fibers became too large, the formation of cell clusters did not occur. The latter was most apparent in the Pall RC100 filter, in which only a small part of the platelets, granulocytes and monocytes was captured in the pre-filter.

The distribution of captured leukocytes and platelets in the main filter (fine layers) of the flat-bed filters depended on the amount of platelets and granulocytes already removed in the pre-filter. For example in the first two layers of the main filter of the Pall RC100 filter, small pores were clogged by platelet aggregates and mixed clusters of platelets and leukocytes, because in this filter a relatively low number of platelets and granulocytes was captured in the pre-filter. As a result of the clogging, lymphocytes were captured in the remaining layers. These cells could easily be recovered from the fine layers by the wash-out procedure. Thus, there is a risk of lymphocyte leakage at the end of a filtration of fresh RBC on the Pall RC100 filter. When a part of the granulocytes and platelets were already captured in the pre-filter, as occurred in the Sepacell R500 and the Cellselect Optima filter, the concentration of captured leukocytes gradually decreased in the main filter.

Leukocyte depletion mechanisms

The difference found in granulocyte and lymphocyte distribution in the cellulose-acetate column filter and the polyester flat-bed filter prompted us to further investigate the underlying mechanisms of leukocyte removal from RBC by electronmicroscopy, with special emphasis on the interaction of blood cells with the fibers, cell-cell interactions and cell damage.

The electronmicroscopic study [15] revealed that depletion of leukocytes from fresh RBC by filtration occurred by three mechanisms: 1) mechanical sieving, 2) direct adhesion, and 3) indirect adhesion onto adhered and spread platelets.

Mechanical sieving

The sieving mechanism was apparent from leukocytes detected in small pores. These leukocytes showed no contact with the fibers. According to their morphology, most of these leukocytes were not activated. All lymphocytes and most monocytes were captured by this mechanism. However, in the Sepacell R500 filter a small part of the lymphocytes showed direct contact with the fibers, which explains the relatively small recovery of these cells from the filter in the wash-out procedure with saline-glucose solution [14]. Based on the functional properties of granulocytes (deformability and adhesion) it was not expected that these cells would be captured by the sieving mechanism. However, a small number of these cells was captured by this mechanism due to cell swelling and morphologic alterations. Granulocyte capture by sieving in the small pores probably occurred in the initial stage of filtration when the fibers were not yet covered with plasma proteins and platelets.

When red cells have their biconcave shape they can easily pass small pores [18]. However, a small part of the red cells, mostly sphero-echinocytes, were captured by sieving, which indicates that these red cells had lost their deformability. It is possible that the filters captured "older" red cells by sieving, because 3% of the original red cells were already echinocytes.

Direct adhesion

The interaction of blood cells with artificial materials depends on the surface characteristics of these materials. Direct adhesion to the fibers of the leukocyte depletion filters was apparent from leukocytes which showed close contact with the fibers grading from contact through small pseudopods up to surrounding of the fibers with large pseudopods. These findings suggest that leukocytes adhered to the fibers by transient and by longlasting adhesion processes [19]. In transient adhesion most cells will be round and thus sensitive for disattachment. In long lasting adhesion the cells are flat, which increases the adhesion tremendously. A large proportion of the granulocytes as well as a small proportion of the monocytes in the small pores of the filters were captured by direct adhesion to the fibers. Especially the directly adhered granulocytes showed morphologic features of activation, indicating that activation is needed for adhesion. The direct adhesion of granulocytes may be complement-mediated or adhesive protein-mediated [20]. Two observations suggest that direct adhesion of granulocytes during filtration is most likely complement-mediated. Firstly, a preliminary study showed that granulocytes, which were incubated with a monoclonal antibody (CD18) against the β-chain of the CR3 heterodimer (complement receptor 3), hardly adhered to the polyester fibers. The CR3 heterodimer has been found to be the main molecule mediating both adherence and aggregation of granulo-

cytes [21]. Second, artificial devices made of cellulose acetate are known to cause complement activation and subsequent granulocyte adhesion and aggregation. In accordance, adhered and aggregated granulocytes were found in the top of the cellulose-acetate column filter.

Indirect adhesion onto adhered and spread platelets

Indirect adhesion of leukocytes was apparent from granulocytes which had adhered onto adhered and spread platelets on the fibers. Spread platelets have an increased expression of the selectin GMP-140, which may mediate adhesion of granulocytes onto platelets in the presence of Ca^{2+} [22]. However, preliminary results showed that the depletion of granulocytes by indirect adhesion could not be prevented by incubation of the platelets with a monoclonal antibody against GMP-140 (CD62). Furthermore, adhered platelets are known to release granule-bound materials such as fibrinogen, fibronectin and von Willebrand factor, which also may act as bridging molecules to polymorphonuclear granulocytes [23]. Although the mechanism of granulocytes by indirect adhesion remains unclear, efficient adhesion of granulocytes onto platelet-covered parts of the filters most likely occurred subsequent to coating of the fibers with plasma proteins and adherence and spreading of platelets. This suggestion is in accordance with the results of Wester et al. [24], who showed that granulocytes and platelets can be found especially in the first 100 ml fraction of red cell filtrates from the Cellselect filter.

Filters

All tested leukocyte depletion filters retained leukocytes by the afore mentioned mechanisms. However, the contribution of each leukocyte depletion mechanism differed for the filters. In all filters which were tested, lymphocytes were retained by a sieving mechanism. In the cellulose-acetate column filter granulocytes were most predominantly captured by direct and indirect adhesion to the fibers. In the pre-filter (coarse layers) of the polyester flat-bed filters granulocytes in the main filter (fine filter layers) mostly occurred by direct adhesion, except for the Sepacell R500 filter in which capture of granulocytes apparently occurred by both mechanical sieving and direct adhesion.

The extent of granulocyte adhesion and activation was different for the filters due to fiber characteristics or surface modifications. In the cellulose-acetate filter most granulocytes were captured by longlasting adhesion. The highest degree of activation and spreading of granulocytes was found in the Pall RC100 filter in which the fibers were modified. In contrast, in the Sepacell R500 filter most granulocytes were captured by transient adhesion. As a consequence these cells are sensitive for disattachment by post-filtration rinsing with saline-glucose.

Disintegrated leukocytes were generally found in the first two layers of the main filter. Disruption of leukocytes and platelets may release enzymes, micro-organisms and cell fragments into the filtrate. As this release is inherent to all filters studied, filtration conditions should include measurements to minimize these phenomena.

In conclusion, the distribution pattern as well as the leukocyte depletion mechanisms observed in these studies suggest that an efficient removal of leukocytes from RBC by filtration depends on the physico-chemical characteristics of the non-woven in the various filters as well as the cellular composition of the RBC. Removal of granulocytes by indirect adhesion in the pre-filter layers (coarse and medium layers) of the flat-bed filters increases the leukocyte depletion capacity of these filters.

The effect of the cellular composition of RBC on the leukocyte depletion mechanisms

The previous filtrations with standard RBC revealed interaction of platelets and granulocytes during leukocyte depletion from fresh RBC. This phenomenon prompted us to investigate the effect of platelet-depletion from RBC on the capture of leukocytes, because nowadays there is an increasing demand for platelet concentrates, which are prepared from whole blood prior to filtration, thus leaving RBC depleted of platelets.

Today, the platelet-rich plasma (PRP) method [25] and the buffy-coat (BC) method [26] are the most widely used methods for the preparation of platelet concentrates. Platelet-depleted RBC obtained by the PRP method contain 95% of the original amount of leukocytes and about 25% of the original amount of platelets, whereas buffy-coat-depleted RBC obtained by the BC method contain 30% of the original amount of leukocytes and 15% of the original amount of platelets (Table 1).

The microscopic data of our studies revealed that platelet depletion prior to filtration did not change the lymphocyte and monocyte distribution pattern in any of the filters [16]. In contrast, the distribution of granulocytes clearly depended

Table 1. The leukocyte and platelet content in RCC, platelet-depleted RCC and buffy-coat-depleted RCC.

Composition	**Red cell concentrates**		
	Standard	**Platelet depleted**	**Buffy-coat-depleted**
Leukocytes $\times 10^9$			
range	1.4 – 5.0	1.3 – 4.4	0.2 – 1.8
mean (n=108)	3.1 ± 0.9	2.7 ± 0.9	0.8 ± 0.3
Platelets $\times 10^9$			
range	55 – 180	5 – 50	0.0 – 30
mean (n=108)	102 ± 25	28 ± 9	4.6 ± 9.3
G:L (n=10)	70:30	70:30	85:15

G = granulocyte; L = lymphocyte.

depended on the platelet content of the RBC prior to filtration. In our previous experiments with fresh RBC [15], granulocytes were mostly captured in the top of the cellulose-acetate column filter by indirect adhesion. In contrast, depletion of granulocytes from platelet-depleted RBC and buffy-coat-depleted RBC occurred in a broader zone of the cellulose-acetate column filter by direct adhesion and mechanical sieving [16]. Platelet-depletion of RBC prior to filtration decreased the capture of granulocytes by indirect adhesion in the pre-filter of the flat-bed filters. The majority of granulocytes was now captured in the small pores of the main filter by direct adhesion and mechanical sieving.

Platelet-depletion of RBC prior to filtration resulted in a less efficient removal of granulocytes from RBC, because the filters had a diminished capacity for granulocyte depletion. A quantitative estimation of this effect indeed showed a positive correlation (r=0.62 to 0.86) between leukocyte depletion capacity of the filters and platelet count in the RBC prior to filtration. Differentiation of the leukocytes retrieved in the 100 ml fractions of the filtrate showed that the capture of granulocytes by indirect adhesion onto adhered and spread platelets was the explanation, because filtrations with platelet- and buffy-coat-depleted RBC resulted in a higher number of granulocytes in the filtrate fractions. As a consequence flat-bed filters were not capable to reduce the leukocyte amount of buffy-coat-depleted RBC beneath the level of 5.0×10^6 leukocytes per unit [27]. In conclusion, our studies explain why buffy-coat removal and subsequent filtration do not have the assumed synergistic effect on the residual leukocyte number in filtered RBC.

Implications for routine preparation of leukocyte-depleted red cell concentrates by filtration

Our studies show that leukocyte depletion from fresh RBC by filtration depends on direct adhesion, on indirect adhesion onto adhered and spread platelets and on mechanical sieving. Therefore, optimal depletion of leukocytes from RBC by filtration will be determined by the choice of the RBC which have to be filtered, the available filters as well as by the filtration procedure.

Component preparation

The functional properties of platelets and granulocytes involved in the adhesion processes are easily lost by activation of the clotting process at blood collection, by cooling blood below 20°C [28] or during storage of blood [29,30]. Therefore, RBC used for filtration should be prepared within 24 h after collection of whole blood, which is kept at 20°C up to the preparation of components. Rapid cooling of whole blood at 20-24°C and subsequent storage at ambient temperature provides optimal conditions for component preparation and contributes to the quality and the standardization of the subsequently prepared blood components [31]. Moreover, the presence of leukocytes during the storage of whole blood at ambient temperature seems to reduce the number of bacteria [32]. In blood bank practice it is logistically preferable to separate whole blood

into plasma, a buffy-coat and a RBC resuspended in additive solution (i.e. SAGM), because 1) the functional properties of the blood cells are well preserved during this preparation, 2) the plasma yield is high, 3) the buffy-coat is an optimal source for the preparation of leukocyte-poor platelet concentrates [26], 4) the removal of the buffy-coat prevents microaggregate generation in RBC upon storage [33], and 5) it prevents the release of internalized bacteria from disintergrating granulocytes during storage [32]. However, RBC obtained by buffy-coat removal contains only ≤ 15% of the original number of platelets and about 30% of the original leukocytes. Therefore, the leukocyte depletion from these RBC by filtration will be restricted to direct adhesion and mechanical sieving [16]. As a consequence, the choice of the filter will be limited to those filters which have a sufficient leukocyte depletion capacity based on the latter mechanisms.

The choice of filters

The leukocyte depletion capacity of the filters for buffy-coat-depleted RBC with on average a concentration of 0.8×10^9 leukocytes and 25×10^9 platelets was only sufficient in the cellulose-acetate column filter, in which the cell suspension passed a long way through the filter bed and the fibers induced direct adhesion of granulocytes [16]. All tested flat-bed filters, which removed the majority of granulocytes from fresh RBC by indirect adhesion onto adhered and spread platelets, failed to reduce the leukocyte amount of buffy-coat-depleted RBC, beneath 5.0×10^6 [27]. Thus optimalization of, in particular, flat-bed filters is needed for the efficient removal of granulocytes from buffy-coat-depleted RBC, because in these filters the contribution of the direct adhesion mechanisms was rather poor. This can be achieved by surface modification of the polyester fibers (as seen in the Pall filters to an extreme degree), by using other materials, which induce mainly direct adhesion of granulocytes to the fibers (like cellulose acetate) and by increasing the number of fine filter layers, which will increase the chance of granulocytes to be captured by mechanical sieving.

Optimal filtration conditions

Priming of the filters with a rinsing solution removes air and possible contaminants from the filter bed. It provides optimal contact between fibers and blood cells, which is physically only possible when all fibers are surrounded by the medium in which the cells are suspended. Furthermore, air-blood interfaces promote foaming which could result in denaturation of proteins and cell membranes. Moreover, air pockets in the filter bed may induce an uneven distribution of the blood flow through the filter, which will result in a lower leukocyte depletion capacity of the filter for, in particular, the first 100 ml of the RBC. Preliminary results of filters loaded with RBC without priming with saline-glucose indeed showed a relatively high leukocyte number in the first 100 ml of the filtrate (200 leukocytes per µl). Therefore, all filters should be primed with a suspension medium prior to filtration to ensure an optimal leukocyte-fiber contact.

The adhesion of leukocytes to the fibers or platelets is a process which requires a sufficient contact time. A flow rate of more than 100 ml per minute way prevent adherence and, in addition, may induce disattachment of blood cells. Previous experiments showed that under similar conditions the flow rate is inversely correlated with the hematocrit of the cell suspension being applied to the filter bed. If the filtration time increases, the risk of leakage of cell remnants from the filter increases, because adhered and activated leukocytes slowly disintegrate in the filter. Therefore the flow rate should be sufficiently low to allow contact between leukocytes and the fibers and for routine use high enough to limit the total duration of the filtration, including pre- and post-rinsing, to less than 30 minutes.

During filtration adherence of leukocytes and platelets and a sufficient deformability of red blood cells are required in leukocyte capture and red cell recovery, respectively. Thus it was expected that a temperature of 20°C would favour both features. However, preliminary results obtained with cellulose-acetate column filter loaded with fresh RBC, showed no difference between filtration of RBC kept at 20°C and RBC stored at 4°C for three hours (after three hours storage at 4°C, the RBC are between 4-6°C). The leukocyte depletion capacity of polyester filters was even higher when RBC stored at 4°C were used. Storage at 4°C promotes aggregation of platelets and increases the expression of GMP-140 on the surface of the platelets [22]. This could have enhanced the leukocyte depletion capacity of filters, because these are highly dependent on indirect adhesion of granulocytes. The deformability of granulocytes and the active adhesion capacity of these cells decreases at 4°C. Therefore, a large part of the granulocytes from RBC stored at 4°C will probably be removed by mechanical sieving due to their rigidity. In conclusion, additional studies under standardized conditions with respect to the composition of the RBC are required to determine the optimal temperature for leukocyte depletion by filtration.

The ultimate goal of filtration is to obtain the highest possible leukocyte depletion next to the lowest possible loss of red blood cells. The latter requires post-rinsing of the filters. The amount of liquid retained in the filters is determined by their void volume. The efficacy of the post rinsing procedure is dependent on the difference in viscosity between rinsing liquid and red cell suspensions. A red cell loss of less than 5%, requires at least an amount of rinsing liquid of 1.5 times the void volume. Excessive rinsing may increase the leukocyte leakage from the filters and in addition increases the final volume of the filtered RBC. To design a filter, that avoids the loss of red cells by reducing the void volume is a dangerous approach, because reduction of the void volume is associated with a reduction in the leukocyte depletion capacity of the filters. Moreover, the design of presently available flat-bed filters already balances on the border between sufficient and insufficient leukocyte depletion capacity [16].

Conclusion

In conclusion, optimal leukocyte depletion by filtration requires a filter with a leukocyte depletion capacity adapted to the cellular composition of the RBC that has to be filtered. Furthermore, the results of any filtration is highly dependent on the quality of the functional properties of leukocytes and platelets involved in the adherence of these cells to the fibers. These properties can only be preserved by standardization of the collection, the storage and the preparation of the components of whole blood. Finally, the leukocyte depletion capacity of any available filter should be defined in relation to the cellular composition of the RBC, that has to be filtered. The simple specification of the leukocyte reduction capacity of a filter in ^{10}log has no significance in the routine practice of blood component preparation. Since satisfactory filtered products depend on the use of standardized techniques, optimal leukocyte depletion will never be achieved under the conditions of bed-side filtration.

References

1. Leonard EF, Turitto VT, Vroman L (eds). Blood in contact with natural and artificial surfaces. Annals of the New York Academy of Sciences, 516, The New York Academy of Sciences, New York, 1987.
2. fleming A. A simple method of removing leukocytes from blood. Brit J Exp Pathol 1928;7:281-6.
3. Greenwalt TJ, Gajewski M, McKenna JL. A new method for preparing buffycoat poor blood. Transfusion 1962;2:221-9.
4. Diepenhorst P, Sprokholt R, Prins HK. Removal of leukocytes from whole blood and erythrocyte suspensions by filtration through cotton wool. I. Filtration technique. Vox Sang 1972;23:308-20.
5. Kikugawa K, Minoshima K. Filter columns for preparation of leukocyte poor for transfusion. Vox Sang 1978;34:281-90.
6. Prins HK, Henrichs HPJ, Conemans JMH, et al. Preparation of leukocyte-poor red cell syspensions from buffy-coat-free red cell concentrates by simplified cotton wool filtration. 17th Congr Int Soc Hemat, Paris 1978;(abstract)1:147.
7. Sirchia G, Parravicini A, Rebulla P, et al. Evaluation of three procedures of the preparation of leukocyte-poor and leukocyte-free red blood cells for transfusion. Vox Sang 1980;38:197-204.
8. Reesink HW, Veldman H, Henrichs HPJ, et al. Removal of leukocytes from whole blood by fibre filtration. A comparison study on the preformance of two commercially available filters. Vox Sang 1982;42:281-8.
9. Rebulla P, Parravicini A, Reggiani E, Sirchia G. The manual preparation of leukocyte-poor red cells for transfusion by a new filter. Transfusion 1985;25:282.
10. Sirchia G, Rebulla P, Parravicini A, Carnelli V, et al. Leukocyte depletion of red cell units at the bedside by transfusion through a new filter. Transfusion 1987;27:402-5.
11. Kickler TS, Bell W, Ness PM, et al. Depletion of white cells from platelet concentrates with a new adsorption filter. Transfusion 1989;29:411-4.
12. Pikul FJ, Farrar RO, Boris MB, et al. Effectiveness of two synthetic fiber filters for removing white cells from AS-1 red cells. Transfusion 1989;29:590-5.
13. Steneker I, Biewenga J. Histological and immunohistochemical studies on the preparation of leukocyte-poor red cell concentrates by filtration: The filtration process on cellulose-acetate fibers. Vox Sang 1990;58:192-8.

14. Steneker I, Biewenga J. Histological and immunohistochemical red cell concentrates: The filteration process using three different polyester filters. Transfusion 1991;31:40-6.
15. Steneker I, van Luyn MJA, van Wachem PB, Biewenga J. Electronmicroscopic examination of white cell reduction by four white cell-reduction filters. Transfusion 1992;32:450-7.
16. Steneker I, Prins HK, Florie M, Loos JA, Biewenga J. Mechanisms of white cell reduction in red cell concentrates by filtration: The effect of the cellular composition of the red cell concentrates. Transfusion 1993;33:42-50.
17. Olijslager J, Hennink WE, Prins HK. Leukocyten filter fase II: Invloed van vezelmateriaal en vezel diameter. Instituten voor kunststoffen, rubber, verpakking en verf TNO, 1986;487:41.
18. Stuart J, Stone PCW, Bareford D, et al. Effect of pore diameter and cell volume on erythrocyte filterability. Clin Hemorheol 1985;5:449-61.
19. Forrester JV, Lackie JM. Adhesion of neutrophil leukocytes under conditions of flow. J Cell Sci 1984;70:93-120.
20. Takemoto Y, Matsuda T, Kishimoto T, et al. Molecular understanding of cellular adhesion on artificial surfaces. Trans Am Soc Artif Intern Organs 1989;35:354-6.
21. Kuypers TW, Koenderman L, Weening RS, et al. Continuous cell activation is necessary for stable interaction of complement receptor type 3 with its counter-structure in the aggregation response of human neutrophils. Eur J Immunol 1990;20:501-8.
22. Hamber SA, McEver RP. GMP-140 mediates adhesion of stimulated platelets to neutrophils. Blood 1990;75:550-4.
23. Adams GA, Feuerstein IA. Kinetics of platelets adhesion and aggregation on protein coated surfaces: Morphology and release from dense granules. ASAIO J 1981;4:90-9.
24. Wester MR, Prins HK, Huisman JG. A new radioimmune assay for detection of small amounts of white cells and platelets in red cell concentrates: Implications for blood transfusion. Transfusion 1990;30:117-25.
25. Slichter SJ, Harker LA. Preparation and storage of platelet concentrates. I. Factors influencing the harvest of viable platelets from whole blood. Brit J Haematol 1976;34:318-24.
26. Pietersz RNI, Reesink HW, Dekker WJA, et al. Preparation of leukocyte-poor platelet concentrates from buffy coats. I. Special inserts for centrifuge cups. Vox Sang 1987;53:203-7.
27. Pieterz RNI, Steneker I, Reesink HW, et al. Comparison of five different filters for the removal of leukocytes from red cell concentrates. Vox Sang 1992;62:76-81.
28. Manara FS, Schneider DL. The activation of the human neutrophil respiratory burst occurs only at temperature above 16°C: Evidence that activation requires membrane fusion. Biochemical Biophysical Res Com 1985;132:696-701.
29. Trutter EJ, Rossouw JJ, Böhm L. Studies on the ultrastructure of blood cells and the microaggregate fraction in stored human blood. Intensive Care Med 1981;7:115-9.
30. Humbert JR, Fermin CD, Winsor EL. Early damage to granulocytes during storage. Sem Hematol 1991;3:10-3.
31. Pietersz RNI, de Korte D, Reesink HW, et al. Storage of whole blood for up to 24 hours at ambient temperature prior to component preparation. Vox Sang 1989;56: 145-50.
32. Högman CF, Gong J, Eriksson L, et al. White cells protect donor blood against bacterial contamination. Transfusion 1991;31:620-6.
33. Prins HK, de Bruyn JCGH, Henrichs HPJ, et al. Prevention of microaggregate formation by removal of "buffycoats". Vox Sang 1980;39:48-51.

LEUKOCYTE DEPLETION OF RED CELL PREPARATIONS: FOURTH FILTER GENERATION LEADS TO 4LOG REDUCTION. HOW FAR CAN/SHOULD WE GO?

E. Richter[1], M. Hosoda[2], T.A. Takahashi[2], S. Sekiguchi[2], G. Matthes[1]

Introduction

Leukocyte depletion became one of the major tasks in blood transfusion within the last years. Both red cell and platelet preparations should be leukocyte depleted prior to transfusion to lower the risk of alloimmunization and CMV transmission [1,2]. Beside freezing and washing with saline, filtration is the method of choice in clinical routine as newly developed filter systems are highly effective [3]. Third filter generation allowed 3log reduction of white cells in stored red cell concentrates but failed to eliminate granulocytes of fresh unrefrigerated blood [4]. We compared in this study the effectiveness of different leukocyte removal filters on filtrations of fresh unrefrigerated and stored refrigerated whole blood (WB) and red cell concentrates (RCC).

Material and methods

We used 73 units of blood from regular blood donations; 29 units of them were used without further processing as WP (CPDA1), 44 were buffy-coat depleted by routine blood bank preparation [5] and used as SAGM-resuspended RCC.

The blood units were leukocyte depleted by filtration either without any refrigeration after donation and kept at room temperature or after overnight storage at 4°C. Additionally 12 RCC were stored for 1 h at 4°C prior to the filtration. We used different filter systems analogous and comparable to earlier described studies [6]. We defined Sepacell R500N and A (Asahi Med., Japan) and Pall RC50 and 100 (Pall Corp., USA) as 3rd filter generation and Sepacell RS200 and Pall BPF4 as 4th filter generation.

Before and after filtration we measured red cell (RBC), white cell (WBC) and platelet (PLT) counts and the blood bag volume. The cell counts were done by Coulter S IV Plus (Coulter Electronics, Hialeah) and flow cytometry using Ortho Cytoron (Ortho Diagn. Systems, Tokyo) following Takahashi et al. [7,8].

1. Institute of Transfusion Medicine, Medical Faculty (Charité), Humboldt-University Berlin, Germany.
2. Hokkaido Red Cross Blood Center, Sapporo, Japan.

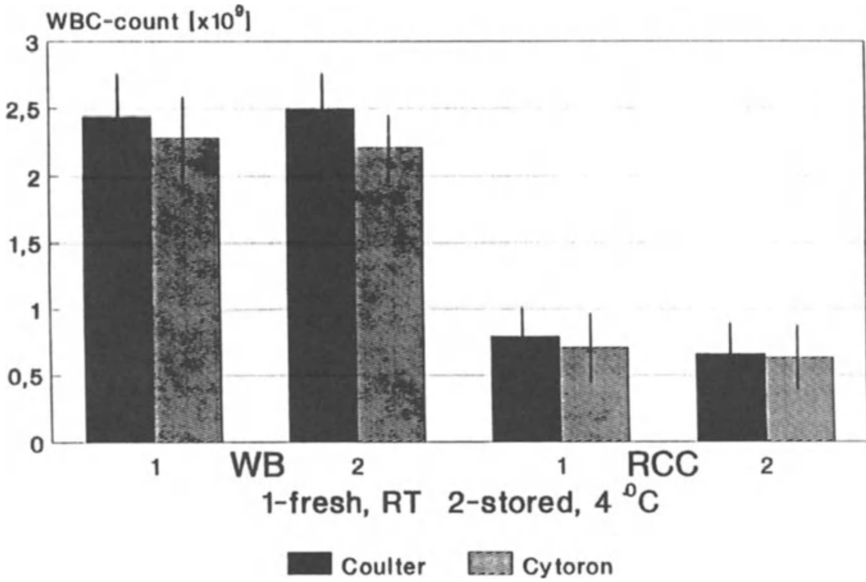


Figure 1. WBC-counts of whole blood (WB) and red cell concentrates before filtration (mean ± SD, n=21).

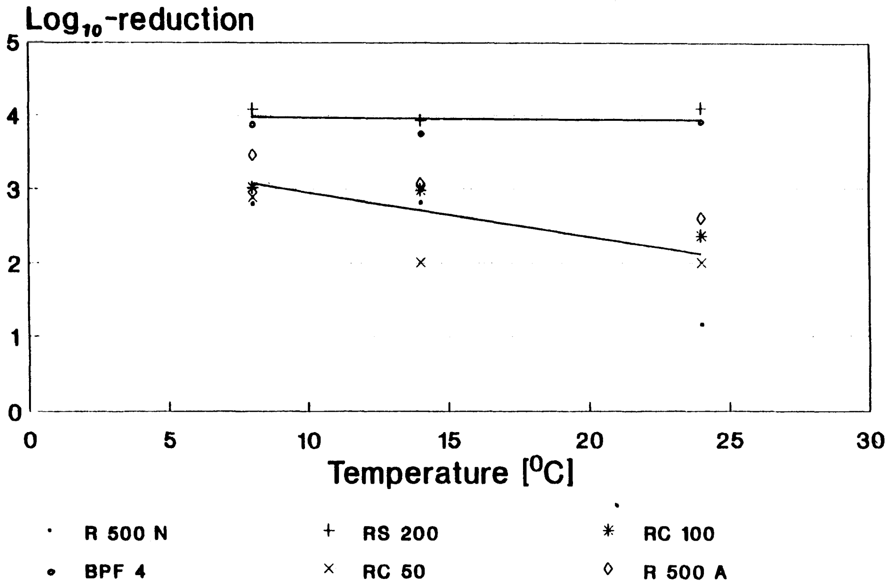


Figure 2. Temperature dependence of the leukocyte removal efficiency of 3rd and 4th filter generation (see Table 3 and 4).

In addition we registered the duration of the filtration procedure and the temperature of the blood unit when the filtration was started. The filtration itself was done following the instructions of each different system.

Results and discussion

The initial WBC-count of WB and RCC were $2.44\pm0.39\times10^9$ and $0.73\pm0.19\times10^9$, respectively. This is 70% WBC-reduction by buffy-coat removal which corresponds well with published data [5]. There were neither differences of the initial WBC-counts of the fresh and 1 day stored blood preparations nor differences in the WBC-counts obtained by Coulter and Cytoron (see Figure 1).

The filtration resulted in a red cell loss of 5 to 18% depending upon the used filter system. However, we found no significant differences between buffy-coat and RCC and fresh and stored blood (RC50 = 6.1±0.7%, RC100 = 10.3±2.3%, BPF4 = 8.3±2.1%, R500N = 18.5±3.7%, R500A = 12.4±1.7%, RS200 = 9.9±2.6%). These data are similar to those of other studies summarized by Meryman [9] regarding the 3rd generation filters. Beside higher leukocyte removal efficiency the 4th generation filters showed also improved RBC-recovery.

The PLT-removal were between 75 and 99% and differed also between the used filter systems, but was lower in fresh blood units especially using Pall filter systems. There were no differences between WB and RCC with Pall filters but there were differences with Sepacell filter systems:

a. Fresh blood cell preparations – WB:

RC 50	= 75.8±1.2%,	RC100	= 79.2±3.7%,	BPF4	= 83.8±3.1%,
R500N	= 99.0±0.3%,	R500A	= 99.0±0.4%,	RS200	= 98.9±0.5%,
RCC	= 96.0±1.8%,	RCC	= 82.1±0.6%,	RCC	= 82.4±2.1%.

b. Stored (overnight at 4°C) blood cell preparations – WB:

RC50	= 91.6±3.8%,	RC100	= 95.6±2.4%,	BPF4	= 95.0±3.4%,
R500N	= 94.0±3.1,	R500A	= 99.1±0.4%,	RS200	= 98.4±0.6%,
RCC	= 95.4±2.1%,	RCC	= 94.1±2.9%,	RCC	= 94.1±1.2%.

These findings seem to be due to platelet adhesion on the filter fibres which is influenced by cold storage and the different fibre materials. Analysis of the white cell counts are summarized in Table 1 to 4. The highest, 4log reduction, leukocyte removal was obtained with the 4th generation filter and stored RCC.

The different storage conditions resulted in different blood product temperatures at the time of filtration. We measured 8°C of overnight stored WB and RCC and 24°C of unrefrigerated room temperature stored blood. One hour storage at 4°C of RCC led to a temperature drop of 10°C to 14°C. There is a significant temperature dependence of the filtration efficiency of the 3rd filter generation (Figure 2) but not using filter systems of 4th generation on filtration of RCC. Those WBC of fresh blood which passed the leukocyte removal filter could be detected as granulocytes [4].

The ability of the 4th filter generation to be efficient even also in fresh blood products is the basis of so called in-line-filter systems. Filter systems which are included in a blood bag system to process not only RCC from WB but also leukocyte-depleted RCC within initial red cell preparation. First of those systems are already in experimental use.

This could be the beginning of a new blood bank strategy to supply only leukocyte-depleted blood products in future.

The increasing costs will be compensated by lower prices if increased numbers of filters are used and by diminishing of the side effects of blood transfusions. Especially reduced rates of alloimmunization [10,11] with all of its complications for further blood supply and reduced rates of infections caused by strong leukocyte-associated viruses like CMV or HTLV-I [12,13]. To be effective in the latter Rawal et al. [14] proposed an early filtration otherwise virus transmission could be caused by viruses released from lyzed WBC during the storage which promotes the idea of in-line-filtration.

As we discuss now 4log reduction of WBC with residual WBC-counts of 10^4 the problem of the sensitivity of counting methods has again to be discussed. Takahashi et al. [6,15] were able to stabilize the results by tenfold concentration of the samples. Newly developed 6log reduction filters needs new counting methods. Only with polymerase chain reaction (PCR) residual leukocytes would be detectable.

The question arises do we need such high leukocyte removal as we know that we still find some percentage of alloimmunization even after transfusion of blood products with very low (10 times below the CILL-dosis) residual leukocyte counts [16] due to the unknown nature of the complete mechanism of -alloimmunization [16,17].

Table 1. Pre- and post filtration WBC-count analysis: fresh whole blood.

	Coulter S IV Plus			**Ortho Cytroron**			
	pre ($\times 10^9$)	**post ($\times 10^6$)**	**rem. (%)**	**pre ($\times 10^9$)**	**post ($\times 10^6$)**	**rem. (%)**	**log-red.**
RC50 (n=2)	2.33 ±0.27	467.6 ±27.9	79.9	1.98 ±0.53	365.9 ±36.7	81.4	0.8
RC100 (n=2)	2.02 ±0.49	130.9 ±18.8	93.4	2.04 ±0.45	104.3 ±5.6	94.8	1.3
BPF4 (n=3)	2.26 ±0.26	22.0 ±8.8	99.0	2.28 ±0.25	20.4 ±2.7	99.2	2.1
R500N (n=2)	2.00 ±0.41	283.4 ±13.4	85.8	1.86 ±0.42	252.1 ±18.6	86.5	0.9
R500A (n=3)	2.39 ±0.33	82.9 ±27.8	96.6	2.23 ±0.32	89.6 ±26.0	96.0	1.4
RS200 (n=4)	2.42 ±0.36	n.d.[1]	n.d.[1]	2.34 ±2.34	1.23 ±0.41	99.9	3.3

1. n.d. = not detectable.

Furthermore the next question will follow which blood product has to be given to which patient. Therefore and because those filters and their production will be much more expensive we think it will be better to continue with a more general application of leukocyte depletion of all blood products.

Table 2. Pre- and post filtration WBC-count analysis: stored whole blood (1d, 4°C).

	Coulter S IV Plus			**Ortho Cytroron**			
	pre ($\times 10^9$)	**post ($\times 10^6$)**	**rem. (%)**	**pre ($\times 10^9$)**	**post ($\times 10^6$)**	**rem. (%)**	**log-red.**
RC50 (n=2)	2.41 ±0.38	n.d.[1]		2.16 ±0.36	8.23 ±0.52	99.62	2.3
RC100 (n=2)	2.42 ±0.22	n.d.[1]		2.18 ±0.42	0.63 ±0.04	99.97	3.4
BPF4 (n=2)	2.40 ±0.57	n.d.[1]		2.27 ±0.44	0.43 ±0.05	99.98	3.7
R500N (n=2)	2.09 ±0.51	4.99 ±0.64	99.77	2.36 ±0.34	0.62 ±0.06	99.97	3.4
R500A (n=2)	2.48 ±0.19	3.31 ±0.61	99.87	2.12 ±0.13	0.65 ±0.06	99.97	3.4
RS200 (n=3)	2.55 ±0.43	n.d.[1]		2.35 ±0.25	0.05 ±0.01	99.99	4.3

1. n.d. = not detectable.

Table 3. Pre- and post filtration WBC-count analysis: fresh SAGM-resuspended red cell concentrate.

	Coulter S IV Plus			**Ortho Cytroron**			
	pre ($\times 10^9$)	**post ($\times 10^6$)**	**rem. (%)**	**pre ($\times 10^9$)**	**post ($\times 10^6$)**	**rem. (%)**	**log-red.**
RC50 (n=3)	1.13 ±0.31	71.11 ±0.51	93.72	1.04 ±0.27	60.10 ±0.60	94.21	2.0
RC100 (n=2)	0.56 ±0.14	2.07 ±0.42	99.63	0.48 ±0.48	2.34 ±0.47	99.51	2.4
BPF4 (n=2)	1.06 ±0.32	3.19 ±0.45	99.70	0.87 ±0.17	0.26 ±0.11	99.97	3.5
R500N[2] (n=2)	2.03 ±0.62	210.9 ±9.9	98.92	2.36 ±0.89	233.1 ±10.7	90.15	1.1
R500A (n=3)	0.89 ±0.18	4.94 ±0.66	99.45	0.57 ±0.10	1.10 ±0.17	99.81	2.6
RS200 (n=4)	0.64 ±011	n.d.[1]		0.53 ±0.17	0.04 ±0.01	99.99	4.1

1. n.d. = not detectable; 2. no buffy-coat removal and SAGM-resuspension.

Table 4. Pre- and post filtration WBC-count analysis: stored SAGM-resuspended red cell concentrate (1d, 4°C).

	Coulter S IV Plus			Ortho Cytroron			
	pre ($\times 10^9$)	post ($\times 10^6$)	rem. (%)	pre ($\times 10^9$)	post ($\times 10^6$)	rem. (%)	log-red.
RC50 (n=3)	0.69 ±0.13	n.d.[1]		0.67 ±0.10	1.09 ±0.26	99.83	2.8
RC100 (n=2)	0.54 ±0.17	n.d.[1]		0.42 ±0.08	0.43 ±0.08	99.89	3.0
BPF4 (n=3)	0.52 ±0.12	n.d.[1]		0.48 ±0.09	0.11 ±0.07	99.98	3.8
R500N (n=3)	0.67 ±0.05	n.d.[1]		0.64 ±0.09	1.12 ±0.18	99.82	2.8
R500A (n=2)	0.82 ±0.16	n.d.[1]		0.84 ±0.23	0.37 ±0.11	99.96	3.4
RS200 (n=3)	0.79 ±0.13	n.d.[1]		0.80 ±0.19	0.08 ±0.02	99.99	4.0

1. n.d. = not detectable.

References

1. Eernisse JG, Brand A. Prevention of platelet refractoriness due to HLA antibodies by administration of leukocyte-poor blood components. Exp Hematol 1981;9:77-83.
2. Brady M, Anderson D, Milan J. Prevention of posttransfusion cytomegalovirus infection in neonates by the use of frozen-washed red blood cells. Clin Res 1982;30:895.
3. Wenz B. Leukocyte-free red cells: The evolution of a safer blood product. In: McCarthy LJ, Baldwin ML (eds). Controversies of leukocyte-poor blood and components. American Association of Blood Banks, Arlington VA 1989:27-48.
4. Richter E, Takahashi TA, Mavrina L, Matthes G, Sekiguchi S. Leukocyte depletion of whole blood and red cell concentrates with PERMSELEKT and SEPACELL R500. 1st German-Japanese Symposium on Transfusion Medicine. 1991; Berlin, May 8th.
5. Strauss D, Seidel B, Meurer W, Markova NA. Das buffy-coat-arme Erythrozytenkonzentrat: Herstellung, Konservierung und Qualitätsbewertung. Folia Haematol (Lpzg) 1980;107:104-24.
6. Takahashi TA, Hosoda M, Sekiguchi S. Leukocyte depletion from red cell concentrates with the Pall RC100, RC50 and BPF4 filters. Jpn J Med Instr 1992;62:68-77.
7. Takahashi TA, Hosoda M, Sekiguchi S. A flow cytometric method to detect residual leukocytes in platelet and red cell concentrates. Jpn J Med Instr 1990;36:429-37.
8. Mogi Y, Hosoda M, Takahashi TA, Sekiguchi S. Flow cytometric analysis of residual leukocytes in filtered blood components. Jpn J Transf Med 1991;37:617-26.
9. Meryman HT. Cleaning up red cells and platelets: Alloimmunization, immunosuppression and disease transmission. In: McCarthy LJ, Baldwin ML (eds). Controversies of leukocyte-poor blood and components. American Association of Blood Banks, Arlington VA 1989:1-26.
10. Brand A. Alloimmunization and refractoriness. In: Smit Sibinga CTh, Das PC, The TH (eds). Immunology and blood transfusion. Kluwer Academic Publishers, Dordrecht/Boston/London 1993;37-44

11. Kickler TS. The challenge of platelet alloimmunization: Management and prevention. Transf Med Rev 1990;4:8-18.
12. Gilbert GL, Hayes K, Hudson IL, James J. Prevention of transfusion-acquired cytomegalovirus infection in infants by blood filtration to remove leukocytes. Lancet 1989;i:1228-31.
13. Sekiguchi S, Takahashi TA. Leukocyte-depleted blood products and their clinical usefulness. In: Brozovic B (ed). The Role of leukocyte depletion in blood transfusion practice. Blackwell Scientific Publications, London 1989:26-34.
14. Rawal B, Yen TS, Vyas GN, Bush M. Leukocyte filtration removes infectious particulate debris but not free virus derived from experimentally lysed HIV-infected cells. Vox Sang 1991;60:214-8.
15. Takahashi TA, Oka S, Nishimura T, et al. Evaluation of a new leukocyte removal filter, the R-350, in a closed system. Jpn J Transf Med 1992;38:17-23.
16. Meryman HT, Mincheff M. Immunomodulation by blood transfusion. In: Smit Sibinga CTh, Das PC, The TH (eds). Immunology and blood transfusion. Kluwer Academic Publishers, Dordrecht/Boston/London 1993:11-20.
17. Meryman HT. Transfusion-induced alloimmunization and immunosuppression and the effects of leukocyte depletion. Transf Med Rev 1989;3:180-93.

IMMUNE TARGETING OF T-LYMPHOCYTES[1]

L. de Leij[2], B.J. Kroesen[1], T.H. The[1], J. Buter[3], N.H. Mulder[2], D.Th. Sleijfer[2], C.Th. Smit Sibinga[4]

Introduction

Immunotherapy is a treatment modality which meets considerable interest. Manipulation of the immune system is based, firstly, on our steadily increasing insight into the various cellular and humoral entities involved in an immune response and, secondly, from the availability of an array of monoclonal antibodies and recombinant cytokines, which offer the possibility to induce and/or modify immune activities.

Modifying an immune response may be useful both for the attenuation of unwanted reactions and for the intensification or even induction of wanted reactions. An example of the former is the treatment of rejection in organ transplant recipients. This can be done by specific suppression of T-lymphocyte activity via antibody treatment or by the application of T-lymphocyte specific drugs like cyclosporin A or FK506. Concerning the latter it appears that, apart from the successes obtained with current anti-microbial immunization protocols, the deliberate enforcement of immune reactions is still in an early experimental stage. An example of this is the enhancement of anti-tumour immunity, which is the topic of this chapter.

In the following a short introduction to anti-cancer immunity is given by discussing the clinical findings with rIL-2 treatment. The lessons which can be learned are, firstly, that immunotherapy has indeed enormous potential and, secondly, that most tumours are not susceptible for rIL-2 boosted "genuine" anti-tumour immune response. Such tumours might benefit from adoptive immunization protocols. One such a protocol can be the application of bispecific monoclonal antibodies for "tumour-targeting" of cytotoxic effector T-lymphocytes.

1. This work has been supported by grant GUKC 89-07 from the Dutch Cancer Society.
2. Department of Immunology, Internal Medicine, University Hospital Groningen, The Netherlands.
3. Department of Medical Oncology, Internal Medicine, University Hospital Groningen, The Netherlands.
4. Red Cross Blood Bank Groningen-Drenthe, Groningen, The Netherlands.

Immunotherapy in cancer

The concept of anti-cancer immunotherapy has been boosted by a study published by Rosenberg and coworkers in 1985 [1]. This study indicated a considerable response rate of metastatic tumour after an intensive treatment of a group of patients with high doses rIL-2 in combination with lymphokine activated killer cells (LAK, i.e. peripheral blood lymphocytes which had been pre-activated ex vivo by culturing in rIL-2 containing medium for 4-5 days). These initial results have been partly corroborated by a number of large clinical studies employing rIL-2 alone or rIL-2 plus LAK [a.o. 2,3].

From these studies it became clear that the addition of LAK had no major impact on the outcome of rIL-2 therapy and, importantly, the renal cell carcinoma (RCC) and melanoma were the best responding tumours. The toxicity of the treatment when given intravenously proved to be severe and limited the number of patients to whom treatment could be given. More recently a subcutaneous route of rIL-2 administration was adopted which lacked the severe toxicity associated with intravenous schedules, but retained its efficacy [4,5]. So rIL-2 treatment can be given without major toxicity and induces good responses in some patients with metastatic RCC or melanoma (10% showed longlasting complete responses and an additional 10% have partial remission). This is an important finding, since it indicates the great potential of immunotherapy in cancer treatment, even in patients with a large tumour burden. Still the question should be raised why most tumour types do not respond to rIL-2 treatment and why also in sensitive tumour types still 80% of tumours are unreactive. To answer such a question the biology of rIL-2 treatment must be investigated. The rIL-2 induced toxicity can be explained by an indirect effect of rIL-2 on lymphocytes, since toxicity appears to correlate with TNF levels in blood [6]. Thus TNF and other cytokines are quickly released in the blood probably by rIL-2 stimulated T- and NK cells. With intravenously applied rIL-2 this may lead to acute problems, since TNF in the peripheral blood causes direct damage to the endothelial cells lining the vessels. In this way the occurrence of a number of serious side effects as induced by intravenous rIL-2, known as the capillary leak syndrome, can be explained.

Anti-tumour activity in vitro appears to be derived mainly from rIL-2 activated NK cells (LAK) showing non-MHC-restricted tumour cell lysis. The anti-tumour activity in vivo, however, appears to be derived from MHC-restricted T-lymphocytes as has been shown in animal studies [7]. Also in humans, both in RCC and in melanoma, MHC-restricted, tumour-reactive T-cells have been isolated from patients after rIL-2 therapy [8-10]. Apparently rIL-2 treatment can start the proliferation of autologous anti-tumour-directed (probably along with many other specificities) T-lymphocytes to an extent where these become able to directly, or indirectly, kill the tumour cells.

These results indicate that only those patients will benefit from rIL-2 therapy who have already at least some pre-existing, tumour-reactive T-lymphocytes. Such a notion sets important limitations to the use of rIL-2 therapy, since autolo-

gous, MHC-restricted anti-tumour reactivity is absent, or at least undetectable, in most cancer types. To achieve still anti-tumour immunoreactivity in these (vast majority of) cancer cases other strategies have to be developed. One such strategy could be the deliberate induction of autologous anti-tumour T-lymphocytes by employing specific immunization protocols with pre-selected, patient-HLA-binding and tumour-specific peptides [11]. An alternative to such an approach is the application of adoptive transfer of anti-tumour reactivity using monoclonal antibody defined anti-tumour specificities. This approach will be discussed in the next paragraphs.

Retargeting of T-lymphocytes with bispecific monoclonal antibodies

During the past years monoclonal antibodies have been generated, which, although not really tumour-specific, are directed against antigens abundantly present on tumour cells and which are expressed only minimally and/or only in a (small) subset of normal cells. Such antigens are called tumour-associated antigens. When monoclonal antibodies directed against such tumour-associated antigens were labelled with a gamma-emitting radionuclide and injected into a tumour-bearing patient, specific gamma camera imaging of tumour has been obtained [12,13], indicating a preferential in vivo binding of antibodies to tumour cells. If such monoclonal antibodies could be endowed with the potential to kill

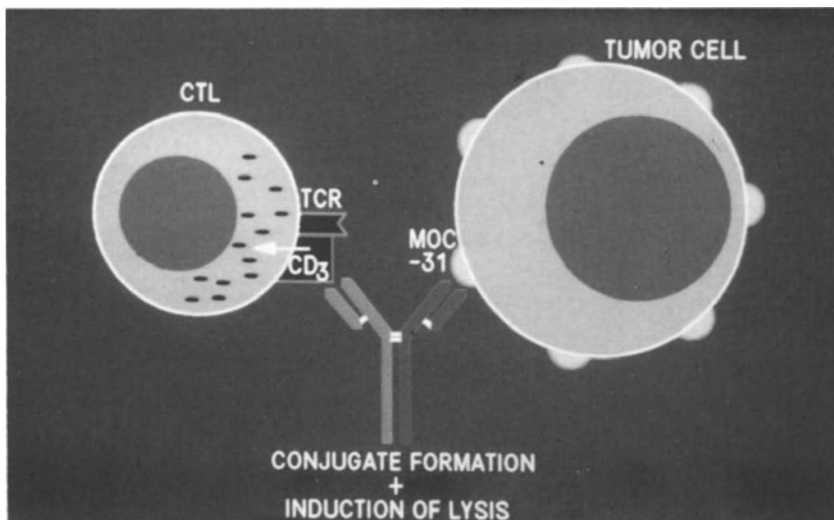


Figure 1. A tumour cell is linked to a cytotoxic T-lymphocyte through the bispecific monoclonal antibody BIS-1. BIS-1 has one specificity (left arm) directed against the CD3-complex on T-lymphocytes, whereas the other specificity (right arm) is directed against the pan-carcinoma-associated antigen AMOC31. As a result of the specific binding of the T-lymphocyte to the tumour cell through its TCR/CD3 complex activation of the T-lymphocyte is induced. At the end of this activation process the lytic machinery of the T-cell is triggered and the tumour cell will be lysed.

these tumour cells, then the injection of these antibodies into a patient might provide adoptive (humoral) immunity. A lot of research is going on to test such a hypothesis. This research ranges from injection of antibody "alone" (effecting lysis via complement activation or ADCC) to the use of the antibody as the tumour-seeking part of all kinds of "immunoconjugates" (effecting lysis via the delivery of toxins, cytostatic agents or radioactive labels). Progress in this field is slow but steady.

An alternative to the use of antibodies "alone" is to include the antibody directed against the tumour-associated antigen in a complex also containing an antibody reacting with cytotoxic effector cells, like for instance T-lymphocytes. In this way these potent, but tumour-unreactive effector cells become "retargeted" to react with and subsequently kill the tumour. An elegant example of such a "two-antibody-sided" complex is a bispecific monoclonal antibody (Figure 1).

To obtain functional "retargeting" the anti-effector-cell reactivity of the bispecific monoclonal antibody needs to be directed against a receptor molecule, which directly or indirectly induces the effector cell to become active. In the case of T lymphocytes a good candidate for such a receptor molecule is the T-cell-receptor/CD3 (TCR/CD3) complex, which, in the normal situation, mediates antigen-specific-T-lymphocyte activity through recognition of antigenic peptides in conjunction with HLA molecules [14]. It has been shown that antibodies reactive with TCR- or CD3-molecules are able to bypass the antigen/HLA restricted activation process by a direct switching on of the activation pathway coupled to TCR/CD3 [15].

Using this principle bispecific monoclonal antibodies containing one specificity for CD3 and another for a membrane antigen on a tumour target have been used successfully in vitro to direct the lytic activity of various cytotoxic T-lymphocytes towards the tumour target cells [16,17]. This so-called "effector cell retargeting" has met increasing interest, since it opens up the possibility to provide adoptive specificity to any T-lymphocyte irrespective of its genuine recognition ability.

Isolation of the bispecific monoclonal antibody BIS-1

We have produced the bispecific monoclonal antibody BIS-1 by fusing hybridoma RIV9 (specificity: anti-CD3; subclass: mouse IgG3; source: generously provided by Dr. H. Kreeftenberg, RIVM, Bilthoven, The Netherlands) with hybridoma MOC31 (subclass: mouse IgG1 [18]) according to a procedure described by de Lau [19]. The resulting hybrid hybridoma (called quadroma) produces, in addition to both parental antibodies, hybrid antibodies among which the bispecific ones. MOC31 is an antibody reacting with a pan-carcinoma membrane antigen with an apparent molecular weight of 40 kDa [18]. This antigen is the same as the one recognized by antibodies AUA-1 [20] and GA377-2 [21]. The antigen is not released into the circulation [22], making it a good target for bispecific monoclonal antibody directed T-lymphocyte recognition.

For clinical studies the BIS-1 producing quadroma was cultured in an endotronics hollow fiber culture system (cooperation with Dr. R. van der Griend, AZU, Utrecht, The Netherlands), which yealded about 1 gram functionally active BIS-1 antibody. Since quadroma-produced bispecific monoclonal antibody are contaminated with among others mono-specific (parental-type) monoclonal antibodies, a lot of purification has to be done before a defined bispecific antibody product is obtained. In the case of BIS-1 the bispecific antibodies (subclass: IgG3/IgG1) could be separated from the parental-type antibodies (subclass: IgG3 or IgG1) by protein A chromatography, tested for functional activity [23] and used for the pilot clinical study described below.

Lymphocyte preparations to be used in clinical studies

Since about 70% of peripheral blood lymphocytes (PBL) are T-lymphocytes and since both CD4- and CD8-positive lymphocytes might be operative in the induction of tumour cell lysis, PBLs appear a good source of effector cells for bispecific antibody mediated therapies. Bispecific antibody mediated killing is mediated only be activated and not by resting BPL, however [23]. Therefore PBL must be pre-activated, either in vivo or in vitro.

In the case of an intended systemic treatment of patients with bispecific monoclonal antibodies, PBL can be pre-activated in vivo by subcutaneous rIL-2 treatment. This last treatment results in considerable activation of PBL within 24 hours. Subsequently the bispecific antibodies can be applied by intravenous injection. Whether this latter treatment is a safe procedure, or not, has to be established yet.

Alternatively, ex vivo pre-activation of lymphocytes can be done. In the case of treatment of malignant effusions, as is the object of the first clinical studies with this new kind of treatment, in vitro pre-activation of isolated PBL is the first option, Since lymphocytes are often poorly present in malignant effusions.

An in vitro protocol which pre-activates mainly the T-lymphocytes is the following. PBL isolated by isopaque ficoll density centrifugation, or when large amounts are needed, lymphosurge [24] are incubated in anti-CD3 (about 1 mg/l) containing medium for 2 days. Subsequently the cells are cultured in rIL-2 (30,000 Cetus U/l) containing medium for three additional days, harvested, incubated with bispecific monoclonal antibody and reinfused.

BIS-1 incubation is done by adding 0.2 microgram/ml BIS-1 IgG to 10^8 cells/ml at 0°C for 30 min. After harvesting by centrifugation, the cells can be resuspended in 0.9% NaCl solution supplemented with 1% human albumin and 1800 U rIL-2/ml, and are ready then to be injected into patients.

Local anti-tumour treatment in carcinoma patients with BIS-1-redirected T-lymphocytes

In a pilot clinical study 7 carcinoma patients with malignant ascites or pleural exudates have been treated locally, i.e. in the peritoneal or pleural cavity, with

ex vivo activated, BIS-1 retargeted-T-lymphocytes. This treatment was given once (5 patients) or twice (2 patients). To evaluate the effect of the addition of BIS-1, 2 other patients received treatment with ex vivo activated T-lymphocytes "only" (i.e. no BIS-1 added), whereas 4 patients were treated with, alternating, activated T-lymphocytes to which BIS-1 was added and activated T-lymphocytes "only".

All treatments showed no or only minor local and systemic (mild fever) toxicity. To evaluate the two types of therapy, samples were taken from the effusions and the peripheral blood at various time points before and after infusion of the T-lymphocyte preparations. Analysis of the samples included immunocytology to visualise both tumour cells and infused lymphocytes. In addition, assessment of CEA, as a tumourmarker, and TNF and sCD8, as markers for immune activation, was done. In the samples, extensive conjugate formation between tumour cells and activated T-lymphocytes was observed already 1 hour after injection of the lymphocytes (Figure 2). In the case of injection of BIS-1 retargeted-T-lymphocytes such conjugate formation was more pronounced than in the case of injection of activated T-lymphocytes "only".

In addition, in the former case conjugate formation is followed by a disappearance or strong reduction of tumour cells in the samples taken after 24-48 hour. In parallel with this cytological indication of tumour disappearance the

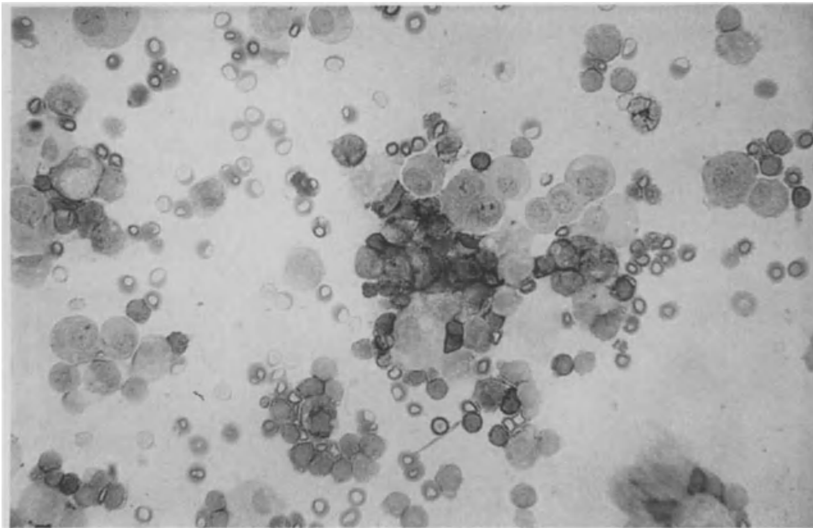


Figure 2. Cytospin preparate made from a sample take from an ascites one hour after injection of BIS-1 retargeted-T-lymphocytes. Staining was done with an indirect immunoperoxidase staining procedure in which anti-CD45 was used to specifically stain the lymphocytes (brown colour, which can be appreciated as a dark lining), whereas the tumour cells are not stained. The nuclei of all cells were weakly counterstained with haematoxylin. The picture shows a cluster of activated cytotoxic T-lymphocytes firmly attached to tumour cells.

tumour marker CEA decreased in the effusion fluid in the case of injection of BIS-1 retargeted-lymphocytes, whereas this was not the case when BIS-1 was omitted from the treatment. In addition to the anti-tumour effect some remarkable immunological reactions took place as a result of the infusion of BIS-1 retargeted-T-lymphocytes. A steep increase in granulocyte numbers became apparent 24-48 hour after infusion of BIS-1 retargeted-T-lymphocytes, which was paralleled by an increase of sCD8 and TNF. After 72 hours sCD8 and TNF returned to base-line level again, whereas granulocytes disappeared and became replaced by macrophages.

Conclusion

The result obtained with rIL-2 treatment in patients with extensive renal cell carcinoma or melanoma show that immune manipulation can be a very powerful form of anti-cancer therapy. It appears however that for such an approach at least some pre-existing T-cell immunity should be present. In those tumour cases which do not meet such a requirement T-lymphocyte retargeting with bispecific monoclonal antibodies appears to be an alternative. The study described above indicates, firstly, that local treatment with bispecific antibody retargeted T-lymphocytes is a save procedure, which is accompanied with only minor toxicity. Secondly, that such a treatment induces a strong local inflammatory reaction and, thirdly, that significant reduction of local tumour can be obtained.

Since metastatic cancer is a systemic disease by definition systemic application of bispecific monoclonal antibodies is the next step to be taken. Phase 1 studies employing intravenous application are currently carried out to answer the question whether such a systemic treatment can be given safely or not.

References

1. Rosenberg SA, Lotze MT, Muul LM, et al. Observation on the systemic administration of autologous lymphokine-activated killer cells and rIL-2 to patients with metastatic cancer. N Engl J Med 1985;313:1485-92.
2. West WH, Tauer KW, Yanelli JR, et al. Constant-infusion recombinant interleukin-2 in adoptive immune therapy of advanced cancer. N Engl J Med 1987;316:898-905.
3. Fisher RI, Coltman CA, Doroshow JH, et al. Metastatic renal cell cancer treated with interleukin-2 and lymphokine activated killer cells. Ann Int Med 1988;100:518-23.
4. Sleijfer DTh, Janssen RAJ, Willemse PHB, et al. Low dose regimen of interleukin-2 for metastatic carcinoma. Lancet 1990;335:1522-3.
5. Stein RC, Malkovska V, Morgan S, et al. The clinical effects of prolonged treatment of patients with advanced cancer with low dose subcutaneous interleukin-2. Brit J Cancer 1991;63:275-8.
6. Fraker DL, Langstein HH, Norton JA. Passive immunization against tumor necrosis factor partially abrogates interleukin-2 toxicity. J Exp Med 1989;170:1015-20.
7. Gallinger S, Hoshin DW, Mullen JB, Wong AH, Roder JC. Comparison of cellular immunotherapy and anti-CD3 in the treatment of MCA-38-LD experimental hepatic metastasis in C57B1/6 mice. Cancer Res 1990;50:2476-80.
8. Alexander J, Rayman P, Edinger M, et al. TIL from renal cell carcinoma: Restimulation with tumor influences proliferation and cytolytic activity. Int J Cancer 1990;45: 119-24.

9. Mitchell MS, Kempf RA, Harel W, et al. Effectiveness and tolerability of low dose intravenous interleukin-2 in disseminated melanoma. J Clin Oncol 1988;6:409-24.
10. Wolfel T, Klehman E, Mueller C, et al. Lysis of human melanoma cells by autologous cytolytic T-cell clones. Identification of human histocompatibility leukocyte antigen A2 as a restriction element for three different antigens. J Exp Med 1989;170: 797-810.
11. Sette A, Grey HM. Chemistry of peptide interactions with MHC proteins. Curr Opin Immunol 1992;4:79-86.
12. Mach JP, Pelegrin A, Buchegger F. Imaging and therapy with monoclonal antibodies in non-haematopoietic tumors. Curr Opin Immunol 1991;3:685-93.
13. Goldenberg DM, Schlom J. The coming of age of cancer radioimmunoconjugates. Immunol Today 1993;14:5-7.
14. Meuer SC, Schlossman SF, Reinhertz EL. Clonal analysis of human cytotoxic T-lymphocytes: T4 and T8 effector cells recognize products of different major histocompatibility complex regions. Proc Natl Acad Sci USA 1982;79:4395-9.
15. Spits H, Yssel H, Leeuwenberg J, de Vries JE. Antigen-specific T cell and antigen-specific proliferating T cell clones can be induced to cytolytic activity by monoclonal antibodies against T3. Eur J Immunol 1985;15:88-91.
16. Staertz UD, Kanagawa O, Bevan MJ. Hybrid antibodies can target sites for attack by T cells. Nature 1985;314:628-31.
17. Lanzavecchia A, Scheidegger D. The use of hybrid hybridomas to target human cytotoxic T lymphocytes. Eur J Immunol 1987;17:105.
18. De Leij L, Postmus PE, Poppema S, Elema J, The TH. The use of monoclonal antibodies for the pathological diagnosis of lung cancer. In: Hansen HH (ed). Lung Cancer: Basic and clinical aspects. Martinus Nijhoff Publishers Boston/Dordrecht/Lancaster 1986:31-48.
19. De Lau WBM, van Loon AE, Heije K, Valerio D, Bast EJE. Production of hybrid hybridomas based on HAT-s/neomycin-r double mutants. J Immunol Methods 1989; 146:906-16.
20. Spurr NK, Durbin H, Sheet D, et al. Characterization and chromosomal assignment of a human cell surface antigen defined by the monoclonal antibody AUA-1. Int J Cancer 1986;38:631-6.
21. Herlyn D, Herlyn M, Ross AH, et al. Efficient selection of human tumor growth-inhibiting monoclonal antibodies. J Immunol Methods 1984;73:157-67.
22. Woo DV, Li D, Mattis JA, Steplewski Z. Selective chromosomal damage and cytotoxicity of 125I-labelled monoclonal antibody 17-1A in human cancer cells. Cancer Res 1989;49:2952-8.
23. De Leij L, de Jonge MWA, ter Haar J, et al. Bispecific monoclonal antibody (Biab)-retargeted cellular therapy for local treatment of cancer patients. In: Crommelin DJA, Schellekens H (eds). From clone to clinic. Kluwer Academic Publishers 1990:159-65.
24. Elias MK, de Leij L, Smit Sibinga CTh, Das PC. Killing capacity of LAK cell transduced lymphocytes harvested by Haemonetics V50 lymphosurge technique. In: Rock G (ed). Apheresis. Progress in clinical and biological research. Vol. 337. New York: Alan R. Liss 1989:469-76.

HUMORAL FUNCTIONS OF IMMUNOGLOBULIN: RELATIONSHIP TO PURIFICATION TECHNOLOGY OF INTRAVENOUS IMMUNOGLOBULIN

P.L. Yap

Introduction

One of the major therapeutic achievements in the field of clinical immunology in the last twenty years has been the development of safe and effective immunoglobulin of therapeutic applications in immunodeficiency, autoimmune disease and infectious diseases [1-3]. However, the use of antibody preparations in the prevention and treatment of various infectious disease has a long history, dating back to 1891, when sheep serum was used by von Behring and Kitasato for the successful treatment of diphtheria in a child. This work had followed the experimental use of equine antitoxin raised against diphtheria toxin but unfortunately, the use of animal antisera in patients was accompanied by many side effects, particularly febrile reactions and serum sickness. Research was also conducted on the use of serotherapy against viral infections and there was also interest in the use of equine antiserum to treat pneumococcal pneumonia. However, the development of effective chemotherapy (in the form of sulfonamides, followed by the penicillin antibiotics) for the treatment of infectious diseases such as pneumococcal pneumonia, discouraged further research and development in this area till the 1940s.

The next important development occurred during the Second World War, when adequate supplied of blood and blood products were needed for the treatment of wartime casualties. The cold ethanol fractionation procedure for human plasma was developed for the production of albumin, and a plasma fraction (called Fraction II & III) which was rich in immunoglobulin G (IgG) was also identified as a by product of albumin purification [4,5]. Serum hepatitis could be prevented by the prophylactic administration of this fraction, as well as other infections and immunoglobulin concentrates for intramuscular administration (IM IgG, also called immune serum globulin (ISG)) soon became standard therapy for the prophylaxis of a number of viral infections including measles, hepatitis A and B, Varicella-Zoster and Rabies, and the treatment of patients with primary hypogammaglobulinemia (X-linked gammaglobulinemia, common variable immunnodeficiency). Unfortunately, only limited amounts of such immunoglobulin concentrates could be administered by the intramuscular route [6]. This was particularly important for patients with primary hypogamma-

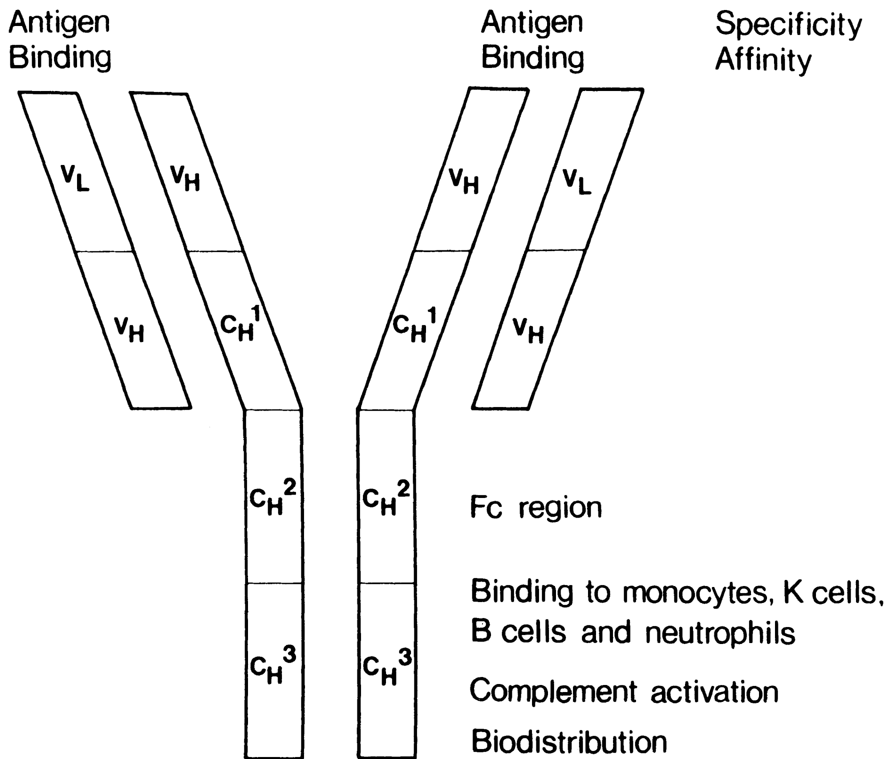


Figure 1. **A general model for the structure of the IgG molecule showing the four chain structure with two heavy (H) and two light (L) chains. Both heavy and light chains have variable (VH and VL) and constant regions (CH1-3, CL) respectively. IgG is divalent, with two antigen binding sites (Fab) and one Fc region where interaction with secondary effector systems occurs. Oligosaccharide groups are found on the CH2 domain of the Heavy chain.**

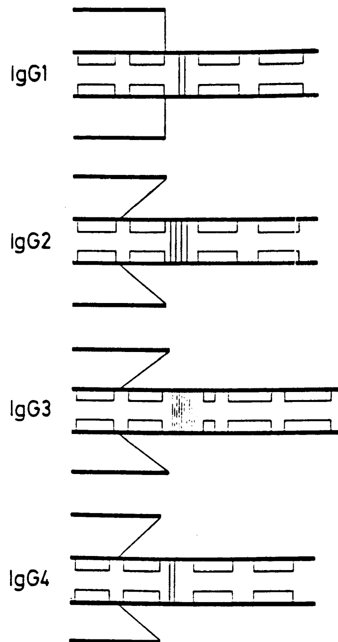


Figure 2. **Structure of the four human IgG subclasses with domains and disulphide bonds indicated. Note the extended hinge region of IgG3 which is believed to correlate closely with its potent effector function activity. The T-shape of IgG4 may explain the inability of this subclass to interaction with the C1q component of the complement pathway. Reprinted with kind permission of Dr M.W. Turner and Oxford University Press.**

globulinemia but severe adverse reactions accompanied attempts at administering higher immunoglobulin doses intravenously [7]. Various methods were therefore devised to reduce the incidence of adverse reactions without compromising the function of the IgG molecule and this review will consider the immunoglobulin isotypes in the human circulation, their function, distribution and subclasses, and the characteristics relevant to the technology for the preparation of intravenous immunoglobulin preparations.

Immunoglobulins: structure and function

The immunoglobulins are the group of protein molecules that possess antibody activity, i.e. the ability to specifically combine with the substance which elicited their formation (antigen). They comprise approximately 20% of the total plasma proteins but are also found in varying proportions in extravascular fluids, in exocrine secretions, and on the surfaces of some lymphocytes.

Immunoglobulins are glycoproteins composed of 82-96% polypeptide and 4-18% carbohydrate with a Y-shaped basic structure (Figure 1). The polypeptide component possesses almost all of the biologic properties associated with antibody molecules. The role of carbohydrate side chain is poorly understood but they may play a role in the secretion of immunoglobulins by plasma cells and in

the biological functions associated with the constant (C) regions of the two heavy chains since carbohydrates are not found in two Light (L) chains or the variable (C) regions of heavy chains (Figure 1) [8].

Antibodies are bifunctional molecules in that they bind specifically with antigen and also initiate a variety of secondary phenomena. These two kinds of activity can each be localized to a particular part of the immunoglobulin molecule: antigen binding occurs at the two Fab ends of the molecule formed from the combined V region of the heavy and light chains. The other secondary activities of the immunoglobulin molecule are localized to the C regions of heavy chains, the Fc end (Figure 1).

Antibodies are involved in a number of biological systems [9]. The two main effector mechanisms are complement induced cell lysis following the formation of antibody-antigen complexes [10], and complement activation and Fc receptor-mediated phagocytosis [11]. In addition, antibodies may directly neutralize biologically active compounds such as bacterial toxins, and they may also prevent attachment of micro-organisms to host cells by agglutination or by binding to adhesion molecules such as pili.

The five immunoglobulin classes (isotypes) differ from each other in the C region of the heavy chain. Immunoglobulin isotypes also differ from each other in size, charge, chemical composition and carbohydrate content as well as concentration and biological properties (Table 1).

Table 1. Biological properties of immunoglobulins found in the circulation.

Function	**IgM**	**IgG1**	**IgG2**	**IgG3**	**IgG4**	**IgA**	**IgD**	**IgE**
Mean serum concentration (g/l)	1.5	6.6	2.4	0.7	0.3	1.5	0.05	0.00005
Complement fixation (classical pathway)	+++	+	+	+++	–	–	–	–
Complement fixation (alternative pathway)	–	–	–	–	–	+	–	–
Interaction with Fc receptors:								
macrophages	–	+	–	+	–	–	–	+
neutrophils	–	+	–	++	+	+	–	–
lymphocytes	+	+	+	+	+	+	+	+
basophils	–	–	–	–	–	–	–	+++
mast cells	–	–	–	–	–	–	–	+++
Placental transfer	–	+	+	+	+	–	–	–
Half-life (days)	10	21	21	7	21	5	3	2

Immunoglobulin G (IgG) constitutes approximately 75% of the total serum immunoglobulins and consists of four subclasses which show structural differences: IgG1, IgG2, IgG3 and IgG4 (figure 2) [12]. IgG1 and IgG2 normally constitute approximately 66% and 24% of the total IgG respectively and IgG3

and IgG4 contribute approximately 7% and 3% respectively of the total IgG. Biological properties differ markedly between the subclasses (Table 1). Only IgG1 and IgG3 effectively fix complement and the subclasses differ in ability to fix complement in the following order: IgG3 > IgG1 > IgG2 > IgG4. IgG4 is completely unable to fix complement by the classical pathway but may be active in the alternative pathway. The relevance of complement fixation to antibody function is that via the complement proteins, immunoglobulin molecules can trigger a cascade system that generates a variety of potent biological molecules including anaphylatoxins and chemoattractants, leading ultimately to the lysis of antibody coated cells. In this process, fragments of the complement proteins may also be bound to the antibody coated cells resulting in their removal via complement receptors on phagocytic cells, without the completion of the complement cascade.

The other main mechanism by which antibodies contribute to the immune defence in the binding of immune complexes to Fc receptor bearing cells such as granulocytes and monocytes/macrophages. The complexes are subsequently phagocytosed and the engulfed material is then degraded. In addition to their established role on phagocytosing cells, Fc receptors are also essential in the antibody dependent cell-mediated cytotoxicity of K (killer) cells (Figure 3).

IgG is the only class of immunoglobulin that can cross the placenta in humans, and provides protection of the newborn during the first months of life. Although IgG may be synthesized by the foetus, the majority of IgG molecules at

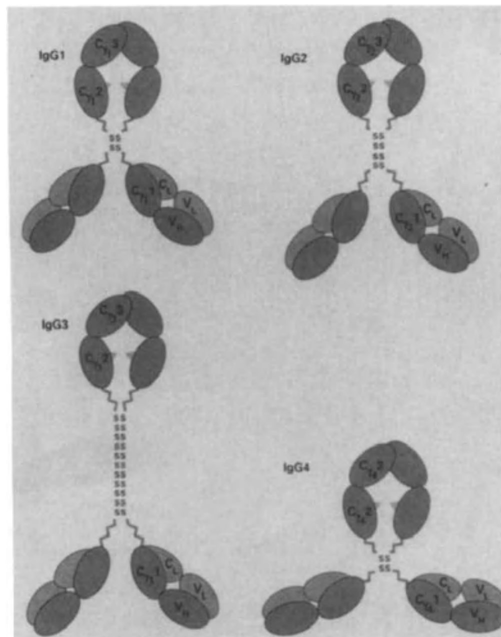


Figure 3.

birth are of maternal origin and are transferred in the last few weeks of pregnancy. The subclasses are not equally transferred transplacentally as IgG2 is transferred more slowly than the other IgG subclasses.

Immunoglobulin A (IgA) exists in serum in the monomeric form, constituting approximately 15% of the total serum immunoglobulins. However, IgA, in the form of secretory IgA, is the main immunoglobulin isotype in secretions and each secretory IgA molecule consists of a dimer of the basic immunoglobulin unit of two light and two heavy chains and one molecule each of secretory component and J chain. Secretory IgA is more resistant to proteolysis than the monomeric (serum) IgA molecule due to the presence of secretory component. the predominance of secretory IgA in saliva, tears, bronchial secretions, and mucous secretions of the small intestine suggests that its principal function may not be to destroy foreign micro-organisms but rather to prevent the entry of these cells and foreign substances to the body.

Immunoglobulin M (IgM) constitutes approximately 10% of the total serum immunoglobulins and normally exists as a pentamer with ten antigen combining sites, instead of two for IgG and serum IgA, and four for secretory IgA. IgM antibody is prominent in early immune responses to most antigens and predominates in certain antibody responses such as "natural" blood group antibodies. IgM antibodies are very effective at agglutinating bacteria due to their pentameric structure and IgM is also the most efficient complement-fixing immunoglobulin, a single molecule bound to antigen being able to initiate the complement cascade due to the five Fc sites per IgM molecule. IgM has a short half life of five days, compared with IgG (Table 1).

Immunoglobulin D (IgD) and immunoglobulin E (IgE) are monomers and are both present in serum in trace amounts. The function of IgD is unknown, but IgE is involved in immediate hypersensitivity type reactions, due to its reaginic activity. Immediate hypersensitivity to specific allergens can be passively transferred by serum and although the serum half life of the IgE is 2-3 days, IgE bound on mast cells via Fc receptors may persist for weeks or even months. The main physiological function of IgE is thought to be the defence against parasite infections, and high serum levels are sometimes seen in patient with certain parasitic diseases. The IgE mediated mechanism for the killing of parasites is thought to involve eosinophils, mast cells and basophils.

The immunoglobulin isotypes differ in their distribution between the intravascular and extravascular compartments, and secretory surfaces of the body. IgG is distributed throughout the intravascular and extravascular spaces, while IgM is mainly confined to the intravascular space. The physiologically important dimer of IgA, secretory A, exerts its biological activity only in mucous membrane secretions, since IgA producing cells normally line mucosal surfaces, and superficial mucosal infections induce a localized immune response restricted to secretory IgA.

The isotypes also differ in their response following an antigenic challenge. During primary immunization, there is an initial lag phase when no circulating antibody can be detected. The initial antibody molecules are of the IgM isotype

and of low affinity. However, IgM antibodies produced in the primary response will effectively agglutinate bacteria and induce complement mediated lysis via the classical pathway. This initial phase is followed by an isotype switch to IgG antibodies which retain the original antibody specificity, followed by a rapid increase in circulating antibody levels which are mainly of the IgG isotype. These IgG antibodies may then disappear or remain at low levels for long periods of time. Secondary immunization with the same antigen results in a rapid increase in antibody levels, but mainly of the IgG class, and the antibodies of the secondary response normally have a high affinity for the antigen. Some IgA antibodies may also be produced. The magnitude of the secondary response is influenced by a number of factors including feedback suppression by antibodies which combine with the antigen and therefore compete with the antigen receptors on the responding B-lymphocytes. Other regulatory mechanisms include the Fc mediated effects of immune complexes formed during the immune response and also idiotype-anti-idiotype interactions [13].

Some protein antigens (allergens) may also induce specific IgE antibodies. Most allergens are derived from plants or animals whereas viruses and most bacteria do not normally induce specific antibodies of the IgE isotype. Most antigens which tend to induce an antibody response of the three major immunoglobulin classes induce a poor or non-existent IgE response, and a combination of several properties is probably required to make a protein allergenic.

Immunoglobulin isotype levels are related to the age of the individual. Initially, the infant's circulating IgG levels reflect the maternal IgG that has been transplacentally acquired, with the lowest levels at six months of age. Subsequently, gradual increases of IgG, IgA and IgM levels occur with the circulating IgA levels being the last to reach adult levels. IgG subclass levels also differ in the rate with which they increase with IgG2 being the slowest subclasses to reach adult levels.

Finally, the immunoglobulin isotypes and IgG subclasses differ in their specific antibody activity. IgM antibodies are usually synthesized during the primary immune response to most antigens and also predominate in immune responses to bacteria and to their products. For the IgG isotypes, subclass differences exist in the antibody responses. Antibodies against protein antigens are mainly of the IgG1 subclass but low amounts of IgG3 and IgG4 may also be formed.

However, repeated exposure to certain antigens will lead to the formation of significant amounts of specific antibodies of the IgG3 or IgG4 subclass and hyperimmunization may result in an IgG3 or IgG4 restricted response. In contrast, antibodies against carbohydrate antigens are restricted to the IgG2 subclass in adult individuals. Adult levels of IgG2 antibodies against carbohydrate antigens are not reached until adolescence, an increase which is paralleled by the rise in total circulating IgG2. Children respond to both protein and carbohydrate antigens by the production of specific IgG1 antibodies but the IgG subclass of antibodies against carbohydrate antigens show a gradual shift from IgG1 to IgG2 as the immune system matures. Young children may also respond to cer-

tain protein antigens (such as viral antigens) by the production of significant amounts of IgG3 antibodies, suggesting that IgG3 antibodies may have a specialized biological function.

Immunoglobulin isotypes and intravenous immunoglobulin preparations

The choice of immunoglobulin isotype for the preparation of antibody concentrates depends upon a number of factors. Of the factors that affect production methodology, yield is the most important, and it is no coincidence that all existing licensed intravenous immunoglobulin preparations consist of IgG, with only traces of IgA and IgM. This is because of the five immunoglobulin isotypes, IgG is present in the highest concentrations in human plasma and will therefore give the best yield in the fractionation process. The plasma concentrations of IgM and IgA are much lower and the monomeric IgA present in the circulation is structurally different from the secretory IgA on mucous membranes. The yield of IgD and of IgE from human plasma is also very low, reflecting their low serum concentrations. There has been no interest in preparing IgD concentrates since no function has been ascribed to this immunoglobulin isotype, and there is no requirement for IgE concentrates to treat parasitic infections at present. Furthermore, an IgE concentrate could potentially cause a passive transfer to immediate hypersensitivity. An experimental IgM enriched intravenous immunoglobulin preparation exists, but data on it is limited at present.

Structural and chemical stability of immunoglobulin molecules are also important characteristics in view of the chemical changes that occur during the manufacturing of intravenous immunoglobulin preparations. Compared with IgM, IgG is more stable. Furthermore, IgG is the isotype with the widest range of antibody specificities and IgG antibodies have the highest affinities for antigen. IgG will only fix complement following binding with antigen compared with IgM (which exists as a pentamer) which can spontaneously activate complement. This is relevant to the tolerance of intravenous immunoglobulin preparations, since some anaphylactic reactions are thought to involve complement activation.

The ability of the antibody molecule to penetrate to the required site of action is also important. IgG molecules are distributed through the intra- and extravascular spaces whereas the majority of IgM molecules are confined to the intravascular space. Secretory IgA exerts its biological activity in mucous membrane secretions only and is therefore not suitable for administration since administered monomeric IgA would not be dimerized and undergo the addition of the J chain and secretory component. Finally, IgG has a much longer half-life in the circulation, compared with IgM and this permits the administration of intravenous immunoglobulin concentrates every 2-4 weeks in patients with primary hypogammaglobulinemia.

Methods used for the fractionation of plasma

Major developments in plasma fractionation occurred before the identification of the immunoglobulin isotypes, although by the 1940s, the chemistry of the γ-globulins (as the immunoglobulins were previously called, due to their electrophoretic mobility) was well known by then. Initially, attempts were made to purify the animal antisera for clinical use because of the problems of serum sickness and salt fractionation was introduced. Although the incidence of serum sickness was considerably reduced, they were not abolished and subsequent developments concentrated on fractionating human plasma. Two major processes were developed between 1946 and 1962 that utilize fractional precipitation of groups of proteins with ethanol, under controlled, low temperature conditions, on an industrial scale. These are the Cohn-Oncley process [4] and the Kistler-Nitschmann process [5], utilized mainly in the US and Europe, respectively. Both processes are based on five variables: ethanol concentration, pH, ionic strength, temperature an protein concentration.

The immunoglobulin product of both cold ethanol fractionation processes was suitable for intramuscular administration [6], but when attempts were made to administer this preparation (IM IgG) intravenously adverse reactions of a variable severity occurred, particularly in patients with primary hypogammaglobulinemia [7]. Additional (and varied) processing steps were therefore introduced for improving the tolerance and efficacy of immunoglobulin preparations from cold ethanol fractionation, when administered intravenously [13-15]. This was of particular importance, since intravenous immunoglobulin preparations raise IgG levels immediately, compared with intramuscular IgG preparations which are painful to administer, limited in administration volume and are associated with a delay of 2-3 days before peak IgG antibody levels are reached in the circulation, with loss, due to proteolysis at the site of infection.

The first major development in identifying additional processing steps was in 1962, when Barandun demonstrated that the adverse reactions were related to non-specific binding of complement, also described as anti-complementary activity, caused by the presence of IgG dimers or aggregates. Attempts were therefore made to overcome the adverse reactions and develop intravenous immunoglobulin preparations by treating the IgG rich product of cold ethanol fractionation with a variety of methods. These initially involved the use of enzymes such as pepsin and plasmin to cleave the Fc site, so as to reduce the interaction with complement. This was followed by methods that avoided Fc cleavage, but modified the Fc end chemically, such as reduction and alkylation, reductive sulphonation of disulphide bonds and treatment with β-propiolactone. Subsequent approaches involved the application of gentle processing conditions after cold ethanol fractionation to remove proteins present as impurities or harmful enzyme activities. These methods included polyethylene glycol precipitation, DEAE-Sephadex adsorption, silica gel adsorption and removal of alcohol by ultrafiltration and stabilization of the IgG molecule at pH 4 with or without traces of pepsin [15].

Effects of additional processing steps on IgG

The initial methods of treating the IgG molecule after cold ethanol fractionation unfortunately affected the function of the IgG molecule adversely. This was particularly the case with pepsin or plasmin treatment, since pepsin-digested IgG molecules lose the Fc portion, leaving a divalent F(ab')$_2$ fragment. Although this fragment can bind the appropriate antigens, all secondary effector functions are lost. Plasmin treatment, on the other hand, cleaves the IgG molecule at different sites and produces three fragments of equal size: 2 Fab fragments and 1 Fc fragment. While 33-66% of the IgG in the preparation is split, the remainder stays intact and represents the active component of this intravenous immunoglobulin preparation. However, as with pepsin treatment, all secondary functions based on the Fc end of the IgG molecule are lost in the cleaved IgG molecules.

Chemical modification by β-propiolactone modifies both the Fc and the Fab portion, although the Fc portion is particularly affected. During reduction and alkylation, disulfide bridges that occur between the polypeptide changes of the IgG molecules are hydrolyzed and irreversibly blocked. Although the molecule is still held together by hydrogen bonds, its functions are slightly altered. Sulphonation reduces a selected number of disulfide bonds between and within the polypeptide chains of the IgG molecules. Since the IgG molecule retains its integrity in vivo after infusion, this step is thought to be reversible. However, all three methods of chemical modification are thought to effect secondary Fc related functions but to a lesser extent than pepsin and plasmin treatment.

Finally, additional processing methods that avoid enzymatic cleavage or chemical modification produce IgG molecules that are chemically intact, or so-called, native. The IgG molecules retain full antigen-binding capacity, Fc-mediated effector function and complement activation after antigen binding in vivo. Fc-mediated effector function is particularly important since it is thought that the efficacy of intravenous immunoglobulin in autoimmune disorders may depend on this property. Polyethylene glycol (PEG) treatment at the end of cold ethanol fractionation, precipitates potentially harmful aggregates and prevents reaggregation. Ion-exchange chromatography (DEAE) isolates monomeric human IgG and eliminates aggregates. Reaggregation of the material is prevented by the addition of albumin and sugars. The incubation of the immunoglobulin rich fraction at the end of cold ethanol fractionation at pH 4, with or without traces of pepsin after alcohol removal by ultrafiltration or chromatography provides the basis for a number of widely used intravenous immunoglobulin preparations. Sucrose or maltose is added as a stabilizer.

Comparison of intravenous immunoglobulin preparations in vitro and in vivo

By 1980, a variety of intravenous immunoglobulin preparations were available. As a result of this, a WHO meeting in 1982 established several guidelines for intravenous immunoglobulin preparations based on information that was avail-

able at that time. The following recommendations were made: 1) that 90% of the preparation consist of monomeric IgG molecules and that polymers should not exceed 5% and fragments not exceed 10%; 2) that antibody activity include virus neutralization, neutralization of bacterial toxins (such as diphtheria and tetanus) and opsonization and induction of phagocytosis; 3) that all four IgG subclasses be present, until more was known about their clinical relevance; 4) that the preparations should mediate intact Fc-related effector functions and finally 5) that the preparations have an adequate biologic half-life.

In the light of the above recommendations, the characteristics of IgG prepared by the different procedures after cold ethanol fractionation were examined and all preparations were found to be mostly monomeric with variable traces of dimers and polymers [13,14]. The distribution of IgG subclasses was also investigated. Most commercially available preparations were found to contain IgG subclasses in proportions similar both to the starting plasma and to a WHO plasma reference. However, some were found to lack of IgG3 or IgG4 but practical significance of this observation is unclear, since patients with IgG subclass deficiency and who suffer recurrent pyogenic infections lack the IgG2 subclass, which is present in all intravenous immunoglobulin preparations.

A range of specific anti-viral anti-bacterial antibodies were found in intravenous immunoglobulin preparations, reflecting the differences between individuals and donor populations contributing plasma towards the large pools used for fractionation. However, the practical significance of these differences in specific antibody levels in unclear, as there is no data showing that they matter in the treatment of patients with primary hypogammaglobulinemia and they are not relevant in applications of intravenous immunoglobulin in autoimmune disease, such as idiopathic thrombocytopenic purpura (ITP).

Intravenous immunoglobulin preparations produced by different methods may vary in ability to activate complement. It is critical that intravenous IgG preparations are free of spontaneous anticomplementary activity, since this spontaneous anticomplementary activity may cause potentially dangerous reactions, particularly in hypogammaglobulinemic patients. Once infused, however, the IgG molecules should be able to activate complement when the corresponding antigen is bound. Not surprisingly, IgG antibodies in pepsin- and plasmin-treated intravenous immunoglobulin preparations activate the complement cascade poorly after exposure to antigen in vivo, compared with so-called "intact" or "native" intravenous immunoglobulin preparations. Chemically modified preparations have an intermediate position in terms of the ability to activate complement.

The requirement that intravenous immunoglobulin preparations should mediate intact Fc-related effector functions was not fulfilled by the preparations based on enzyme treatment with pepsin or plasmin and these preparations are therefore no longer used significantly. It was possible to show that chemical modifications to the Fc end of the molecule did affect second Fc-related functions but to a much lesser extent. As a result of this, one of the intravenous immunoglobulin preparations, based upon reduction and alkylation has now been

superseded by a preparation based upon pH 4 treatment, from the same manufacturer.

Finally, the survival of infused IgG molecules in vivo is crucial, especially for replacement therapy in patients with primary hypogammaglobulinemia. Because biologic half-life is mediated by a site on the Fc portion of the IgG molecule, preparations in which this site is digested or altered have a shortened half-life. In pepsin-treated preparations, IgG is degraded and eliminated in less than 48 hours, and in plasmin-treated preparations, the Fab pieces are eliminated within hours. Chemically modified preparations may have shortened survival times, or they may not readily equilibrate between intravascular and extravascular compartments. More recently, additional information about the half-life of antibodies of the IgG isotype has been studied by measuring the decline in specific antibody levels in serum of patients who have received high-dose intravenous immunoglobulin infusions. These newer tests have shown that the half-life of IgG molecules, which is assumed to be about 22 days, may actually be several days longer.

Conclusions

The concentrated immunoglobulin fraction obtained by the cold ethanol fractionation process has proven suitable for intramuscular use and has provided a beneficial therapeutic agent for the prophylaxis and treatment of infection. Intravenous immunoglobulin preparations that are well tolerated have been developed by additional (and varied) processing steps after cold ethanol fractionation. Of the different preparations, those involving enzyme cleavage or chemical modification are not widely used or generally available, since they do not have fully preserved secondary effector functions through the Fc end of the IgG molecule. All licensed intravenous immunoglobulin preparations are currently based upon IgG, and these preparations have been shown to be effective in patients with immunodeficiency, and some autoimmune diseases and specific infections. Other than for a single IgM-enriched preparation, none of the other immunoglobulin isotypes will undoubtedly concentrate on either finding new applications for intravenous immunoglobulin therapy or the development of hyperimmune polyclonal or monoclonal IgG or IgM preparations.

References

1. Berkman SA, Lee ML, Gale RP. Clinical uses of intravenous immunoglobulins. Sem Hematol 1988;25:140-58.
2. National Institutes of Health. Intravenous immunoglobulin: Prevention and treatment of disease. NIH Consensus Development Conference. JAMA 1990;264:3189-93.
3. Yap PL (ed). Clinical applications of intravenous immunoglobulin therapy. Churchill Livingstone, Edinburgh 1992.
4. Cohn EJ. Blood proteins and their therapeutic value. Science 1945;101:51-6.
5. Kistler P, Nitschmann H. Large scale production of human plasma fractions. Vox Sang 1962;7:414-24.

6. Janeway CA. The development of clinical uses of immunoglobulins: A review. In: Merler E (ed). Immunoglobulins, biological aspects and clinical uses. National Academy of Sciences, Washington DC 1970:3-14.
7. Barandun S, Kistler P, Jeunet F, Isliker H. Intravenous administration of human gammaglobulin. Vox Sang 1962;7:157-74.
8. Amzel LM, Poljak RJ. The three dimensional structure of IgG. Ann Rev Bioch 1979; 48:961-97.
9. Winkelhake JL. Immunoglobulin structure and effector functions. Immunochemistry 1978;15:695-714.
10. Benacerraf B, Sebestyen M, Cooper NS. The clearance of antigen antibody complexes from the blood by the reticulo-endothelial system. J Immunol 1959;82:131-7.
11. Unkeless JC, Eisen HN. Binding of monomeric immunoglobulins to Fc receptors of mouse macrophages. J Exp Med 1975;142:1520-33.
12. Oxelius VA, Immunoglobulin G (IgG) subclasses and human disease. Am J Med 1984;76:7-18.
13. Romer J, Morgenthaler J, Schertz R, Skvaril F. Characterization of various immunoglobulin preparations for intravenous application.Vox Sang 1982;42:62-73.
14. Romer J, Spath PJ, Skvaril F, Nydegger UE. Characterization of various immunoglobulin preparation for intravenous application. Vox Sang 1982;42:74-80.
15. McCue JP, Hein RH, Tenold R. Three generations of immunoglobulins G preparations for clinical use.Rev Infect Dis 1986;8(suppl 4):S374-81.

IMMUNE ADSORPTION CHROMATOGRAPHY FOR PROTEIN PURIFICATION

J.P. Tharakan[1], C.A. Williams[2], W.N. Drohan[2]

Introduction

Immune adsorption chromatography (IAC) for the purification of proteins in on the threshold of becoming the separation method of choice in the bioprocess industry. This technology results in extremely pure protein preparations, even with a single process step. However, several problems remain. These include: 1) low throughput, due to compression and crushing of resins with attendant flow restrictions; and 2) low monoclonal antibody (MAb) efficiency and utility, due possibly to steric hinderances, diffusional limitations, and MAb accessibility.

In this report we focus on studies that have been conducted in our laboratories to address the various issues involved in the scale-up of IAC. These studies began with the development of an immunoaffinity process for the purification of human coagulation factor IX (FIX) [1]. Following the original process development, our investigations focused on the optimization of the IAC process through the characterization of five commercial IAC resins [2], the examination of the effects of column flow configuration, whether radial or axial [3], various process operational parameters [4], and the effect of three MAb coupling chemistries on the efficiency of the MAbs [5].

The system that we studied is utilized for the IAC purification of human coagulation FIX. A similar process is used for the purification of the antithrombotic agent protein C (PC) [6]. The American Red Cross has developed IAC methods to separate both FIX and PC from plasma to high levels of purity, using MAbs targeted against either FIX [1] or PC [6]. Both of these processes utilize divalent-metal-ion-dependent MAbs that bind the protein of interest in the presence of certain divalent metal ions [7]. Elution is accomplished by simple chelation of the divalent metal ions using either 10 mM EDTA or 20 mM citrate buffer.

1. Howard University, Department of Chemical Engineering, Washington DC 20059.
2. Holland Laboratory, American Red Cross, Plasma Derivatives Department, Rockville, MD 20855.

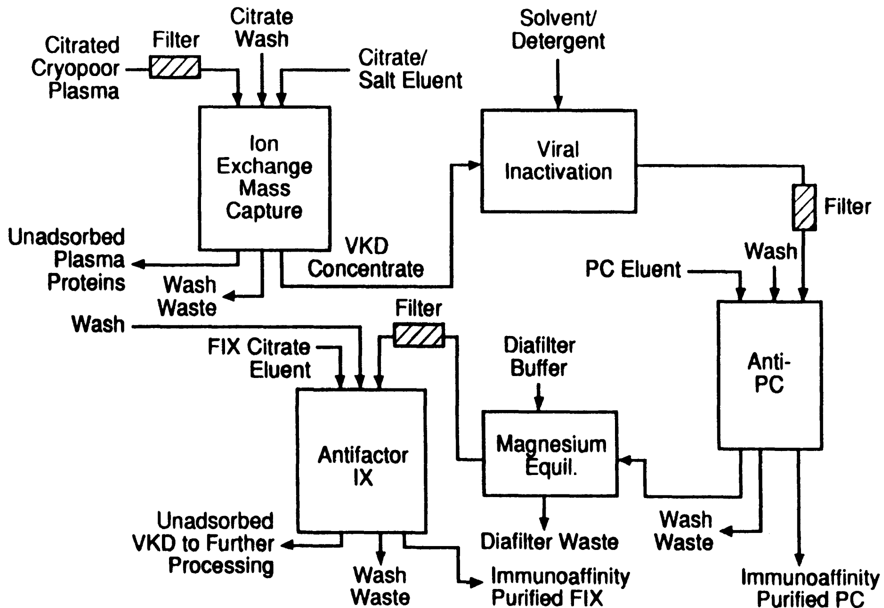


Figure 1. Process schematic for immunoaffinity purification of factor IX and protein C.

This report will focus primarily on the results obtained with the purification process for FIX, but will also include conclusions drawn from our experience with the purification process for PC.

Materials and methods

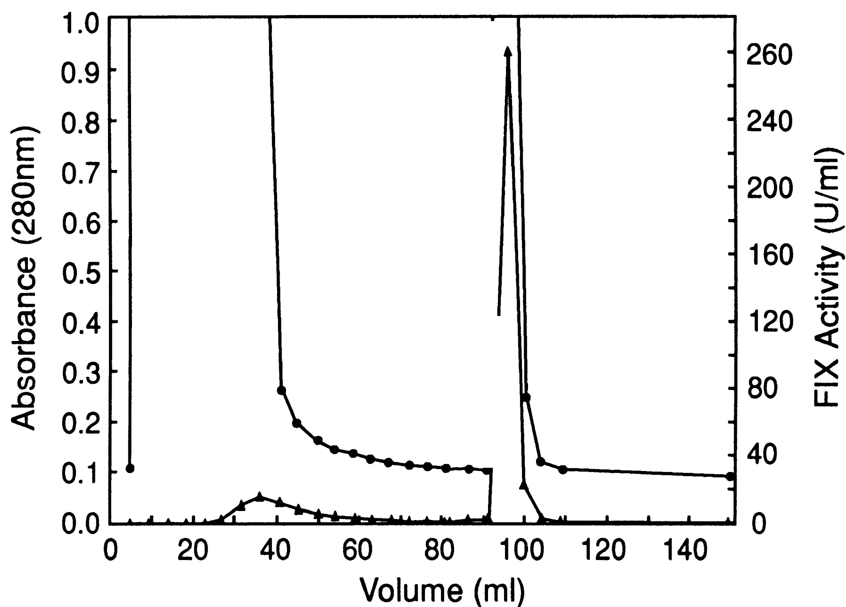
Materials and reagents

Human plasma was obtained from volunteer donors to the American Red Cross. Sepharose CL-2B and CNBr-activated Sepharose 4B were purchased from Pharmacia, Piscataway, NJ. All chemicals were reagent grade and were purchased from Sigma Chemical Co., St. Louis, MO.

MAbs were produced from BALB/c mice as previously described [8]. The purification of these MAbs was accomplished by precipitation of the centrifuged and filtered ascites fluid with ammonium sulfate, followed by dialysis and ion-exchange chromatography on DEAE-Sephacel. The details of these procedures have been published elsewhere [1,6].

Assays

– *Protein concentration:* Protein concentrations were measured by absorbance at 280 nm.



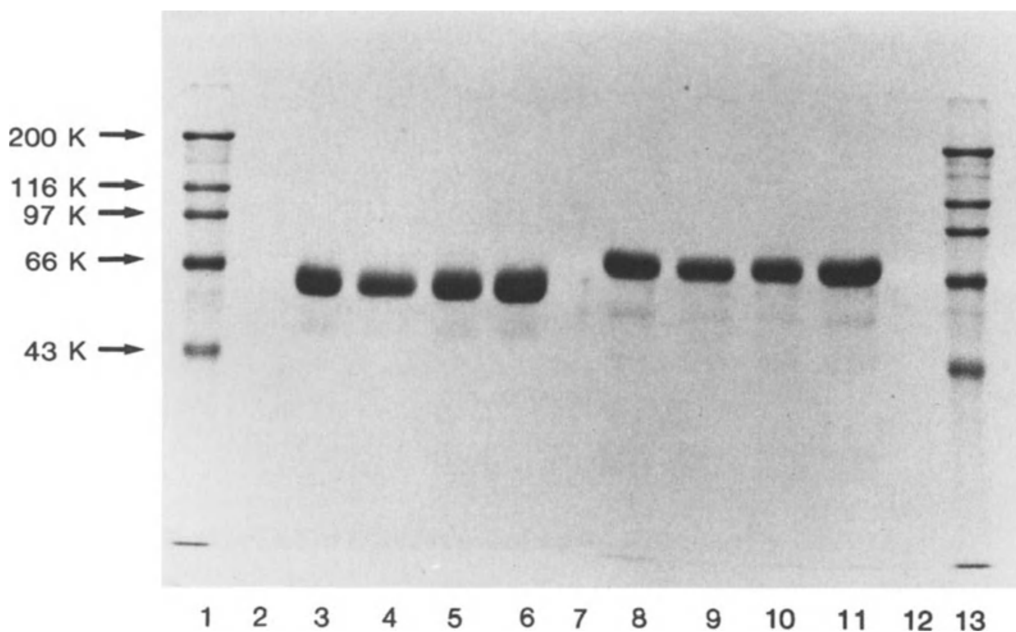


Figure 3. SDS-PAGE of immunoaffinity-purified factor IX (4% stacking gel, 9% separating gel, stained with Coomassie Brilliant Blue R-250). Lane 1, MW standards; lane 2, empty; lanes 3-6, non-reduced factor IX products from four separate experimental runs; lane 7, empty; lanes 8-11, reduced factor IX products from the same four runs as in lanes 3-6; lane 12, empty; and lane 13, MW standards. (Reproduced from Tharakan et al. [1] by permission of S. Karger.)

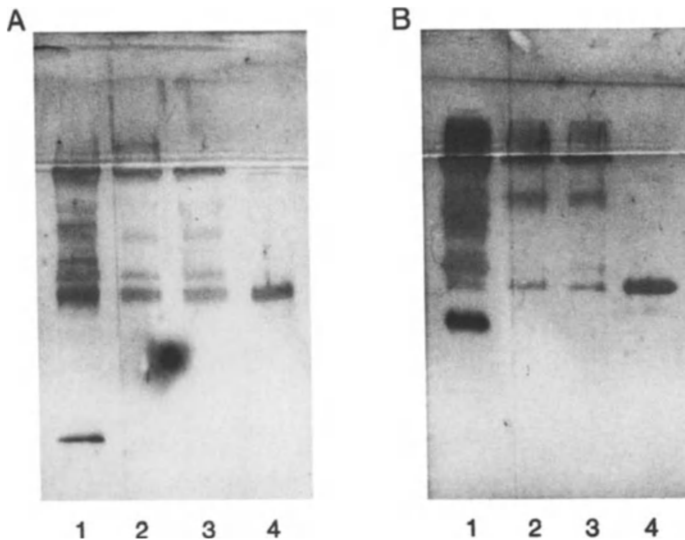


Figure 4. SDS-PAGE of four different factor IX products (8-25% gradient gels, silver-stained, PhastSystem, Pharmacia LKB Biotechnology). Gel A, non-reduced; gel B, reduced. Lane 1, factor IX complex; lane 2, FIX heat-treated; lane 3, FIX solvent/detergent treated; and lane 4, FIX monoclonal antibody purified.

– *FIX activity:* FIX activities were measured using a clotting assay according to the method of Biggs [9] as modified by Miekka [10].

– *PC activity:* PC activities were measured according to the method of Odegaard et al. [11]. The PC in each test sample was activated by Protac and the resulting activated PC was measured using the chromogenic substrate S-2366. Further details of the assay are available on Orthner et al. [12].

Parameters investigated

– *Effects of process parameters on FIX purification:* FIX was purified under various conditions. The general process schematic is shown in Figure 1. In one set of experiments, the concentration of the MAb on the resin was varied and the effect of MAb density was investigated. The effect of flow rate of the feed was studied by varying flow rate and examining the efficiency of the MAb. Finally, the effect of the concentration of antigen in the feed was varied to investigate the effect this would have on the ability of the MAb to bind the FIX. Some of the details of these experiments have been presented earlier [4]. Additional details are described in this report.

– *Physical and biochemical effects:* We have investigated the effects of flow on both natural and synthetic resins [2]. We studied the effect that increasing flow rates through various commercial resins had on the ability of the packed resin column to support the flow as well as on the extent to which the resins

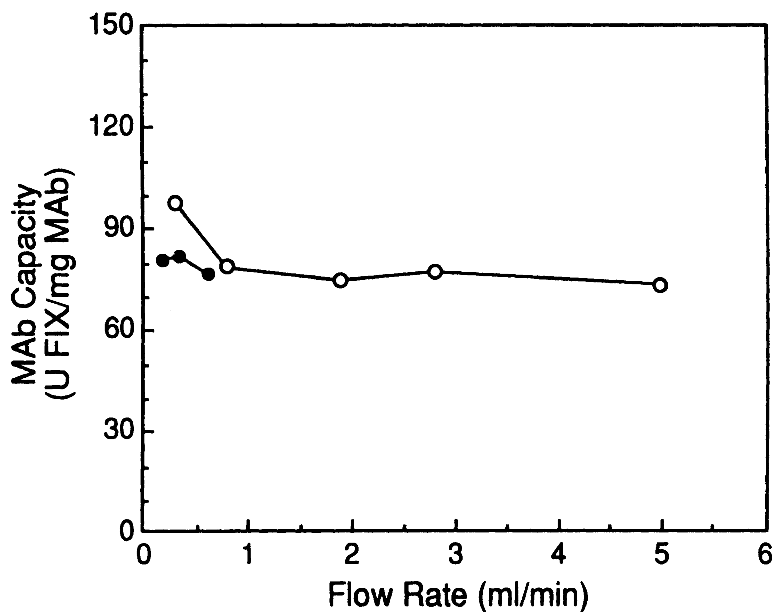


Figure 5. Effect of feed flow rate on MAb capacity (U FIX/mg MAb). 2% agarose as immobilization resin (●); 6% agarose as immobilization resin (○).

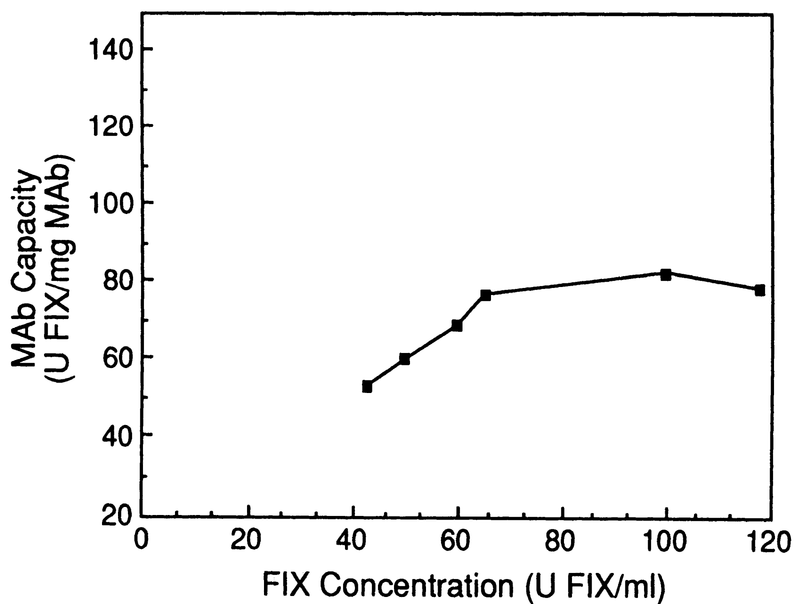


Figure 6. Effect of FIX feed concentration on the MAb capacity using 2% agarose as immobilization resin.

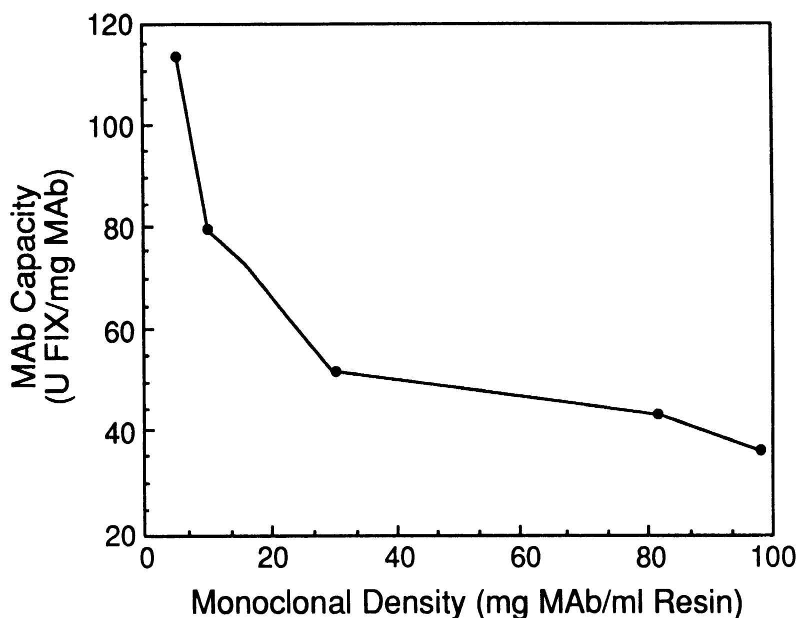


Figure 7. Effect of MAb resin density on MAb capacity using 2% agarose as immobilization resin.

were compressed when flow was applied. Implications of these data for scale-up of IAC is discussed.

– *Coupled chemistry:* The effects of several coupling chemistries were investigated, including a comparison of FMP-, hydrazide-, and CNBr-activated resins [5,12] on the purification ability of the MAbs against both FIX and PC. Additionally, a chemistry involving aldehyde group-reduction by $NaCNBH_3$ to form secondary amine linkages was studied [2]. In the case of the PC MAb, hydrazide- and CNBr-activation were compared in terms of the ability of the respective MAbs to bind their respective antigens as well as the activity of the purified protein obtained [12].

Results and discussion

Process development

The IAC process for the purification of FIX resulted in the production of FIX of high purity. The FIX eluted in a single peak (Figure 2) and SDS-PAGE analysis (Figure 3) of the elution pool revealed a single band when run under both reduced and non-reduced conditions. Western blot analysis and factor II, VII and X and PC activity assays indicated that these contaminants were not present at detectable levels. In addition, a Western blot analysis against mouse IgG yielded a negative result, suggesting that the amount of mouse IgG that may have leached from the IAC resin during the purification was negligible.

The improvement of the product throughout the various generations can be seen clearly from the SDS-PAGE of the first (factor IX complex), second (coagulation FIX-heat-treated and coagulation FIX-solvent/detergent-treated), and third (coagulation FIX-monoclonal antibody purified) generation products (Figure 4).

Process parameter variation

In order to optimize the efficiency of the MAb and the process, the effects of feed flow rate, feed FIX concentration, and MAb density was studied. Our results indicated that the feed flow rate (Figure 5) and the feed FIX concentration (Figure 6) did not have a significant effect on the efficiency of the MAb (defined as units FIX bound/mg MAb immobilized). The effect of MAb density, however, was quite pronounced (Figure 7). At low MAb density (approximately 0.5 to 1.0 mg MAb/ml resin), the efficiency of the MAb was high, capturing as high as 140 units of FIX/mg of MAb, or about 88% of the MAb was active in capturing FIX. As the density of the immobilized MAb increased, the efficiency of the MAb in capturing FIX decreased. The decrease in MAb efficiency was rapid at first, dropping to 80 units FIX/mg MAb (or approximately 50% active MAb) at 1.7 mg MAb/ml resin; subsequently, the slope of the drop in MAb efficiency dropped. The highest MAb density studied, 9.7 mg MAb/ml resin, resulted in a MAb efficiency of 25 units FIX/mg MAb, or 15%.

These results suggested to us that the immobilized MAb provided steric hinderances to FIX adsorption, with the hinderance increasing as the density of the immobilized MAb increased.

Resin characterization

In an effort to improve process efficiency, we studied the abilities of various resins to function as support matrices for IAC. Resins examined included a 2% cross-linked agarose, two 6% cross-linked agaroses, and two synthetic polymers [2]. We examined the effect of flow rates on the compressibility of these resins and the ability of the MAb immobilized on the resins to bind FIX. Our studies indicated that a 6% cross-linked agarose (Actigel-LAD Superflow, Sterogene Bioseparations Inc., Arcadia, CA) was the best of the three cross-linked agarose resins, in terms of its ability to support high pressures and linear flow rates, with a maximum linear flow rate of 25 cm/min. In addition, this resin performed satisfactorily when the ability of the immobilized MAb to bind FIX was examined, resulting in a matrix with 70% of immobilized MAb active. A 2% cross-linked agarose (Sepharose Cl-2B, Pharmacia LKB Biotechnology, Piscataway, NJ) also provided a resin that had 70% of immobilized MAb active; however, this resin was only able to support a 5 cm/min linear flow rate before undergoing compression which caused cessation of flow.

The two synthetic polymers were able to support higher flow rates than either the 2% or the 6% cross-linked agarose. However, the percent of active immobilized MAb was low in both cases, ranging from 1 to 38%.

Coupling chemistry

The chemistry utilized for the coupling of the MAb to the affinity matrix may be critical in determining the amount of MAb that remains active after the process. In addition, MAb orientation may determine the amount of antigen that may bind, as immobilization through the Fc portion would potentially yield a more active MAb than one immobilized through either Fab region of the MAb. It has been suggested that hydrazide immunosorbents will preferentially immobilize MAbs through the Fc portion of the molecule, thus leaving both Fab regions available for binding antigen [13]. Our investigations utilized MAbs against both PC and FIX. Immobilization was performed either by the manufacturer of the resins or in our laboratories, using hydrazide- or CNBr-activated resins. Our results showed that there was no noticeable improvement using the hydrazide resins. With MAb against FIX immobilized on the resins, the hydrazide chemistry yielded a 36% active MAb population, while immobilization through CNBr chemistry resulted in 54% active MAb. With the MAb against PC, both hydrazide and CNBr chemistries resulted in affinity matrices with average MAb activities of $40 \pm 5\%$ and $30 \pm 9\%$, respectively.

Conclusions

An IAC process has been developed at the American Red Cross to isolate FIX from human plasma to a high degree of purity and specific activity. This process can provide a FIX preparation that contains no detectable levels (by coagulation assays) of factors II, VII or X, or of PC, and no detectable amounts of leached murine IgG (by Western blot and ELISA). The metal-ion dependent MAb enables the use of non-chaotropic buffers for FIX elution and increases the reusability of the immunosorbent matrix.

Investigation of the effects of various parameters suggests that, for the FIX immunosorbent, MAb efficiency is not significantly affected by feed flow rate and feed antigen concentration. This is a benefit for processing, as throughput times may be increased without loss of antigen, and pre-treatment, either concentration or dilution, of the feed material is unnecessary. Thus, both these findings indicate that decreases in process time may be obtainable. Our studies also showed that increases in process flow rate must be able to be tolerated by the resin. A 6% cross-linked agarose was shown to be capable of supporting higher flow rates and back pressures.

MAb density, on the other hand, significantly affects the efficiency of the MAb. Lower MAb coupling densities result in higher specific utilization of the attached MAb. This is a definite advantage in scale-up as the major cost of a large scale IAC process is the MAb. Reduction of the amount of MAb required will decrease the start-up cost of the process and more importantly the cost of the final product.

Finally, the studies we have undertaken illustrate the development and optimization of an immunoaffinity chromatography process for the purification of factor IX. The types of investigations undertaken demonstrate a simple and

straightforward set of studies that can be undertaken in IAC process development. The application of these principles has led to the optimization of an immunoaffinity purification process for human protein C. Consequently, we feel that immunoaffinity column chromatography has widespread applicability in the purification of proteins.

Acknowledgements

The author would like to acknowledge the help and support of the following colleagues in the studies: Drs. D.B. Clark, S.I. Miekka, and C.L. Orthner, and H.E. Behre, M.A. Belizaire and B.L. Kolen. The bulk of this work was performed in the Plasma Derivatives Department of the Holland Laboratory at the American Red Cross. Some of the experiments on the parameter effects were conducted in J.P. Tharakan's laboratory in the Department of Chemical Engineering at Howard University.

References

1. Tharakan JP, Strickland D, Burgess W, Drohan WN, Clark D. Development of an immunoaffinity process for factor IX purification. Vox Sang 1990;58:21-9.
2. Tharakan JP, Highsmith F, Clark D, Drohan WN. Physical and biochemical characterization of five commercial resins for immunoaffinity purification of factor IX. J Chrom 1992;595:103-11.
3. Tharakan JP, Smith B, Hawkins K, Welsh G. An investigation of the effects of feed flow rate and antigen concentration in immunoaffinity chromatography of factor IX in axial and radial flow columns. 203rd ACS Natl Mtg, Division of Biochemical Technology, San Francisco, CA, April 1992, Abstract No. 0132.
4. Tharakan JP, Clark D, Drohan WN. Effect of feed flow-rate, antigen concentration and antibody density on immunoaffinity purification of coagulation factor IX. J Chrom 1990;522:153-62.
5. Highsmith F, Regan T, Clark D, Drohan WN, Tharakan JP. Evaluation of CNBr, FMP and hydrazide resins for immunoaffinity purification of factor IX. BioTechniques 1992;12:418-23.
6. Orthner CL, Madurawe RD, Velander WH, Drohan WN, Battey FD, Strickland DK. Conformational changes in an epitope localized to the NH_2-terminal region of protein C. J Biol Chem 1989;264:18781-8.
7. Velander WH, Madurawe RD, Orthner CL, et al. Process implications for metal-dependent immunoaffinity interactions. Biotech Prog 1989;5:119-25.
8. Wang H, Steiner J, Battey F, Strickland D. An immunoaffinity method for the purification of human factor IX. Fed Proc 1987;46:2119.
9. Denson KWE. Appendix 2: Techniques. In: Bigss R (ed). Human Blood Coagulation, Haemostasis and Thrombosis. Blackwell Scientific Pub, Oxford 1972:614.
10. Miekka SI. Use of albumin and Tween as stabilizers to prevent activity loss during clotting assays of coagulation factor IX and X concentrates. Thromb Haem 1987;58: 349.
11. Odegaard OR, Try K, Andersson TR. Protein C: An automated activity assay. Haemostasis 1987;17:109-13.
12. Orthner CL, Highsmith FA, Tharakan JP, Madurawe RD, Morcol T, Velander WH. Comparison of the performance of immunosorbents prepared by site-directed or random coupling of monoclonal antibodies. J Chrom 1991;558:55-70.
13. Cress MC, Ngo TT. Site specific immobilization of immunoglobulins. American Biotechnology Laboratory 1989;February:16-9.

DISCUSSION

P.C. Das, H.T. Meryman

H.T. Meryman (Rockville MD, USA): Dr. Richter's question is an important one: At what point has one removed sufficient leukocytes to prevent alloimmunization. It is difficult to answer this question since there has been only one clinical study, that by Sirchia and Rebulla, in which only red cells were administered[1]. In all of those studies, we see a reduction in the incidence of alloimmunization as the number of residual white cells is reduced down to about 10^7. With improved leuko-depletion techniques, the number of residual leukocytes has been reduced down to 10^6, 10^5 and even 10^4. Yet we see no further significant reduction in the incidence of alloimmunization. One interpretation could be that the depletion of leukocytes to the order of 10^7 may be sufficient to prevent alloimmunization by antigen-presenting cells in donor blood and a further reduction in the number of donor leukocytes will have no further effect on immunization from that source. The alloimmunization that continues to be seen even with greater leukocyte depletion may be the result of the very large amount of class I antigen present on platelets. What we need is more data like that of Sirchia and Rebulla on the effectiveness of leukocyte depletion of red cells in the absence of a heavy load of class I antigen. This could tell us at what point the removal of white cells is sufficient and whether the immunization seen at very low leukocyte counts is indeed helpful to know the extent of leukocyte depletion that would prevent alloimmunization from that source since this might make it unnecessary for us to continue to seek more and more leukocyte depletion at greater and greater cost perhaps to no avail. Would any of the panel or someone from the audience wish to add to this discussion?

P. Rebulla (Milan, I): I would just add a few words to what you said. In my presentation I reported that 2 thalassemic patients out of 14 admitted to our study became alloimmunized to HLA after some years of regular transfusion of RBC containing no detectable leukocytes. In this study leukocytes were counted with a microscopic manual chamber method with a lower detection limit of $3\text{-}5\times10^6$ leukocytes per RBC unit. One patient produced very weak HLA anti-

1. Sirchia G, Rebulla P, Mascaretti L, et al. The clinical importance of leukocyte depletion in regular erythrocyte transfusions. Vox Sang 1986;51(suppl 1):2-8.

bodies, the other strong anti-HLA-A2 antibodies. What are the reasons for alloimmunization in such patients given so few leukocyte? In addition to considering all possible biological explanations, one has to mention the chance that, during a 15-year study such as the one I refer to, other factors could have caused alloimmunization, such as receiving unfiltered or badly filtered RBC during holidays in other centres, etc. Although we investigated all these possibilities and the best evidence indicated that they were not present in our cases, we still have to consider them.

Other issues are the accuracy and precision of counting postfiltration residual leukocytes in routine conditions, such as our clinical study, which are poorly known. Certainly, the variability and overall error of counting at such low values are not trivial. Thus, I agree on the need you mentioned of re-evaluating very critically several of the trials done in the past, and of performing new ones in which the issue of transfusing RBC in chronic anemia or in surgery is considered separately from that of transfusing RBC and platelets in hematological malignancies.

C.Th. Smit Sibinga (Groningen, NL): I think, we have come to a point at which we have to realize, that we have to differentiate in our thinking. You quite rightly brought that up. The answer to the question of alloimmunization is not any longer in the absolute amount of residual white cells, but more importantly in what type of cells are these residual cells. Are they still active antigen presenting, are they anergic, do or do not they elicit any longer a reaction? The absolute count is really not anymore the issue and as dr. Steneker has elegantly shown in the efficacy of filtration, you need another type of cells to really trap white cells. If we combine these observations and see that, when we store red cells in the first place for a little longer period of time, two to three weeks, and have it filtered, then apparently the residual cells that come off, have no real competence anymore in the immune stimulation. Therefore, if you do this with red cells which were not buffy-coat depleted, but still contain platelets which may adhere to the filter, you may end up with may be a little higher residual number of cells while eventually having a much better product in terms of the immune prevention. Finally, we have to differentiate quite clearly to what category of patients we are using these products. Because distinctly there is a difference in the base line immune system of multiply transfused thallassemic patients as compared to the heavily immunocompromized oncology, hematology and bone marrow transplant patients. So, I would like to share some thoughts with the panel on this differential way of thinking, which we have to implement today.

H.T. Meryman: To the questions that you have raised, the age of the blood perhaps needs to be added. If it is true that blood products stored for two weeks or more are not going to induce alloimmunization, in some situations this may be more convenient way of dealing with the problem than by filtration. Some

studies to determine whether blood storage prevents alloimmunization in practice would be useful.

I. Steneker (Amsterdam, NL): What I showed you was only fresh red cell concentrates but I did also some capacity studies with two and three weeks old red cell concentrate. I saw that there was a 50% reduction in leukocyte capacity of the filters when we used older blood. So there is another mechanism or maybe a mechanism lacking if you use old blood for filtration. I think you need first a systematical study how old blood filtration is going on before you can say which cells are coming out and how they are. So, it is very important that you know the factors of filtration, the temperature, the storage age, because all affect the three mechanisms. It is also very misleading if you are talking about 2- or 3- or 4log reduction, because that is not true for all the factors. So first of all we should know how the filters act in several cases before you can give a clinical evaluation or even a comparison between the filters.

H.T. Meryman: Yes, but if the stored blood is not immunogenic then the filtering becomes irrelevant.

C.F. Högman (Uppsala, S): I would like to add to this uncertainty about what should be done or should not be done some information about a study, which has recently been carried out in Linköping in our country by Ledent[1]. What she found was, that the time for the filtration is of very great importance. She tried to mimick what is going on when you do a bedside filtration. Sometimes you may give the red cells rather quickly, other times you may take one hour and another time you may take two hours for the transfusion. Quite surprisingly it was found that when the time for filtration was one hour to two hours, the filtration efficacy was unreliable. So then many leukocytes passed through the filters, whereas when the filtration was only ten minutes the wanted results were achieved. I think that the conclusion would be, that we certainly need standardization and particularly, if we could have filters which are not sensitive to such variations it would result in a far better outcome. I think that what we have found clinically in our country is that at the beginning we had quite good effect of a policy of filtration of our products but recently we have had an increase in HLA immunized patients. The likely reason for this is, that we have some kind of errors in the procedures.

H.P. Olthuis (Nijmegen, NL): Dr. Claas, you told us about the effect on kidney transplant of HLA-DR shared blood transfusion in advance. Can we apply that technique for HLA or other platelet transfusions in patients. So, take a transfusion which is HLA-A,B and DR shared and then depress the immunosystem of the patient receiving the platelet transfusion; what do you think of that?

1. Ledent E, Berlin G. Inadequate leukocyte removal by bedside filtration of erythrocyte concentrates (to be published).

F.H.J. Claas (Leiden, NL): I think it is worthwhile to start such a kind of study. I think if you really can make such a study in a randomized way and give people first an HLA-DR shared transfusion. Then according to the theory you can transfuse afterwards whatever you want and then you will half the alloantibody formation. I think that is worthwhile to check. I do not see a reason why it is not chosen for, but you have to do it to be sure.

L. de Leij (Groningen, NL): Dr. Claas, I think it is interesting to hear about the immunizing or tolerizing effect of blood transfusions. You hypothesized that in the periphery there is killing of cells, which may be reactive to the graft. Are you thinking also of the thymus in this kind of tolerization, because the thymus is in fact to place, where people get tolerized against their own antigens.

F.H.J. Claas: Yes, it might be that the thymus plays a role, but in the model I proposed there is no need for the thymus, because instead of the tolerization you only find in the thymus, which is just what you say clonal deletion or whatever; that does not play a role in the model I proposed. In the model I proposed it is a kind of immunization leading to actual downregulation of the immuneresponse. I do not dare to use the term suppressor cell. You have a kind of active downregulation of the immuneresponse and I do not think that for that kind of tolerance there is a need for the thymus. But I think for instance with regard to the tolerance we find for the maternal HLA antigens, it might well be that that kind of mechanism may play a role; for instance either maternal cells when they are early in the pregnancy present and influence the repertoire in the thymus, that it might play a role in the tolerance against the maternal antigens instead. Not only an active downregulation of the immuneresponse plays a role, but also a kind of say clonal deletion or anergy. That is what we are studying at the moment by checking how heterogeneous the immuno repertoire is if you look at the response to maternal antigens versus paternal antigens. So it might tell me that in that case the thymus does play a role.

C.Th. Smit Sibinga: Dr. Racadot, I was very intrigued by your presentation. Is there any mathematical model to be applied today or do you foresee a mathematical model to calculate how many active T-cells need to be still present in the transplant to prevent the graft rejection phenomenon, where on the other hand one avoids the reactions, which one tries to eliminate by the purging.

E. Racadot (Besançon, F): We do not have a mathematical model, but we know that a dose of 10^6 per kg is able to produce acute GvHD. So, we must give to our patients does of T-cells which are lower than these doses.

C.Th. Smit Sibinga: You showed the effects of the monoclonal antibodies on the various epitopes with the specific effects on the CD9 and 10. But you also showed a tremendous suppressive effect when adding the CD24. What actually is it that causes this suppression?

E. Racadot: CD24 is a pan B; it is also present on granulocytes and on younger cells. So, its addition causes many problems in bone marrow purging. For this reason, it is not possible to use this monoclonal antibody to treat effectively in man.

A. Brand (Leiden, NL): Dr. Claas and others from the panel: What is starting to worry me is, that primary immunization with the generation of filters we are now using at this moment is around 10%. Of course, we are confronted with all types of 2nd, 3rd, 4th, 5th generation of filters claiming increasing removal of leukocytes. However, we have no clinical test to demonstrate the difference in reduction of primary immunization from 10% to 8% or to 6%. I think we need thousands of patients in those clinical studies. As Dr. Rebulla said, we also have to take into account the human error, which is of course present everywhere. Can we think of an in vitro assay in which we can estimate whether any type of activation of primary immunization has occurred to monitor the filters we are using, to monitor our approach and to circumvent that we have to conduct trials now in blood transfusion randomizing thousands of patients in each arm?

H.T. Meryman: I do not know whether these are questions that can be feasibly answered, but I suspect that this might be something that the BEST working party of the ISBT could be concerned with in the future. This group has so far concerned itself with the question of leukocyte counting, which, of course, is fundamental to studies of leukocyte depleted products. But I would suspect that the Working Party might begin to address the questions of how and when filters should be used to the extent that there are feasible ways to answer these questions. Some of them, as you say, would clearly require studies of such a magnitude as to be virtually impractical.

I would like also to refer to the question that was asked to Dr. Claas. That exchange went by so rapidly that perhaps many of us did not fully digest the implications. The question was, if you use a DR haplotype-identical blood such as that from a parent, does this induce an immunosuppression so that subsequent transfusions no longer need to be selected for haplotype match? It was my impression that this might well be true, which presents rather a nifty way of avoiding immunization and inducing a tolerance to subsequent transfusions. Am I correct in my interpretation of that?

F.H.J. Claas: Yes, that is what I meant. That is an approach you could use. It is a completely different approach. Instead of prevention of immunization you are really trying to achieve downregulation. The data which I showed are of course statistical data and it is not an all or nothing phenomenon. We still have some gentle immunization but may be you should select beforehand the proper combinations. If I could come back to the question Dr. Brand just raised. I think we have at the moment some in vitro assays available, with which we could start studying the triggering mechanism of the immune response. There are assays in which you can measure the amount of helper cells and you can even divide them

in primed versus naive helper cells. So, I think one of the studies which could be done is looking at the effect of the different preparations on the donor specific helper cells frequency and whether you really see an increase of the primed helper cells. I think that are studies which are feasible.

C.Th. Smit Sibinga: Dr. de Leij presented fascinating work in which we have been involved sofar. The question comes up what is likely to be the next step in identification of specific tumour cell markers; in other words what is the next type of tumour to be attacked by applying bispecific monoclonal retargeting technology.

L. de Leij: Well actually every antibody which is tumour specific or reacting with tumour associated antigen and there is a lot of knowledge accumulating at the moment about for instance monoclonal antibodies against melanoma, colon carcinoma and lymphomas. These are not really specific but are fairly well associated with tumours. Maybe these antigens could be targets for this kind of approach. I know of a number of groups working with these antigens to try to construct bispecific monoclonal antibodies and to use them in preclinical and starting clinical studies.

C.Th. Smit Sibinga: But if they are fairly specific what other target cells would then be in the game when you retarget the activated lymphocytes and what would be the damage thereof.

L. de Leij: The extent to which normal tissue damage is acceptable is worth considering. Take the example of B-cell lymphomas; you have for instance the CD20 molecule which is present on all lymphoma cells and which is only present on matching B-cells and which is absent on bone marrow cells. So, if you destroy, in addition to the lymphoma, the B-cells, these can be generated again. This is one example of a non-tumour-specific but rather cell-type specific antigen which can be used for this kind of therapy. The other example, which I want to mention is the epithelial associated antigen, which we are working with and which is probably non-accessible in normal tissues. For instance when you inject intravenously iodine-labelled monoclonal antibody against this antigen, then you can see fairly specific tumour localization of the antibody. So probably the antigen is shielded off from the circulation in normal tissue, whereas in tumour tissue shielding off is broken down. Then the antigen is accessible to the antibody and probably also to bispecific antibody therapy. We are just starting a phase I study with systemic treatment of patients with this type of antigens. We do not know yet if there is any toxic side-effect.

C.Th. Smit Sibinga: This definitely may lead to a reduction in the need for systemic stimulation of lymphocytes, because there is a much more specific targeting of lymphocytes to the tumour. Normal LAK-cells have a fairly broad targeting aray and need quite some stimulation systematically with

interleukin-2, which is toxic and is very costly. Could you give us a guess to what extent we can anticipate a reduction in this stimulation.

L. de Leij: Well actually I do not know. I think time will tell, because we now have started with these types of experiments. One thing you should keep in mind is that there is a retargeting of T-cells in which one T-cell can only kill one tumour cell. So, many T-cells are needed to kill a lot of tumour. A possibility to overcome this is that you give more often bispecific antibody during therapy as a possibility to reload the T-cell. In the mean time we need to sustain a level of activation of the T-cells. So, you have to give those patients IL-2. However, the amount of IL-2 which you need is much lower than given during for instance a normal IL-2 treatment. Some toxic side effects will occur but my guess is that this will be not very much. So, it will be worthwhile to try.

P. Rebulla: Dr. Yap, what is the rate of success of the intravenous gammaglobulin in acute and chronic ITP. Is the mechanism just a reticulo-endothelial blockade or is it anything different?

P.L. Yap (Edingburgh, UK): First of all, as far as acute ITP goes, you should expect 70 or 80% success for intravenous immunoglobulin. The problem is that all the series published, have had different criteria for success, different criteria for acute ITP. Someone used different doses like 2 grams per kilogram, 1 gram per kilogram and so on. But about two thirds of the patients do respond. For chronic ITP it is probably between a third to a half of the patients that should respond. The difficulty here is to define what should be a practical clinical response, in other words if you push the platelet count above 25 or 30,000 that may be clinically quite satisfactory, whereas other people would define the response to be going above 50,000. So, you have to look it over in the papers because they differ. The actual mechanism of intravenous immunoglobulin and ITP has not been finally proven. There is a whole series of theories. I think it will only be proven when somebody is brave enough and is willing to invest money in making an Fc fragment immunoglobulin preparation. Because if somebody could actually make a pure Fc preparation and inject it, then I think that would prove the Fc receptor blockade theory. There was one study published, in which a monoclonal antibody was made against the Fc receptor[1]. That was shown to be quite effective. Unfortunately, apparently there is a very serious side-effect. Since this came from the US no second paper has been published, but I was told the patient became quite ill.

T.H. The (Groningen, NL): Dr. Yap, also from the practical, clinical point of view with respect to patients with hypogammaglobulinemia you mentioned the

1. Clarkson SB, Bussel JB, Kimberly RP, Valinsky JE, Nachman RL, Unkeless JC. Treatment of refractory immune thrombocytopenic purpura with an anti-Fcγ-receptor antibody. N Engl J Med 1986;314:1236-9.

importance of the half-life of the immunoglobulin in the patients. So, the question is should we monitor the immunoglobulin levels in the patients and what are your thoughts about the levels and monitoring the specificities against the infectious agents.

P.L. Yap: I think this is quite a contentious area. The first difficulty is that different manufacturers want to sell as much intravenous immunoglobulins as possible. So what they propose is, that you give as much as you can and as frequently as possible. The other way of looking at it, of course, is to look at the factors that affect half-life. The natural half-life of the antibody, which as I said earlier is three weeks. But there are other factors such as for instance if your patient has a very active lung infection or chronic lung damage you do find the half-life is reduced. There are a number of other factors which affect half-life, which I cannot go into. What I think is the best way of monitoring patients is to actually give them a dose, which is consistent with freedom from infection and maintains the IgG levels at or near the lower end of the normal range. So, for instance in my laboratory the IgG ranges from 5 to 13 grams per litre and what we find is that if we give a dose of 200 mg per kg every three to four weeks, that in fact is quite sufficient to support the patient. There is also a trend towards giving the same dose but more frequently. If you push the dose up say at weekly intervals, then you do not need to give so much each time and you reduce the effects of the half-life degradation because the period is shorter and in fact the trend is now to giving something like a 100 mg per kg every week by home therapy and teach patients to give it to themselves. That is probably the ideal state, if the patient co-operates.

P.C. Das (Groningen, NL): Dr. Drohan, it was an elegant presentation, excellent work, but my question is how are you going to face the licensing authorities since the technology used in immunopurification involves animal materials and hybridoma cell lines. What sort of difficulty do you think you will be blessed with from the FDA.

W.N. Drohan (Rockville, MD, USA): I think that there are two areas which will come under particular scrutiny by the FDA. One is the possibility for transmission of any infectious agents which might be present in the milk of these transgenic animals. The other issue concerns the post-translational modifications to the protein being produced. I showed some data which suggest that human protein C can be produced in these pigs in a biological active form, but that it may be slightly different from protein C found in human plasma. The question will have to be answered in clinical trials.

H.T. Meryman: Why did you pick the pig instead of the cow.

W.N. Drohan: We picked the pig instead of the cow for a number of reasons. A pig has a gestation period of about three months, three weeks, and three days

and becomes sexual mature at about six months, while a cow has a gestation period of about nine months. In addition, a cow usually has only one offspring at a time, while a pig might have ten or twelve, so we could have more genetic material available for our studies. Initially, we did not expect to use pigs as a production system. We were only doing exploratory experiments. However, we found that the quantities of protein C they produced were enormous, as much as 2000 micrograms per ml of milk, and we are now looking at some of these animals as potential founders for a production herd.

C.Th. Smit Sibinga: Just to follow this up, I know that some time ago the Scots went for the sheep. That is because they had so many sheep on their hills, a very convenient animal. Would that be an alternative?

W.N. Drohan: John Clark and his group in Scotland have been very successful in producing proteins in the milk of transgenic sheep[1]. In fact as much as 50% of the protein in the milk of some of their sheep is alpha-1-antitrypsin, which is equivalent to 30 to 35 grams per litre. This is an impressive quantity of material. I believe you will see a lot more work in the future on the development of transgenic animals for the production of proteins intended for clinical use.

1. Wright G, Carver A, Cottom D, et al. High level expression of active human alpha-1-antitrypsin in the milk of transgenic sheep. Biotechnology 1991;9:830-4.

III. LABORATORY ASPECTS

IMMUNOLOGICAL PRINCIPLES IN ROUTINE QUALITY CONTROL, IMMUNOCHARACTERISTICS AND FUNCTION TESTS

Viral safety of blood and blood components

J.G. Huisman

Advances in HIV diagnostics

Topics related to specificity

The human immunodeficiency virus (HIV) is the causative agent of AIDS [1]. The nucleic acid sequence of the HIV-1 proviral genome has been obtained and the location of various protein coding regions within the viral genome has been determined [2]. Of particular interest is that portion of the HIV genome known to encode a precursor protein pr55 gag that is cleaved and processed into other mature proteins, p17, p24, and p15. The HIV-p24 gag protein known as the HIV-1 core antigen, has an apparent relative molecular mass of about 24 KDa and is a major constituent of the cone shaped viral capsid.

The p24 antigen of HIV is of particular interest as early marker in HIV infection [3]. Other studies have indicated that one of the first evidences of anti-HIV antibody formation (seroconversion) in infected persons is the appearance of anti-core antibodies, next to antibodies to the envelope protein gp160 (gp120/41). Detecting the presence of either the p24 protein or such antibodies therefore appears to be a good strategy for early detection of HIV-1 infection.

The development of sensitive immunoassays for detecting anti-HIV antibodies has been limited by difficulties with respect to the specificity [4,5,6]. Especially in low-risk populations, almost all reactions represent most likely false-positive results. A specimen found initially reactive by a screening test should be retested in duplicate using the original sample. A positive reaction is highly predictive of the presence of HIV-1 antibodies in people at high risk for HIV-infection.

Confirmatory testing

The immunoblot technique is frequently used to study antigen-antibody interaction, especially when the antigen is not available in a pure form. An example of value of this technique is offered by the analysis of HIV-antibodies. Transfer of electrophoretically separated viral proteins to a solid matrix (e.g. nitrocellulose), followed by incubation of strips thus obtained with sera of HIV positive patients, makes it possible to differentiate between specific and non-specific antibodies. This has also permitted the identification of disease specific, so-called marker antibodies [7].

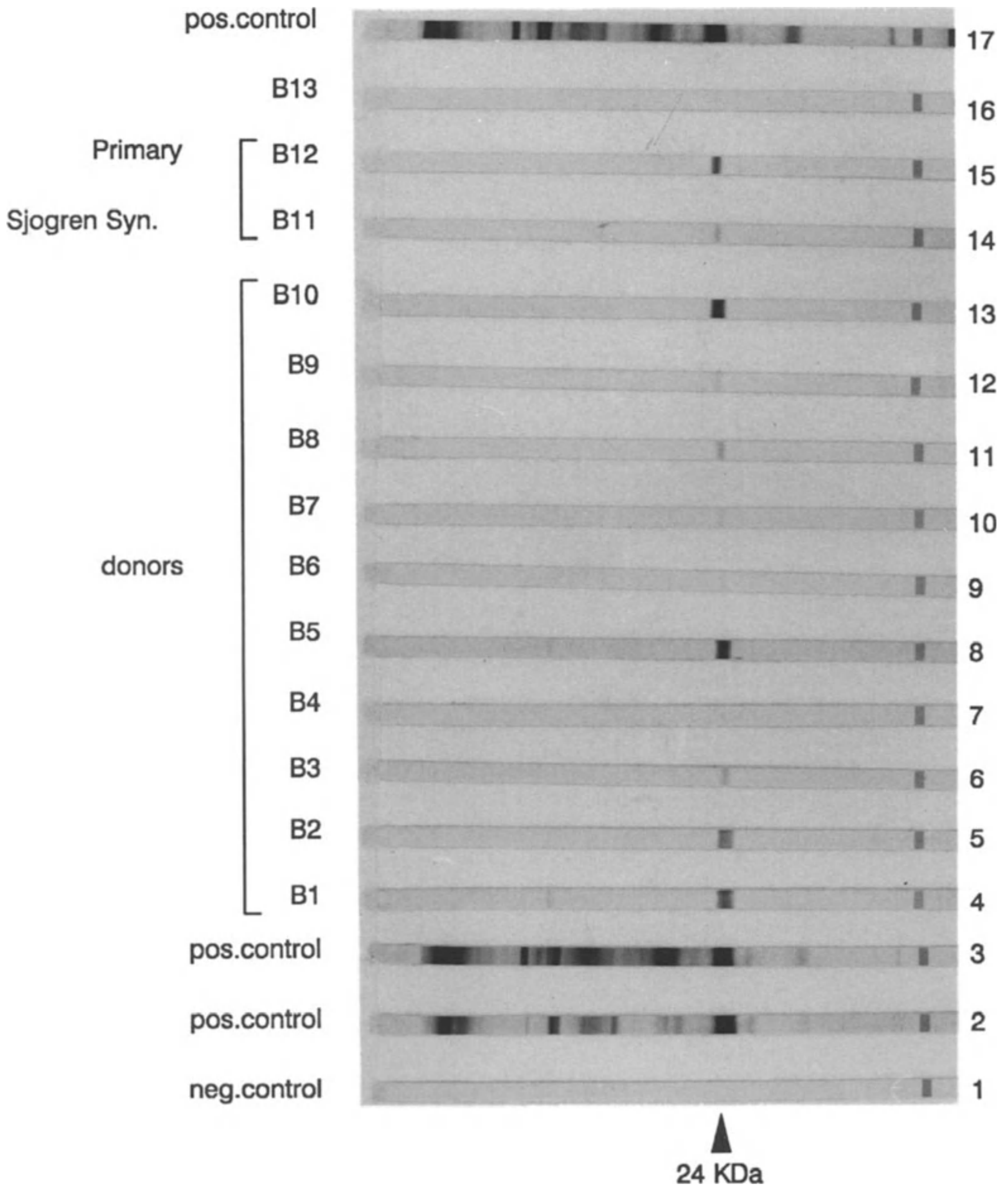


Figure 1. Reactivity of sera from a panel atypical reacting serum samples (diluted 1:50) on immunoblot strips (Biotechnology). From bottom to top: lane 1 seronegative control; lane 2, 3 and 17 seropositive control; lane 4-16 sera B1-B13 of "p24-only reactors". B11 and B12 are sera from patients with primary Sjögren syndrom. B3 and B13 are sera from the same donor which were collected with an interval of one year.

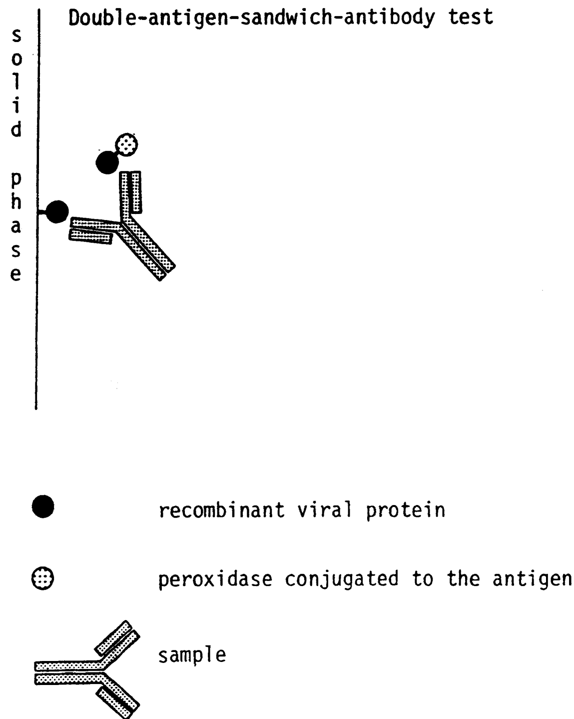


Figure 2. A schematic representation of a double-antigen sandwich antibody assay.

In immunoblot (IB), used as a standard confirmatory test, also false-positive reactivity has been observed [8]. These cross-reactivities are predominantly directed against the core proteins. As demonstrated in Figure 1, the reactivity of most indeterminate serum samples are directed against p24 only. Such results, in which only reactivity against one HIV protein is detected, have been characterized as immunoblot indeterminate [10]. However, reactivity of serum antibodies against p24 only, is also observed for seropositive donors early in HIV infection. Thus, in follow up studies, several approaches must be used to detect HIV-1 infection in these donors: indirectly by new generations of enzyme-linked immunosorbent assay (ELISA), radioimmunoprecipitation assay (RIPA) based on recombinant viral proteins [11] and polymerase chain reaction (PCR) with HIV-1 specific primers [12]. Direct testing for HIV infectivity can be carried out by cocultivation of donor derived lymphocytes with permissive cells [9]. Furthermore from look-back studies of blood donations it can be studied if seroconversion occurred in any of the recipients of blood with a persistent and isolated anti p24 reactivity.

Non specific reactions to HIV have been observed also in a high percentage in sera of patients with Sjögren's syndrom (SS). These observations have recently been extended to patients with systematic sclerosis, another autoimmune disease.

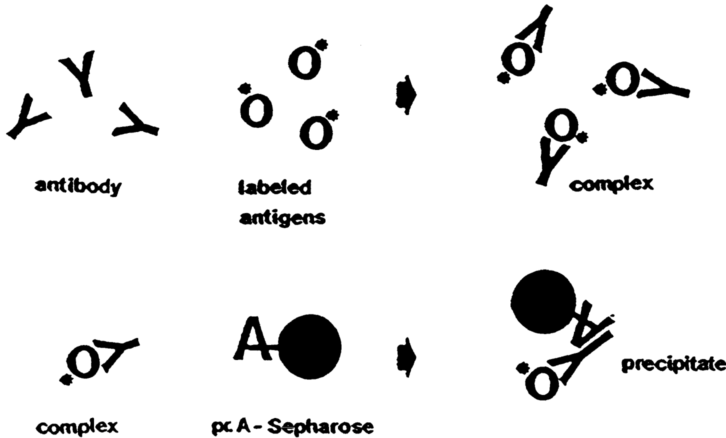


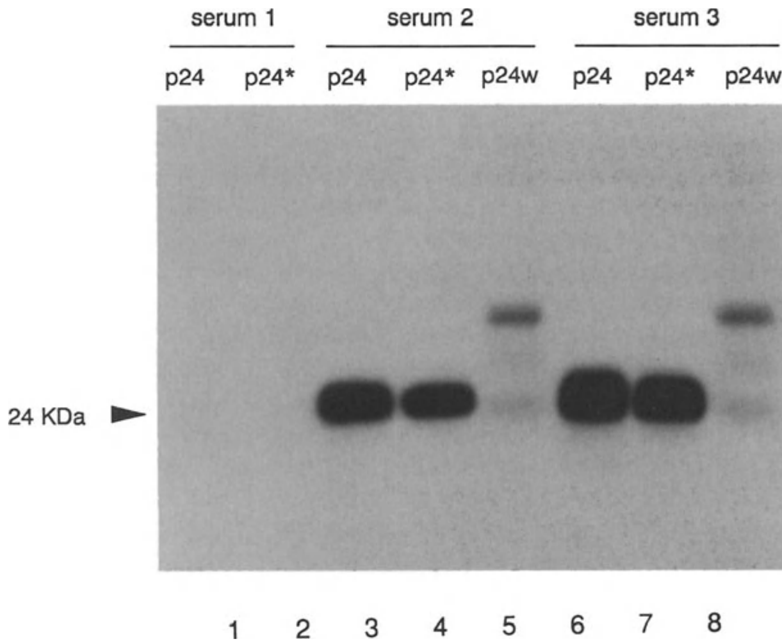
Figure 3. A schematic representation of a radioimmuno-precipitation assay. Serum specimen and radiolabelled antigen are incubated, followed by the binding of immunocomplexes to protein G, covalently linked, to Sepharose beads. Unbound labelled antigen can be removed by washing. The amount of bound antigens can be measured in a gammacounter.

These findings have sparkled the suggestion that a HIV-like retrovirus could represent a viral agent in the etiology of SS [13]. In general, in cases of indeterminate reactivity additional analysis is required. Consequently, large scale screening in low-risk populations will result in many expensive supplemental and confirmatory testing mostly with negative results; furthermore unnecessary and traumatic counselling with the donor is needed. For these reasons, it is important to understand the basis underlying cross-reactivity and to develop alternative conclusive screening assays and confirmatory tests.

We studied in detail the nature of cross-reactivity of atypical reacting serum samples in HIV-immunoblot. Serum samples that were designated as being p24-only reactors were analyzed for reactivity in third generation enzyme immunoassays performed in a double antigen sandwich format (Figure 2) as well as in liquid phase radioimmunoassay [11] based on HIV-recombinant proteins (Figure 3).

The following three purified recombinant proteins were used:

1. Full length HIV-1 p24 expressed in Escherichia coli (amino acids 1-232);
2. p24* Chimaere of HIV-1 p24 (amino acids 1-225), gp41 (amino acid 78-100), a two amino acid linker glycine, proline and amino acid 226-232 of p24;



Figu.. 4. Reactivity of serum samples with ^{125}I-labelled recombinant HIV-viral proteins in an immunoprecipitation assay followed by SDS-PAGE and autoradiography. Lane 1 and 2 reaction with serum of "p24-only reactor" (1) with p24 and p24* respectively; lane 3-5 reaction with a seropositive control serum (2) with p24, p24* and p24W respectively; lane 6-8 reaction with a seropositive control serum (3) with p24, p24* and p24W respectively.

3. p24W Chimaere of HIV-1 p24 (amino acids 1-232) and a N-terminal part of gp41, kindly provided by Dr. R.J.S. Ducan, M.A., Ph.D., Wellcome, Beckenham, England BR3 3BS).

HIV-proteins were labelled exogenous with ^{125}I. Serum was incubated overnight at 4°C with the radioactive HIV-protein in RIPA-buffer containing 50 mM Tris-HCI, pH 8.0, 500 mM NaCl, 0.1% $^{v}/_{v}$ TX-100, 0.02% $^{v}/_{v}$.

Tween-20 and 1% $^{w}/_{v}$ BSA (Sigma, St Louis, USA). The immuno-complexes were bound to proteins G-Sepharose beads (Pharmacia/LKB, Biotechnology AB, Uppsala, Sweden), centrifuged, washed four times with PBS containing 0.1% Tween-20 and the radioactivity was measured in a gamma-counter. For an autoradiogram the bound immunoprecipitates were eluted from the Sepharose beads by means of boiling for 5 min in sample buffer containing: 0.125 M Tris HCl pH 6.8, 17% glycerol, 10% β-mercaptoethanol, 4% sodium dodecylsulphate and analyzed by polyacrylamide slabgel electrophorese and autoradiography, using Kodak XAR film. The antibody reactivity of a panel of

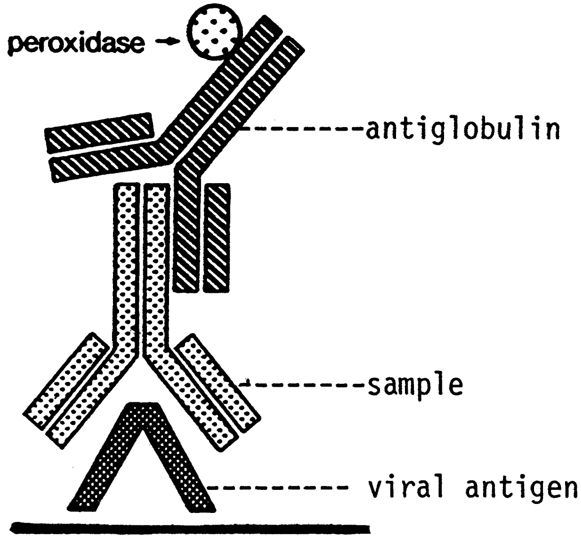


Figure 5. A schematic representation of an indirect antibody assay.

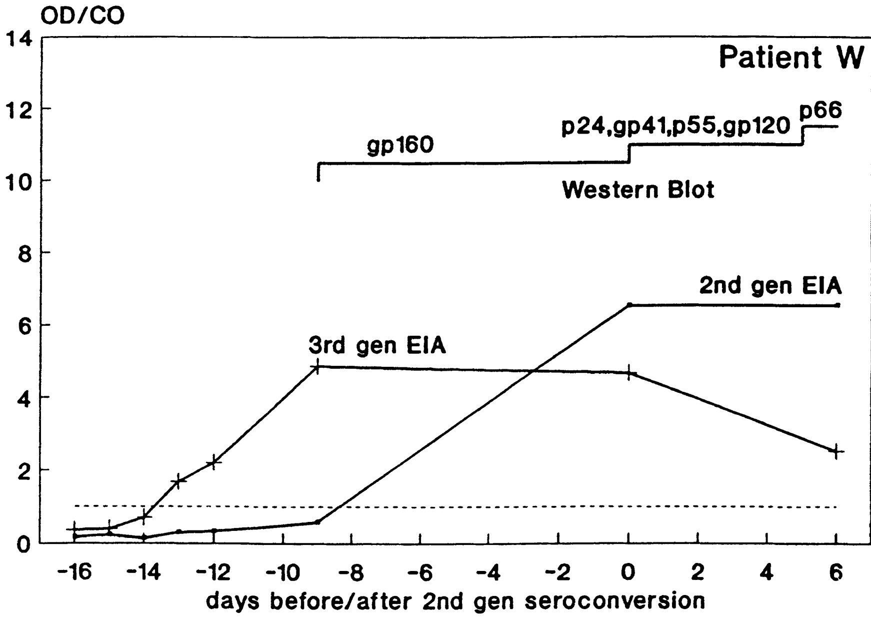


Figure 6. Early detection of HIV. Anti-HIV responses in a primary HIV-infection.

indeterminate serum samples with different HIV-recombinant proteins in RIPA and double-antigen sandwich EIA was studied.

No reaction was found with any of the HIV-1 recombinant proteins for the sera of the "p24-only reactors". As apparent from a representative autoradiogram, shown in Figure 4, sera from HIV-infected individuals reacted strongly with the radiolabelled recombinant HIV-proteins, whereas two sera of p24-only reactors were found negative. This result indicates that first: the RIPA format is specific in detecting true HIV-positive samples and secondly, the test clearly discriminates between positive samples and "p24-only reactors" or negative samples.

In the 3rd generation EIA anti HIV-antibodies will react with the recombinant HIV-1 env and gag protein coated on a solid phase. The specific anti-HIV antibodies are detected by incubating the solid-phase-antigen-antibody complex with the same recombinant antigen mixture conjugated to a probe and presented in a fluid phase (see Figure 2). This format discriminates between phase positive ("sticky") antibodies, false positive antibodies which recognize cryptic epitopes exposed on a solid phase and true positive antibodies.

From these experiments we conclude that reactivity of antibodies from immunoblot indeterminate p24-only reactors is apparently strictly dependent on the immobilization of the protein molecules in WB. Moreover, the p24-only reactivity on WB strips for p24 persists, whether or not recombinant p24 proteins or viral lysate were used. Again, the immobilization of antigen to a solid phase appears to be a prerequisite for reactivity. As a consequence, the antibodies of p24-only reactors probably cross-reacted in WB with epitopes which were not exposed on native p24 as presented in RIPA and in the 3rd generation EIA-format. To locate the epitopes, the antibodies of p24-only reactors were tested for their reactivity in PEPSCAN with overlapping peptides of p24 in a search for a common epitope [11].

Topics related to sensitivity in HIV diagnostics

HIV-infections are diagnosed by the detection of either antibodies reactive with HIV proteins or HIV p24 antigen directly in serum or plasma. Several studies however, have documented HIV infected subjects who were seronegative or lacked serum p24 prior to seroconversion [14,15]. These "silent" HIV-1 infections have been demonstrated by detecting HIV in cultured peripheral blood lymphocytes (PBLs) or detecting HIV-RNA in PBLs and/or by enzymatic amplification of HIV-DNA by PCR. Thus, there seems to be a potential for, and the demonstration of, HIV transmission by blood transfusion from these seronegative donors, and there is a need for improved screening assays. Although there is reasonable doubt about some of these observation (see below) of "silent" HIV infection. It was suggested that the sensitivity of antibody test is influenced by the denaturing of proteins as a consequence for the performance of the immunological assays (e.g. SDS, boiling and treatment with a reductant of viral proteins in immunoblot assay; immobilization of viral antigens onto a solid matrix in EIA). Therefore, one can theorize that there should be some immune respon-

ses to HIV of antibodies which recognize "native" non-denaturated HIV-proteins.

To examine these questions we tested HIV antibody EIAs based on recombinant proteins in a indirect antibody assay. In this format specific antibodies bound to fixed antigens are detected by a second antibody conjugated with a probe (2nd generation EIA) (Figure 5). In the 3rd generation of HIV antibody EIAs specific antibodies are detected by the same antigen(s) conjugated with a probe and presented in a liquid phase (double-antigen sandwich principle). The results obtained with both screening tests were compared to immunoblot analysis of electrophoretically separated SDS denatured viral proteins blotted on nitrocellulose, to HIV-antigen p24 test, and to HIV-RNA analysis using enzymatic nucleic acid amplification by PCR or NASBA [12,16]. Bloodsamples were analyzed from an individual who seroconverted to HIV-1. Samples were collected from before seroconversion with two days interval starting shortly after primary infection. The very short intervals between the samples enabled us to describe the immunological and virological events as examined by several assays. Figure 6 shows the antibody reactivity in EIAs of different formats and immunoblot analysis. The 3rd generation EIAs detected antibody conversion a few days earlier than did the second generation assay or immunoblot. Using a larger panel of seroconverters Zaayer et al. [17] demonstrates the extreme sensitivity of 3rd

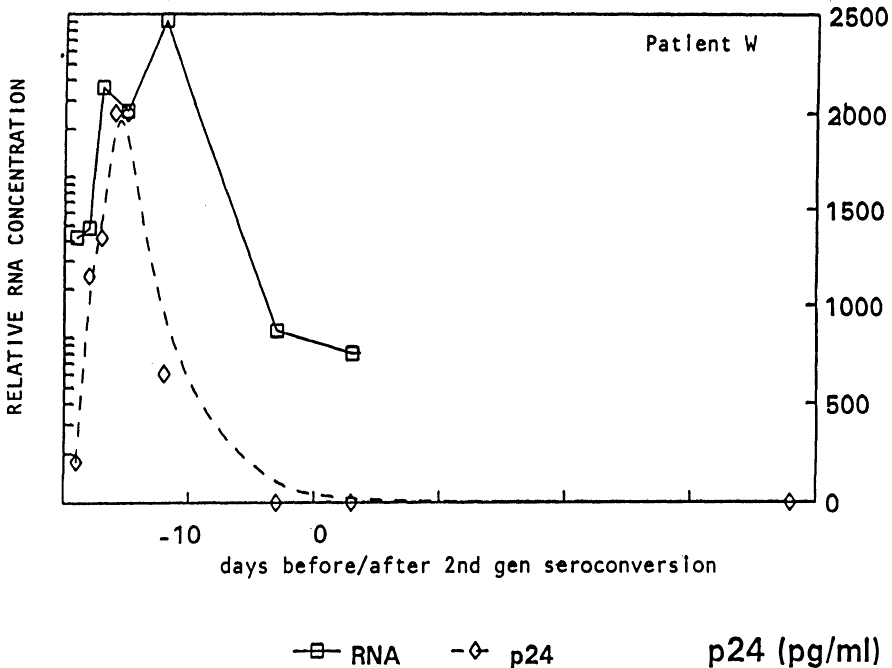


Figure 7. Early detection of HIV. Virological and anti-HIV response in a primary HIV-infection.

generation EIAs in early detection of antibodies to HIV. Since both HIV-p24 and HIV-RNA were also detectable even before seroconversion these analysis could be considered as a supplemental test to confirm EIA seropositive samples but negative in standard confirmatory test: the immunoblot (Figure 7).

Molecular diagnostics: A new way to early detection of HIV-exposure?

The polymerase chain reaction (PCR) is a technique for the enzymatic in vitro amplification of a specific RNA or DNA nucleotide sequence. Several investigators have used PCR to diagnose HIV infection but the sensitivity and specificity of PCR as a diagnostic test is still subject of many laboratories. It remains uncertain whether routine PCR screening is necessary or desirable as a replacement of immunological detection of HIV-exposure. Much depends on the frequency of HIV-infection in several groups at risk for HIV. This, of course, is the only category to answer these questions, due to the very low incidence of HIV in low-risk populations.

Some answers concerning these questions were obtained previously by studying people who participate in follow-up cohort studies. A PCR based detection study was performed on a Los Angeles homosexual cohort and a very high percentage (up to 23%) of HIV-PCR positive, HIV-antibody negative persons was found [14]. In an additional study among the same risk groups at four US study sites (Chicago, Pittsburgh, Baltimore and Los Angeles) it was also stated that the HIV-positive PCR can occur up to 42 months before seroconversion [15]. In a Boston/San Francisco homosexual cohort study [18] however and in French homosexual and hemophiliac cohort studies [19] very low incidences of such PCR positive, serological negative cases were found.

The length of the "open window phase" can be approximated in seroconverters by studying seronegative samples retrospectively, starting at the closest time point before seroconversion and proceeding by going backwards in time with three monthly intervals [20]. In a retrospective study 15 homosexuals who seroconverted for HIV-1 were selected and their cells, stored at three months before seroconversion, were used for DNA isolation and PCR analysis.

In two of the 15 persons a clear positive PCR result was obtained in the cellular samples, indicating the presence of HIV-1 DNA. An important finding was that the sera belonging to both time points also showed positive cDNA-PCR results, indicating the presence of viral RNA. Although the antibody test for HIV-1 (3rd generation EIA) was repeatedly negative at both time points, the HIV-1 p24 antigen test was positive in both cases (Table 1).

The results of this study are in sharp contrast to the study by Imagawa et al. who reported 23% PCR positives in HIV-1 seronegative samples. Lower percentages were reported in a Boston/San Francisco study, where 7 out of 208 seronegative samples (3.4%) were initially PCR positive [18]. four of these persons became seropositive after about twelve months, one was lost for follow up and two were considered false positives because all follow up samples were PCR negative and these persons remained healthy and seronegative for three years. In a large cohort of seronegative drug users only 4 of

1008 (0.4%) individual samples were reliably PCR positive. Two of these four seroconverted shortly after testing.

As mentioned before Bruisten et al. [20] found in two persons PCR positive blood samples only at three months before seroconversion. At these time points the HIV-p24 antigen test was also positive, indicating that these persons were at the primary infection stage.

Two main conclusions can be drawn from that study. Firstly, that serological data, including both HIV-1 antibody and the HIV-1 antigen test, are highly concordant to the nucleic acid detection data. The second conclusion is that in the Amsterdam situation the length of the "open window phase" is usually shorter than three months and is in accordance with the results from Horsburgh et al. [18], who estimate the length of the open window phase to be 2-4 months.

The persons whom we studied were chosen as representatives of very high risk groups. We expect that the frequency of discordant HIV-1 detection by immunological analysis in low risk population will be much lower than in high risk. Considering the fact that HIV-p24 antigen could be performed standard, the risk of not detecting a primary HIV-1 infection by performing only serological assays is small, but not non-existent. HIV PCR testing can thus in suspected cases be of important additional value.

Table 1. HIV-1 PCR results of "open window phase" study.

Participant	**x**	**HIV-Ag**	**Cell PCR**	
		reactivity	**nested pol**	**nested gag**
F	–9	–	–	–
	–6	–	–	–
	–3	+	+	+
	+1	–	+	+
I	–6	–	–	–
	–3	+	+	+
	+1	–	+	+

X = – number of months before seroconversion; + = number of months after seroconversion.
Ag = HIV-1 p24 antigen; + = detectable; – = not detectable.
ND = not determined.
(Data kindly provided by Dr. S.M. Bruisten and co-workers, CLB Amsterdam. Cf. ref.20)

Conclusive remarks

Sensitivity and specificity of assays to detect HIV-infection.

Antibody markers

– There is an improved sensitivity of screening tests due to augmented detection of IgM antibodies. This resulted from the choice of the assay format of 3rd generation antibody assays and the use of recombinant proteins.

– The advantage of the double-antigen sandwich assays and RIPA are: both do not require antiglobulin reagents. This prevents FP reactions due to "sticky" sera bound to the solid phase. The viral antigens are presented in a more "native" configuration, this avoids cross-reactivity to neo-epitopes expressed from immobilized viral proteins.

– There is an urgent need to re-evaluate the value of immunoblot assay as a confirmatory test in HIV-diagnosis. A radioimmunoassay, although restricted to laboratories equiped with isotope facilities, might be an alternative immunoassay.

Viral antigen-/nucleic acid markers

– The concordance of HIV-detection by PCR and viral antigen in seronegative high risk groups implies that both assays can be used as a supplemental test in early detection of HIV-infection. In addition, PCR analysis is applicable for confirmatory purposes in case of individuals with questionable HIV-diagnosis.

HCV-related topics

The genome of hepatitis C virus (HCV), the etiologic agent of posttransfusion non-A non-B hepatitis was recently identified. The lack of culturing procedures for HCV, prompted researchers to develop expression systems of viral genes. In a relative short period of time immunoassays became available and several studies have shown a clear correlation between antibody response against these expressed viral proteins and state of disease. The confirmation of HCV-infection can be achieved by immunoblot analysis using recombinant proteins or amplification of HCV-RNA from properly collected plasma samples. Moreover, in the serological silent period or to establish whether an individual with anti-HCV antibodies had recovered from the infection, HCV-RNA detection appears to be the only marker for proper diagnosis.

The importance of HCV-RNA detection and as confirmation is nicely illustrated if one compares the HCV-reactivity in donors and patients with single anti-HCV reactivity in the recombinant immunoblot (4-RIBA). In patients a relative high number is PCR-HCV-RNA positive. In contrast most donor samples are PCR negative. This is in concordance with what one can observe in HIV diagnosis.

As expected, a comparison of HCV-RNA reactivity in donors and patients with multiple anti-HCV reactivity in the recombinant immunoblot revealed that in both groups a high percentage is PCR positive (data kindly provided by Dr. Lelie and Dr. Cuypers, CLB, Amsterdam, The Netherlands).

Conclusion

New developments in biotechnology have provided reagents that have improved immune-screening methods for potentially infected blood. But investigators look hard at new diagnostic procedures based on the principles of molecular biology. A basic shift from immunological principles to molecular diagnostic is expected to take place in the coming years.

Acknowledgements

The author wishes to thank his colleagues Drs. Sylvia Bruisten, Marco Koppelman, Theo Cuypers, Hans Zaayer and Nico Lelie from CLB for their contribution and support.

I also appreciated the collaboration with Dr. Cees van der Poel of the Bloodbank of Amsterdam and Dr. Hans Langedijk of the Central Veterinary Institute in Lelystad, The Netherlands.

Mrs. Lili de Ridder, Monnickendam, The Netherlands is gratefully acknowledged for preparing the manuscript.

References

1. Barre-Sinoussi F, Chermann JC, Rey F, et al. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 1983;220:868-71.
2. Ratner L, Haseltine W, Patarca R, et al. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature 1985;313:277-84.
3. Alter HJ, Epstein JS, Swanson SG. Prevalence of human immuno deficiency virus type 1 p24 antigen in US blood donors: A measurement of efficacy of testing in donor screening. N Engl J Med 1990;232:1312-7.
4. Huisman JG, Winkel IN, Lelie PN, et al. Future of confirmatory testing. Sensitivity of immunoblot analysis and RIPA for the detection of antibodies to LAV/HTLV-III. In: Petricciani, Gust, Hoppe, Krijnen (eds). AIDS: The safety of blood and blood products. London: John Wiley & Sons Ltd, New York 1987:199-209.
5. Van der Poel CL, Reesink HW, Tersmette M, Lelie PN, Huisman JG, Miedema F. Blooddonations reactive for HIV in Westernblot but non-infective in culture and in recipients of blood. Lancet 1986;ii:752-3.
6. Reesink HW, Lelie PN, Huisman JG, et al. Evaluation of six enzyme immunoassays for antibody against human immunodeficiency virus.Lancet 1986;ii:483.
7. Lange JMA, Paul DA, Huisman JG, et al. Persistent HIV antigenemia and decline of HIV core antibodies associated with transition to AIDS.Brit Med J 1986;293:1459-62.
8. Jackson JB, MacDonald KL, Cadwel J, et al. Absence of HIV infection in blood donors with indeterminate Western blot tests for antibody to HIV-1. N Engl J Med 1990;322:217-22.
9. Tersmette M, Lelie PN, Van der Poel CL, Miedema F, Huisman JG. Confirmation of HIV seropositivity: Comparison of a novel radioimmunoprecipitation assay to immunoblotting and virus culture. J Virol Med 1988;24:109-16.
10. Centers for Disease Control. Interpretation and use of the Western blot assay for serodiagnosis of human immunodeficiency virus type 1 infections. MMWR 1989;38 (suppl S7):1-7.
11. Langedijk JPM, Vos WFHM, van Doornum GJJ, Rotman HAJ, Meloen RH, Huisman JG. Identification of cross-reactive epitopes recognized by HIV-1 false positive sera. AIDS 1992;6:1547-8.
12. Bruisten SM, Koppelman MHGM, van der Poel CL, Huisman JG. Enhanced detection of HIV-1 sequences using polymerase chain reaction and a liquid hybridization technique. Vox Sang 1991;61:24-9.
13. Talal N, Dauphin'e MJ, Dang H, Alexander SS, Hart DJ, Garry RF. Detection of serum antibodies to retroviral proteins in patients with primary Sjögren's syndrome (autoimmune exocrinopathy). Arthritis Rheum 1990;33:774-81.

14. Imagawa DT, Lee MH, Wolinsky SM, et al. HIV-1 infection in homosexual men who remain seronegative for prolonged periods.N Engl J Med 1989;320:1458-62.
15. Wolinsky SM, Rinaldo CR, Kwok S, et al. Human immunodeficiency virus type 1 (HIV-1) infection a median of 18 months before a diagnostic Western blot. Evidence from a cohort of homosexual men. Ann Int Med 1989;111:961-72.
16. Kievits T, van Gemen B, van Strijp D, et al. NASBA, isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection. J Virol Med 1991;35:273-86.
17. Zaayer HL, van Exel-Oehlers P, Kraaijeveld T, Altena E, Lelie PN. Early detection of antibodies to HIV-1 by third generation assays. Lancet 1992;ii:720-2.
18. Horsburgh CR jr, Ou C-Y, Jason J, et al. Concordance of polymerase chain reaction with HIV antibody detection. J Infect Dis 1990;162:542-5.
19. Lefrère JJ, Mariotti M, Vittecoq, et al. No evidence of frequent HIV-1 infection in seronegative at-risk individuals. Transfusion 1991;31:205-11.
20. Bruisten SM, Koppelman MHGM, Dekker JT, et al. Concordance of HIV detection by PCR and by serological assays in a Dutch cohort of seronegative homosexual men. J Infect Dis 1992;164:620-2.

THE EMERGING ROLE OF MHC MOLECULES AS SIGNAL TRANSDUCING RECEPTORS

T.A. Chatila

Introduction

Products of the MHC class I and class II region function to present peptide antigens to CD8 and CD4 T-cell subsets, respectively. In general, MHC class I molecules present peptides derived from different cellular compartments. MHC class I molecules present peptides derived from the cytosol and transported to the endoplasmic reticulum by specialized peptide porter proteins to combine with nascent MHC class I proteins. MHC class II molecules present peptides generated in the vesicular compartment and derived from exogenous proteins that have been taken up by antigen-presenting cells by internalization.

It has been recently appreciated that in addition to their classical role in presenting antigens to T cells, both MHC class I and II molecules may also serve to transduce signals that regulate the functions of MHC bearing cells. The following passages will discuss evidence in support of this new role for MHC molecules and its relevance in health and in disease.

Evidence for signal transduction via MHC class II molecules

The capacity of Ia molecules to deliver signal(s) that modulate cell function was initially suggested by studies using MAbs and which demonstrated an inhibitory effect of these MAbs on B cell proliferation and immunoglobulin (Ig) production [1]. Other studies demonstrated that anti-Ia MAbs facilitated the progression of B cells primed with anti-μ+IL-4 from the G_0 to the G_1 phase of the cell cycle and allowed these B cells to synthesize immunoglobulins in response to IL-5 and IL-6 [2]. Anti-Ia MAbs have also been demonstrated to induce the secretion of IL-1 and the accumulation of IL-1 mRNA in monocytes [3].

The identification of Ia molecules as receptors for superantigenic bacterial toxins and retroviral gene products shed new light on the role of Ia molecules as signal transducers. A number of disease-causing bacterial toxins derived from Staphylococcus aureus, Streptococcus pneumonia and Mycoplasma arthritidis have been demonstrated to bind to Ia molecules and behave as superantigens by engaging T-cell receptors in a Vβ or Vδ restricted fashion [4]. Another class of superantigens are the Mls antigens. These are the products of murine mammary

tumour viruses and play an important role in the elimination of developing T cells which bear the Vβ TCR chains involved in the recognition of these superantigens. The interaction of superantigens with Ia molecules is best understood for the Staphylococcal exotoxins (SE). These include Staphylococcal exotoxin A (SEA), SEB, SEC, SED, SEE, and Staphylococcal toxic shock syndrome toxin 1 (TSST-1). SE preferentially bind to HLA-DR and HLA-DQ [5] and different SE may bind to different sites on individual Ia molecules SE bind to a site on Ia molecules outside of the antigen groove. Mutations of Ia α_1 residues that affect presentation of nominal antigen to T cells fail to affect the presentation of superantigens [6].

SE reproduce many of the previously described effects of Ia MAbs on Ia^+ mononuclear cells. SE are amongst the most potent inducers of IL-1 and TNF production in monocytes, more potent on molar basis than lipopolysaccharides (LPS) [7-10]. We have shown that treatment of peripheral blood monocytes and of the monocytic cell line THP-1 with SEB, TSST-1 or with anti-Ia MAb induced rapid accumulation of IL-1-β and TNF-α mRNA [11]. Nuclear run-on transcription assays demonstrated that Ia ligands cause transcriptional activation of the IL-1β and TNF-α genes that did not require de novo protein synthesis. Induction of TNF-α and IL-1β mRNA expression by Ia ligands was accompanied by proteins synthesis and secretion of the respective monokine.

SE have also been demonstrated to deliver activation signals via Ia to human B cells and to Ia^+ activated human T cells. In the case of B cells, we have demonstrated that SE synergize with anti surface IgM antibodies (anti μ) and with the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) in inducing T cell-independent B cell proliferation [12]. The capacity of SE to provide a superantigenic bridge between T cell receptor molecules on T cells and Ia molecules on B cells allows for the progression of a MHC unrestricted cognate T/B cell interaction between large numbers of T and B lymphocytes leading to B cell proliferation and Ig production [13]. The capacity of SE to trigger MHC unrestricted interaction between T and B lymphocytes could potentially activate self reactive T and B cells leading to the onset of autoimmunity.

In the case of human T cells, we have shown that in addition to their capacity to activate T cells in a TCR Vβ restricted fashion, SE can deliver activation signals via Ia molecules to Ia^+ human T clones [14]. In the presence of accessory cells each of 11 T cell clones tested (9 αβ TCR, 2 γδ TCR) proliferated to all of three toxins tested (TSST-1, SEA and SEB). Synergy between signals delivered via Ia and antigen receptors was also noted in T cells, similar to our previous observation on the synergy between Ia and surface Ig signals in B cells. We have demonstrated that anti-Ia MAbs synergize with suboptimal concentrations of anti CD3 MAb in inducing Ia^+ T cells to proliferate and to express the Il-2, IL-3, IFN-γ and TNF-α genes [15].

TSST-1 and other superantigens also transmit activation signals via Ia molecules that result in the activation of HIV gene expression and viral protein secretion in Ia^+ monocytic cells. We have recently demonstrated that TSST-1 and related staphylococcal superantigens potently activate the transcription of chlor-

amphenicol acetyl transferase gene coupled to HIV long terminal repeat and transfected into the human monocytic cell line THP-1 [16]. These toxins also increased viral protein secretion from chronically infected monocytic cell line U1. Superantigen-induced activation of HIV expression was mediated by activation of the nuclear transcription factor NF-κB, and was upregulated by toxin-induced endogenous TNF-α secretion. These results indicate that superantigens produced by microorganisms as well as putative superantigens encoded by HIV may activate latent HIV infection in monocytic cells as well as in B cells and Ia^+ T cells in patients infected with HIV, leading to disease progression. This is in addition to capacity of superantigen/Ia complexes to engage the TCR of virally infected T cells, leading to activation of virus production.

Further evidence for signalling via Ia molecules was provided by the demonstration that SE, Ia MAbs and soluble CD4 all induce enhanced adhesiveness of Ia^+, but not Ia immune cells including T cells, B cells and monocytes [17]. Cell aggregation induced by Ia ligands is detected within minutes following the addition of ligand, and is remarkably sustained over long periods of time (reaching up to several days). Cell adhesion triggered via Ia molecules has been determined to proceed via two pathways: one dependent on LFA-1 and its counterreceptors ICAM-1 and ICAM-2 and another that was independent of LFA-1 expression and did not involve any of the integrins [18]. The contribution of each pathway to resultant adhesion varies depending on the cell type: LFA-1 dependent cell adhesion predominates in high density tonsillar B cells and activated human T cells while LFA-1 independent adhesion predominates in EBV-transformed B cells and in B cell lines. LFA-1 dependent adhesion induced via Ia is effected at the level of LFA-1 molecules and not its counterreceptors, and probably reflects a state of enhanced adhesiveness acquired by LFA-1 molecules in the wake of Ia signalling (see below). No conclusion can be currently drawn as to the mechanism of LFA-1 independent adhesion as the molecules mediating this function are currently unknown.

Induction of intense cell adhesion upon signalling via IA molecules is a physiologically relevant event that has direct bearing on capacity of T cells and Ia^+ antigen presenting cells to engage in cognate interaction. Antigenic stimulation is associated with enhanced adhesion between T cells and antigen presenting cells. A state of enhanced cell adhesion is indeed required for successful initiation of an immune response as antibodies directed against LFA-1 and its counterreceptors inhibit cognate T cell-antigen presenting cell interaction. Binding of ligands to the T cell antigen receptor transiently activates the adhesion function of lymphocyte function associated molecule 1 (lfa-1; CD11a/CD18), thus providing one mechanism for induction of enhanced cell adhesion during immune recognition [19]. The capacity of Ia molecules to activate upon their engagement sustained lfa-1-dependent and independent adhesion pathways provides a reciprocal mechanism for the induction of sustained adhesion between T cells and Ia^+ antigen presenting cells.

Signalling via Ia molecules may also occur in non-immune cells which have been induced to express Ia molecules by lymphokines. Such signalling may con-

tribute to the pathophysiology of systemic diseases in which superantigens have been implicated such as toxic shock, staphylococcal food poisoning, scarlet fever and possibly Kawasaki's disease and rheumatoid arthritis. In this regard, SE induce the production of the inflammatory cytokines IL-6 and IL-8 in human type II (fibroblast-like) synoviocytes, which express MHC class II molecules following treatment with IFN-γ [20]. This mechanism may contribute to the capacity of bacterial toxins particularly M. arthritidis miogen (MAM) and TSST-1 to trigger joint disease in experimental animals and in humans.

At the biochemical level, early work has demonstrated the capacity of anti-Ia MAbs to induce accumulation of cAMP and translocation of the serine threonine kinase protein kinase C (PKC) from cytosol to nucleus in B cells [2]. The transduction of this signal via Ia was shown to requires intact α and β chain cytoplasmic domains because Ia murine B cell lymphoma cells transfected with truncated Ia α and β chains failed to mediate the translocation of PKC from cytosol to nucleus [21]. More recently, we and others have demonstrated that anti-Ia MAbs and SE induce enhanced tyrosine phosphorylation in monocytes and in T and B lymphocytes. Activation by Ia ligands of tyrosine phosphorylation in turn leads to initiation of secondary intracellular activation events. These include phosphoinositide turnover, Ca^{2+} mobilization and protein kinase C activation.

The relationship between signal transduction events triggered by Ia ligands and the effector functions of these ligands such as cell adhesion and monokine gene transcription has been examined using highly selective inhibitors of protein kinases. Only those Ia ligands that induced tyrosine phosphorylation were found capable of inducing cell adhesion [18]. Induction of cell adhesion by Ia ligands was inhibited by antagonists of PTK and PKC, but not by antagonists of cyclic nucleotide-dependent protein kinases [18]. These results indicated that PKC and PTK play an obligatory role in mediating enhanced cell adhesion triggered by Ia ligands. A similar requirement for PTK and PKC activation emerged in studies on the induction of monokine gene transcription by Ia ligands [22,23]. Induction by superantigens of monokine gene transcription was inhibited by PKC and PTK antagonists, but not by cyclic nucleotide antagonists. Overall, these observations implicated PTK and PKC as protein kinases critical for the effector function of Ia ligands.

We have recently identified members of the src family of PTK as principal candidates for Ia-activated PTK. The src family of protein tyrosine kinases include eight members: src, lyn, yes, fyn, fgr, hck, lck and blk [24]. Multiple src type kinases are expressed in lymphocytes and monocytes. src type kinases expressed in T cells include fyn, yes and the T lymphocyte-specific lck; src type kinase expressed in B cells include lyn, fyn, and the B lymphocyte-specific blk. Monocytes express src, lyn, and the monomyelocytic cell-restricted fgr and hck. We have shown that stimulation of human peripheral blood monocytes with SE induced rapid and selective activation of the src-related protein tyrosine kinases (PTK) fgr and hck [25]. SE also induced the activation of fgr and lyn in B cells. PTK activation by SE required Ia expression, as it was inhibited by anti-Ia MAb

and was not observed in Ia-B cells. Stimulation of src PTK by SE was strongly potentiated by T cells bearing toxin-specific Vβ chain but not by TCR-negative mutant cells. This indicated that crosslinking of Ia/toxin complexes by TCR molecules resulted in enhanced signalling Ia molecules. It also suggested that crosslinking by antigen specific TCR molecules of peptide antigen-presenting Ia molecules may result in a similar activation of src-type PTK in antigen presenting cells.

Evidence for signal transduction via MHC class I molecules

A role for MHC class I molecules as signal transducing structure has been provided by studies using MAbs to MHC class I molecules or to β2-microglobulin as well as soluble CD8 molecules, all of which were demonstrated to induce activation events in treated cells. The scope of activation events induced by MHC class I ligands is remarkably similar to that outlined for Ia ligands. For example, crosslinking by MAbs of MHC class I molecules on T cells has been demonstrated to result in intracellular Ca^{2+} mobilization and, in synergy with phorbol esters or IL-2, promoted cell proliferation [26-28]. Signals delivered via MHC class I molecules also result in the induction of cell adhesion in treated lymphocytes and monocytes [29]. As is the case with Ia mediated cell adhesion, adhesion mediated by MHC class I molecules is mediated by LFA-1 dependent and independent mechanisms, and is inhibited by inhibitors of PTK and PKC. This indicated a role for these protein kinases in mediating functional responses effected via MHC class I molecules. From all these studies, it would appear that the signalling pathways activated via MHC class I molecules are very similar to those coupled to Ia molecules.

Relevance of MHC signalling to disease states

A role for aberrant Ia signalling in disease states is best illustrated by the case of superantigen-mediated diseases, where products produced in the wake of Ia signalling such as TNF and IL-1 actively participate in the precipitation of disease states. Aberrant signalling via MHC molecules is also likely to play a role in autoimmune diseases and in graft-versus-host diseases. Appreciation of the role that signalling via MHC molecules plays in the pathophysiology of these and other diseases of the immune system will hopefully help in the design of more effective therapies for these disorders.

References

1. Clement LT, Tedder TF, Gartland GL. Antibodies reactive with class II antigens encoded for by the major histocompatibility complex inhibit human B cell activation. J Immunol 1986;136:2375.
2. Cambier J, Lehman KR. Ia-mediated signal transduction leads to proliferation of primed B lymphocytes. J Exp Med 1989;170:877-86.

3. Palacios R. Monoclonal antibodies against human Ia antigens stimulate monocytes to secrete interleukin-1. Proc Natl Acad Sci USA 1985;82:6652-6.
4. Marrack P, Kappler J. The staphylococcal enterotoxins and their relatives. Science 1990;248:705-11.
5. Scholl PR, Diez A, Karr R, Sekaly RP, Trowsdale J, Geha RS. Effects of isotypes and allelic polymorphism on the binding of staphylococcal exotoxins to MHC class II molecules. J Immunol 1990;144:226-30.
6. Dellabona P, Peccoud J, Kappler J, Marrack P, Benoist C, Mathis D. Superantigens interact with MHC class II molecules outside of the antigen groove. Cell 1990;62: 1115-21.
7. Ikejima T, Dinarello CA, Gill DM, Wolff SM. Induction of human interleukin-1 by a product of Staphylococcus aureus associated with toxic shock syndrome. J Clin Invest 1984;73:1312-20.
8. Parsonnet J, Hickman RK, Eardley DP, Pier GB. Induction of human interleukin-1 by toxic shock syndrome toxin-1. J Infect Dis 1985;151:514-22.
9. Parsonnet J, Gillis ZA, Pier GB. Induction of interleukin-1 by strains of Staphylococcus aureus from patients with nonmenstrual toxic shock syndrome. J Infect Dis 1986; 154:55-63.
10. Parsonnet J, Gillis ZA. Production of tumor necrosis factor by human monocytes in response to toxic shock syndrome toxin-1. J Infect Dis 1988;158:1026-33.
11. Trede N, Geha RS, Chatila TA. Transcriptional activation of monokine genes by MHC class II ligands. J Immunol 1991;146:2310-5.
12. Fuleihan R, Mourad W, Geha RS, Chatila TA. Engagement of MHC class II molecules by staphylococcal exotoxins delivers a comitogenic signal to human B cells. J Immunol 1991;146:1661-6.
13. Mourad W, Scholl P, Diez A, Geha RS, Chatila TA. The staphylococcal toxin TSST-1 triggers B cell proliferation and differentiation via MHC unrestricted cognate T/B cell interaction. J Exp Med 1989;170:2011-22.
14. Spertini F, Spits H, Geha RS. Staphylococcal toxins polyclonally activate major histocompatibility complex class II+ human T cell clones. Proc Natl Acad Sci USA 1991;88:7533-7.
15. Spertini F, Chatila TA, Geha RS. Signals delivered via MHC class II molecules synergize with signals delivered via TCR/CD3 to cause proliferation and cytokine gene expression. J Immunol 1992;149:65-70.
16. Fuleihan R, Chatila TA, Geha RS. Superantigen mediated activation of HIV gene expression in monocytes. FASEB J 1992;6:A1978.
17. Mourad W, Geha RS, Chatila TA. Engagement of major histocompatibility complex class II molecules induces sustained, LFA-1 dependent cell adhesion. J Exp Med 1990;172:1513-6.
18. Fuleihan R, Spertini F, Geha RS, Chatila TA. Role of protein kinase activation in the induction of B cell adhesion by MHC class II ligands. J Immunol 1992;149:1853-8.
19. Dustin ML, Springer TA. T cell receptor cross linking transiently stimulates adhesiveness through LFA-1. Nature 1989;341:619-24.
20. Mourad W, Mehindate K, Schall TJ, McColl SR. Engagement of major histocompatibility complex class II molecules by superantigens induces inflammatory cytokine gene expression in human rheumatoid fibroblasts-like synoviocytes. J Exp Med 1992;175:613-6.
21. Nabavi N, Chogawala Z, Myer A, et al. Antigen presentation abrogated in cells expressing truncated Ia molecules. J Immunol 1989;142:1444-7.
22. Scholl PR, Trede N, Chatila TA, Geha RS. Role of protein tyrosine phosphorylation in monokine induction by the staphylococcal superantigen toxic shock syndrome toxin-1. J Immunol 1992;148:2237-41.

23. Trede NS, Scholl PR, Geha RS, Chatila TA. Early activation events induced by the staphylococcal superantigen toxic shock syndrome toxin-1 in human peripheral blood monocytes. J Immunol 1992:(in press).
24. Bolen. Signal transduction by the src family of tyrosine kinases in hemopoietic cells. Cell Growth & Differentiation 1991;2:409-14.
25. Morio T, Geha RS, Chatila TA. Engagement of MHC class II molecules by staphylococcal superantigens activates src-type protein tyrosine kinases. (Submitted for publication).
26. Geppert TD, Walcholtz MC, Davis LS, Lipsky PE. Activation of human T4 cells by crosslinking class I MHC molecules. J Immunol 1988;140:2155-64.
27. Geppert TD, Walcholtz MC, Patel SS, Lightfoot E, Lipsky PE. Activation of human T cell clones and Jurkat cells by cross-linking class I molecules. J Immunol 1989; 142:3763-72.
28. Dasgupta JD, Egea E, Relias V, Iglesias A, Gladstone P, Yunis EJ. Involvement of major histocompatibility class I molecules antigens in T cell activation. Eur J Immunol 1990;20:1553-61.
29. Spertini F, Chatila TA, Geha RS. Engagement of MHC class I molecules induces cell adhesion via both LFA-1 dependent and LFA-1 independent pathways. J Immunol 1992; 148:2945-9.

FLOW CYTOMETRY IN TRANSFUSION MEDICINE[1]

B. Houwen, L.K. Lebeck

Introduction

Flow cytometry, in recent years, has emerged from a research setting to assume an active role in clinical pathology. The technology offers a rapid reproducible and quantitative technique for assessing large numbers of single cells in suspension using a combination of measurements. The analysis of individual cells can be performed 1) by using the intrinsic properties of unlabelled cells to measure the dispersion or scatter of focused laser light; 2) by the effects of unlabelled cells on polarized laser light; and 3) by making use of fluorescent probes that label the cell surface and/or intracellular components of live or fixed cells [1-3]. The development of these techniques has resulted in new and powerful insights in cell biology. The fact that this technology depends on single cells in suspension makes it almost a natural for applications within transfusion medicine.

In this overview we will discuss applications of flow cytometry in immunohematology. We shall highlight areas where flow cytometry assays have proven utility and discuss other areas where this technology may become useful. We will also discuss some of the pre-conditions and factors that should be considered before establishing a flow cytometry facility.

Quality assurance

With flow cytometry taking on the role of a routine technology, the development of quality assurance protocols has become essential. Clinical use of flow cytometry requires adequately trained and qualified operators, as well as quality control procedures relevant to the tests performed. Without these qualifications, flow cytometric data will be at best unreliable and at worst potentially dangerous. This need for qualified and trained staff applies to the entire analytical process: from pre-analytical handling of specimens, the instrumentation and analysis it-

1. The experimental work on the detection of fetal red blood cells described in this paper was supported in part by a research grant from TOA Medical Electronics Co., Ltd., Kobe, Japan.

self, to the validation of data. It is also of vital importance that established criteria for sample collection, labelling, transport and storage are met.

The successful use of flow cytometry as a clinical analytical tool is further dependent upon accurate sample identification; proper sample preparation; appropriate (fluorescent) reagents; correct instrument calibration and data analysis. Quality control procedures should be applied to all of these. Similarly, proper reagent performance, the use of positive and negative controls, checks for background and autofluorescence are essential for a proper understanding and interpretation of flow cytometric data.

Manufacturers provide several means for instrument performance checks. These include the use of micro-beads, both fluorescent and non-fluorescent, and in some cases, stabilized or lyophilized cell lines [4]. For the majority of clinical tests however, no performance standards are available, and the individual laboratory must establish criteria for accuracy and reproducibility. A guideline for establishing such criteria for lymphocyte surface antigen testing may be found in the proposed standard H42-T, published by the National Committee for Clinical Laboratory Standards (NCCLS) [5]. Another source of information and training is the Laboratory Training Network, a cooperative training system sponsored by the Association of State and Territorial Public Health Laboratory Directors (ASTPHLD) and the Centers for Disease Control (CDC) in the United States.

Enrollment in external Proficiency Testing Programs is another means for clinical laboratories to check whether their flow cytometry facility meets expected performance levels. In several countries such as the United States and Canada it is mandatory for laboratories that report results of lymphocyte CD4 counts, to take part in external Proficiency Testing Programs.

Performance criteria

Flow cytometers are used to detect and measure qualitative cellular properties, that are subsequently converted to numbers using analytical algorithms. These numbers enable statistical analysis of the data, comparison from one specimen to another, and the development and implementation of specific performance criteria. Such criteria are, in part, influenced by the degree of accuracy and precision for a given assay and the instrument's processing capability. For instance the number of events a flow cytometer is capable of handling in a given time period is determined by several parameters. Sample composition, including cellularity and viscosity, the fluidics and electronics of the analyzer, the signal to noise ratio for a specific assay, all influence the cell throughput rate of a flow cytometer.

Several methods exist to measure the diagnostic performance of an assay. Assay precision is usually expressed as the standard deviation and/or coefficient of variation (CV) from a target or mean value. When flow cytometry results are compared with those obtained by other methods, standard statistical analyses include correlation coefficient for precision and regression analysis for accuracy. Accuracy can also be measured by true/false tables; sensitivity and specificity;

the predictive values for positive and negative test results; and by receiver operator curve (ROC) analysis [6-8]. Occasionally it will be possible, as in simple cell enumeration, to use a concentration endpoint as the performance criterion. Obviously, the choice of methods depends on the situation.

It is important for the clinical user to review carefully the performance criteria for specific tests, when available, and to realize that the data shown in published papers may be representative of a highly specialized research environment and may not be readily reproducible in the clinical laboratory setting.

Cost aspects

Flow cytometry technology, in general, is rather expensive [9]. This is due to a number of factors that all contribute to high start-up costs and operating expenses. The virtual necessity to have available one or more well trained, qualified operators is one such factor, and raises the question of the intended utilization rate of the flow cytometer. Although it will often be possible to use the operator's time elsewhere in a laboratory, a flow cytometer facility usually will be uneconomical if used less than 50% of time. This is due to significant fixed costs such as depreciation, maintenance and laboratory overhead. Therefore it follows that the lower the intended utilization of the flow cytometer, the higher the cost per test.

There are some ways to influence the operating costs. As can be seen from a cost breakdown for a fictitious laboratory (Figure 1), labour amounts to about

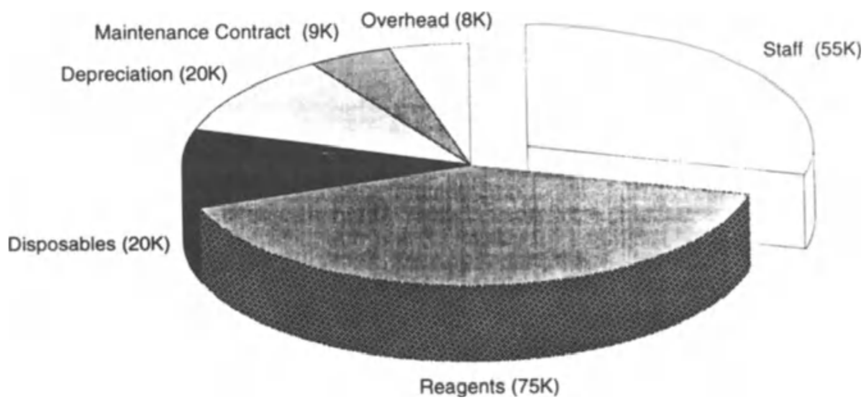


Figure 1. Annual operating cost estimates of a flow cytometer. The values are expressed in 1992 US$. For instance: "Staff 55k" represents $55,000.00 per year needed for staffing.

30% of the overall costs for a flow cytometry facility. Several manufacturers are offering semi-automated devices that assist in the pre-analytical handling and staining of specimens for specific test protocols. Particularly when these tests are performed frequently, such devices may enable significant time and labour savings. Similarly, automated front ends which perform automated aspiration of specimens will allow the analyzers to be run in a "walk away" mode and can result in significant savings in personnel costs.

However, several of the cost factors indicated in Figure 1 are not easily reduced without affecting the quality of the tests: maintenance contract, control materials, external proficiency testing program subscription. These costs, when calculated on a cost per test basis, will decline with increasing numbers of tests performed. Other cost factors represent fixed costs per test and will vary little regardless of the number of tests performed. These include: monoclonal antibodies, isotype controls, disposables, reagents.

Given the high intrinsic costs of operating a flow cytometry laboratory, many laboratories feel a strong incentive to perform tests on a fee for service basis whenever possible. The laboratory should review which applications it wishes to pursue as part of its clinical testing program and should be careful and realistic in its projections of expected workload and revenue. Depending on the local pay scale for technical staff and on reimbursement for tests we feel that in general a flow cytometry facility becomes cost-neutral at approximately 20 reimbursable tests per weekday, and beyond that probably will create a modest revenue.

Under conditions when fee for service is not a possibility, the laboratory will be a cost center, whose budget may have to be derived from overall funding. In such situations the addition of a flow cytometry facility can cause considerable strain on pre-existing finances.

Applications

For this overview we have focused on applications that we feel are relevant and appropriate for transfusion medicine, and have separated such flow cytometry assays into four main categories: 1) related to immune mechanisms; 2) nucleic acid content analysis; 3) phenotyping for diagnosis, monitoring; and 4) enumeration of cells/cell types.

For each of these categories (see Table 1) we will discuss potential applications in the broad sphere of transfusion medicine. Whether it will be practical to implement each of the various applications at your specific site is dependent on local factors, such as the type of facility, its caseload, clinical programs and needs, and the availability of other flow cytometry facilities.

Table 1. Applications of flow cytometry in transfusion medicine.

Cell type	Immune mechanism related
RBC	Auto-immune hemolytic anemia (AIHA) Transfusion reactions
WBC	Anti-neutrophil; monocyte; lymphocyte antibodies
Platelets	Idiopathic thrombocytopenic purpura (ITP) Platelet donor selection HPA (Pla) antibody detection
Cell type	**Nucleic acid content analysis**
RBC	Reticulocyte enumeration Reticulocyte maturation index Parasite detection
WBC	DNA ploidy Cell proliferation/cell cycle (Ki-67; PCNA)
Platelets	Platelet maturation index
Cell type	**Phenotype associated**
RBC	Antigen detection D^u, delution, chimera Paternity testing/genetic testing Fetal-maternal hemorrhage
WBC	Lymphokine activated killer (LAK) cells Tumor infiltrating lymphocytes
Platelets	Activation state Platelet storage lesion
Cell type	**Quantitative aspects**
RBC	Screening for leukocyte removal efficacy Red cell survival in vivo Red cell mass
WBC	Enumeration of cell types and subpopulations Stem cell harvesting (CD34), survival in vitro
Platelets	Screening for leukocyte removal efficacy

Assays related to immune mechanisms

Hematological disorders caused by immune mechanisms often involve cell membrane bound antibodies and are usually diagnosed and monitored by manual tests. However, flow cytometric analysis of red cell surface bound antibodies in auto-immune hemolytic anemia (AIHA) has certain advantages over manual methods. These include improved sensitivity and the possibility to quantitate antibody on the red cell surface [10-13].

Flow cytometric based testing for HLA antibodies has shown a 100-fold greater sensitivity than standard lymphocyte toxicity tests [14]. This technique is therefore useful in detecting transplant recipients with a high probability of graft rejection, who would go undetected with lymphocytotoxicity tests [15-17]. HLA

antigen panel typing itself can also be performed using flow cytometry [18] and may lend itself to automation. Another application is the test for HLA-B27 antigen in individuals suspected of ankylosing spondylitis [19,20].

Antibodies to white blood cells can be categorized as transfusion related alloantibodies directed against HLA, monocyte or granulocyte antigens, as well as autoantibodies, often granulocyte specific [21-24]. Granulocyte-specific antibodies, sometimes detected after transfusion of blood or blood products, may represent passively transferred antibodies, derived from donor plasma, or may occur in the recipient as a reaction against donor granulocyte specific antigens [22]. In neonates immune granulocytopenia can develop as a result of transfer of maternal (IgG class) antibodies directed against the fetus' granulocytes across the placenta [25]. Several autoimmune disorders are associated with the developments of secondary granulocytopenia, for instance: systemic lupus erythematosus, autoimmune thyroid disease and Felty's syndrome [22]. In all these instances anti-neutrophil antibodies can be detected by flow cytometry either in patient or maternal serum or as bound to the cell surface and will confirm the immune nature of the granulocytopenia.

However, the detection of antibodies directed to white blood cells is complicated by the cell surface Fc receptor related binding of immunoglobulins on neutrophils and monocytes [26]. Although this problem can be avoided either by blocking or by prolonged incubation of neutrophils and monocytes at 37°C, it is cumbersome and may be the main reason why tests for the detection of anti-neutrophil or anti-monocyte antibodies are not routinely included in the work-up of patients with neutropenia or leukopenia.

In idiopathic thrombocytopenic purpura (ITP), flow cytometry as well as other techniques (including radioactive labelling) have been used for the qualitative and quantitative analysis of antibodies bound to the platelet surface [27,28]. In general the results from different techniques for demonstrating platelet associated IgG are similar: in 50-100% of patients IgG levels are found to be elevated. The problem with these assays is not so much the sensitivity but the lack of specificity for ITP. False positive results may be encountered in disease states such as septicemia, hypergamma-globulinemia, systemic lupus erythematosus and lymphoproliferative disease [29]. False negative results have also been reported. A second inherent problem with this assay is caused by adherence of platelet non-specific immunoglobulins or immune complexes to the platelet surface.

Although the antigen(s) involved in ITP have not been defined, recent studies have shown that platelet glycoproteins IIb-IIIa or its activated complex may be the target of ITP associated antibodies. However, the same studies have also shown that antigen epitope specificity differs from case to case [30,31]. The potential of platelet associated antibody tests for the clinical diagnosis of ITP remains for all of these reasons somewhat uncertain [32].

Platelet donor selection in patients demonstrating alloantibodies is facilitated by flow cytometry, as it has the capability of analyzing large panels of donors and rendering quantitative answers. The flow cytometric approach affords a

level of sensitivity lacking in other platelet crossmatch assays [28, 33,34]. In neonatal thrombocytopenia, flow cytometry provides an alternative method for the investigation of the mother's serum. Accurate and reproducible quantitative detection of anti-PLA alloantibodies and monitoring of antibody titers is greatly facilitated by this technology [35,36].

Several other assays are directly or indirectly related to immune disorders. As a screening and prognostic test for acquired immunodeficiency states, such as in human immunodeficiency virus (HIV) infection, enumeration of helper T-lymphocytes (CD4+) has been used widely [37-40]. Assays measuring phagocytic capability and involving granulocyte oxidative metabolism, although highly suitable for flow cytometric analysis, we believe are more of interest in those investigating host defense mechanisms, rather than to those in transfusion medicine in general [41,42].

Nucleic acid content analysis

Originally nucleic acid content was used only to determine mitotic cell cycle progression and DNA ploidy [41,42]. These applications are still widely used and cell cycle analysis is now even carried out on cells liberated from paraffin embedded tissue blocks in efforts to derive prognostic information from the DNA content of archival tumour tissues [43]. Whether cells are actively cycling can also be determined by labelling cells with antibodies directed against specific nuclear antigens expressed only in proliferating cells (Ki-67 antigen; PCNA or proliferating cell nuclear antigen) [44,45]. Yet another method for determining cell proliferative state is the BrdU incorporation rate into DNA, also measured by flow cytometry [46].

Extensive studies have shown the usefulness of flow cytometry for the analysis of DNA (aneu-)ploidy of normal and tumour cells [47-49]. The high percentage of DNA aneuploidy in patients with childhood ALL makes this an excellent tool for monitoring residual disease, particularly in hypocellular bone marrow [50]. However, for transfusion medicine it may be of greater interest to look at the applications involving nucleic acid content of cells such as reticulocytes and platelets, and, although not yet clinically applicable, to monitor closely the recent developments in parasite detection using flow cytometry.

Although known for a long time, the fact that reticulocytes contain variable amounts of RNA, and that the amount of RNA is correlated directly with reticulocyte maturity, has only recently been "rediscovered" [51,52]. Flow cytometric analysis of the RNA content of reticulocytes has led to new insights in the kinetics of bone marrow recovery after ablative, cytotoxic chemotherapy and bone marrow transplantation. Clinical studies, carried out by several investigators at different sites have shown conclusive evidence that a decrease in reticulocyte maturity is usually the first sign of bone marrow recovery, and that this precedes changes in reticulocyte, leukocyte and neutrophil counts by several days (Figure 2) [53,54]. Reticulocyte maturity expressed by a "reticulocyte maturation index (RMI)" may therefore develop into an important clinical tool. In addition, flow

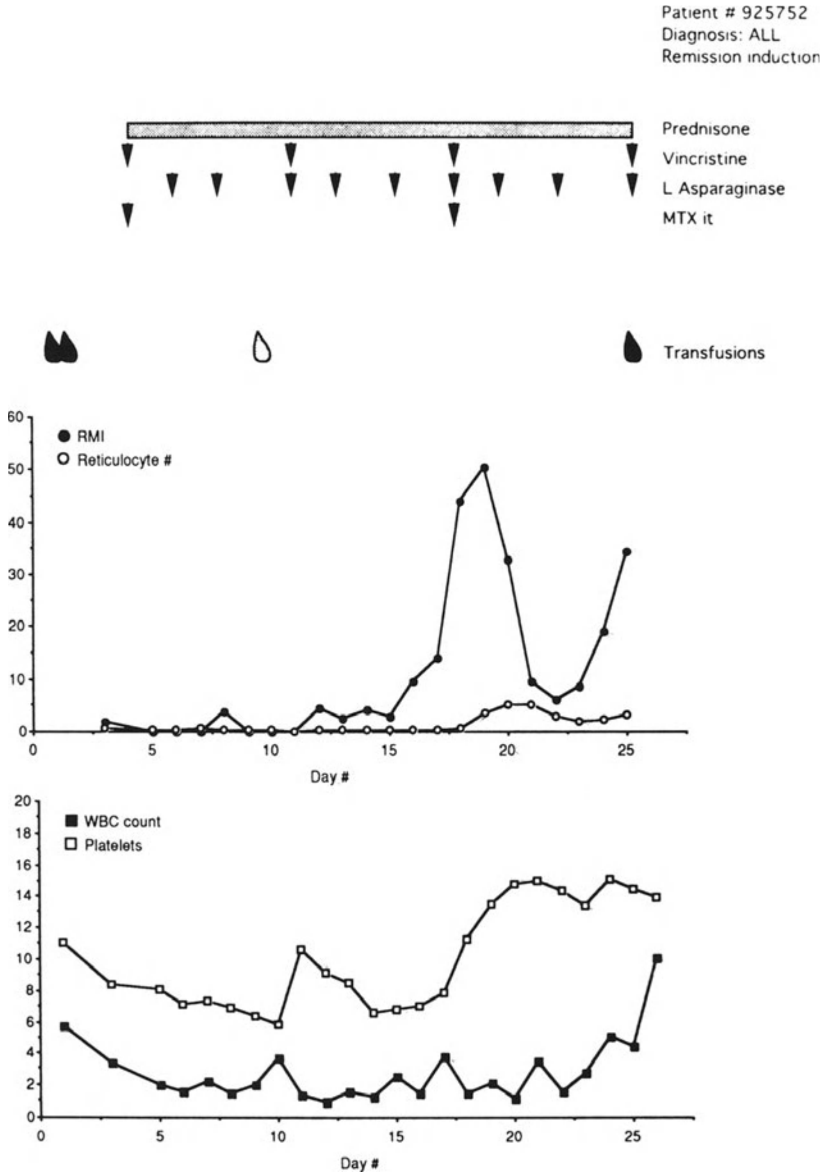


Figure 2. The graph represents the remission induction treatment schedule and peripheral blood parameters of a 9 year old patient with newly diagnosed acute lymphocytic leukemia (ALL). RBC and platelet concentrate transfusions are indicated by closed and open droplet-symbols, respectively. Reticulocyte maturation index (RMI), reticulocyte count ($\times\ 10^9$/L), WBC and platelet counts ($\times\ 10^9$/L) are shown in relationship to the treatment administered.

cytometric enumeration of reticulocytes has resulted, for the first time in history, in a reliable, reproducible and clinically applicable method for counting these cells. Because of this it is now also possible to establish clinically useful lower and upper limits for reticulocyte counts.

This same technology applied to platelets offers a new method to measure platelet turnover, as well as platelet aging in vitro. The RNA content of platelets can be accurately measured and used in very much the same manner as reticulocyte RNA and reticulocyte maturity derived from this parameter [55,56]. As an added advantage, the labelling of platelet associated RNA enables platelets to be accurately distinguished from other small elements in whole blood or blood component preparations. This improves the accuracy of the platelet count particularly in situations where interference by RBC or WBC fragments, small fibrin clots, etc. can create problems.

Recent work on flow cytometric based parasite detection in blood has obvious implications for transfusion medicine [57,58]. Much of this work is still in the experimental stages and in most methods it is yet impossible to distinguish between parasites and other sources of nucleic acids. However, there are clear indications that this may develop into an important application in the near future and may constitute a rapid screening procedure for some clinically important blood borne parasites.

Diseases that lend themselves for flow cytometric detection include malaria, trypanosomiasis and Chagas' disease. In terms of disease frequency and mortality rates malaria and Chagas' disease top the list. In the rural areas of South America and parts of Central America the incidence of Chagas' disease is 50%. Other intracellular parasites that can appear in the peripheral blood are Leishmania and the primarily non-human parasites Babesia and Theileria [59].

The use of dyes not requiring ultra-violet wavelengths for excitation, and the availability of parasite species specific, and perhaps stage specific monoclonal antibodies may bring flow cytometric based parasite detection in whole blood within the reach of clinical and blood transfusion center based facilities. As an alternate to flow cytometry the potential for DNA/RNA probe based molecular diagnostics should be mentioned. The question which technique will become most widely used will depend on the specificity, sensitivity and cost of each type of assay. Currently, the costs of molecular probe techniques are prohibitive for routine screening purposes. Also, the sensitivity and specificity of the molecular techniques are somewhat in question, mainly due to observed contamination problems of polymerase chain reaction (PCR) based methodologies, resulting in false positive results.

Phenotype associated assays

Flow cytometry has long been used for determining cell lineage and enumeration, especially of WBC in leukemia and lymphoma [60,61]. The availability of multi-colour analysis has given tremendous expansion to the usefulness of flow cytometry for abnormal cell analysis as well as for the analysis of altered or acti-

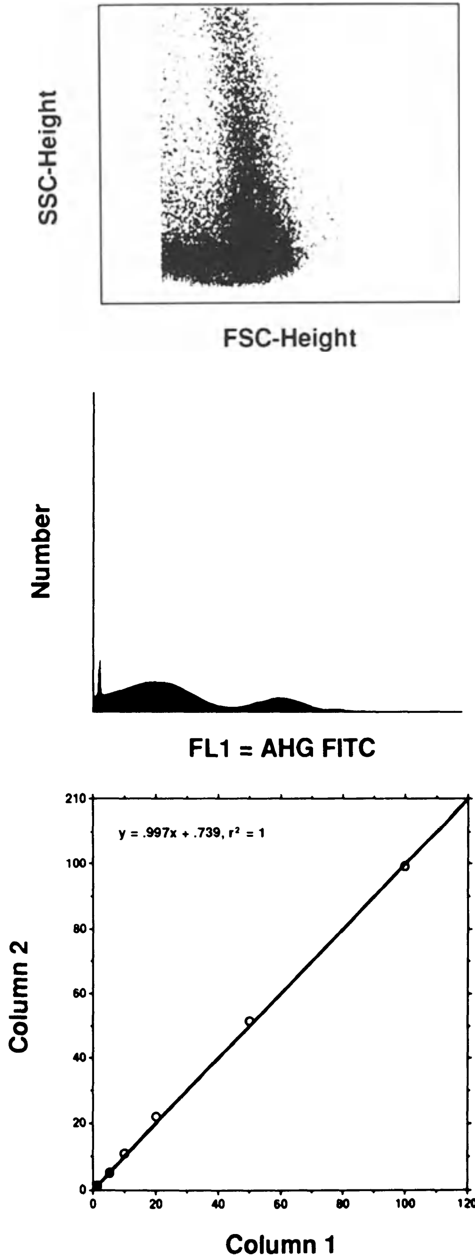


Figure 3. Staining of RBC suspensions with polyclonal anti-D followed by secondary labelling with AHG-FITC (1:8 dilution, Atlantic Antibodies) [83]. Top: Cytogram of forward vs. side scatter of stained RBC suspension. Center: Single parameter fluorescent histogram of 25% Rh+/Rh– artificial RBC mixture. FL1 = log green fluorescence. Bottom: Regression plot of expected vs. test values. Column 1 indicates the known dilution of Rh+/Rh– artificial cell mixtures, column 2 the testresults obtained from flow cytometric assay.

vated state of normal cells. In transfusion medicine the technique is particularly useful for bone marrow harvests in autologous reinfusions, where detection of residual disease is an issue [62]. Panels used for leukemia detection have been described in many papers and we will not attempt in this overview to list immunophenotyping markers [63-66]. In general, it has been found that immunophenotyping is more useful in lymphoid leukemia than in the myeloid types.

The identification of genes causing resistance of cancer cells to multiple drugs (mdr genes) was followed by the discovery of the mdr1 gene product called P-glycoprotein, that functions as a drug efflux pump [67,68]. Subsequently antibodies directed against P-glycoprotein have enabled investigation of cell surface P-glycoprotein expression on human leukemia cells [69]. Although differences in expression have been found in leukemias with various outcomes, the clinical usefulness is still uncertain.

The characterization of other cell populations, such as tumour infiltrating lymphocytes and lymphokine activated killer (LAK) cells can be done using appropriate combinations of monoclonal antibodies [70,71]. The blood bank is often involved in interleukin-2 (IL-2) induced LAK cell procurement for therapeutic applications through apheresis procedures. However, in recent years the use of this elaborate treatment modality in oncology patients has lost some ground. Currently, LAK cells are also being used as a substrate for gene insertion and gene therapy [72].

All of the above mentioned flow cytometric applications, with the exception of LAK cell procurement, are usually under the purview of clinical hematology/oncology or of immunology specialty laboratories and are mentioned here, in passing, mainly for completeness.

A unique characteristic of red blood cells is the expression of membrane antigenic zygosity [73]. Flow cytometric determination of antigen zygosity has been successfully performed with Rh, Kell, Kidd, Ss and Duffy antibodies and applied to paternity cases and family studies [74-76]. The test protocol, based on the amount of specific antibody bound as measured by a secondary fluorescent anti-human immunoglobulin (AHG) reagent, is straightforward and less time consuming than manual antibody titrations. A similar procedure can be used to evaluate antigen deletions or RBC phenotypic variants based on the differences observed in fluorescence marker intensities between patient and normal cells [77].

Flow cytometry is also being used to evaluate cells transfected with known RBC antigens for use in routine antibody identification panels [78,79]. The benefit of such transfected cells would be that one could choose a "host" cell without RBC antigens that may confound antibody identification. For instance, a cell line expressing only Jk^a but lacking Rh and ABO antigens could be engineered.

An area of the clinical laboratory where flow cytometry has been applied sporadically is in the detection of fetal cells within the maternal circulation [80,81]. The ability to evaluate large numbers of RBC's in a relatively short

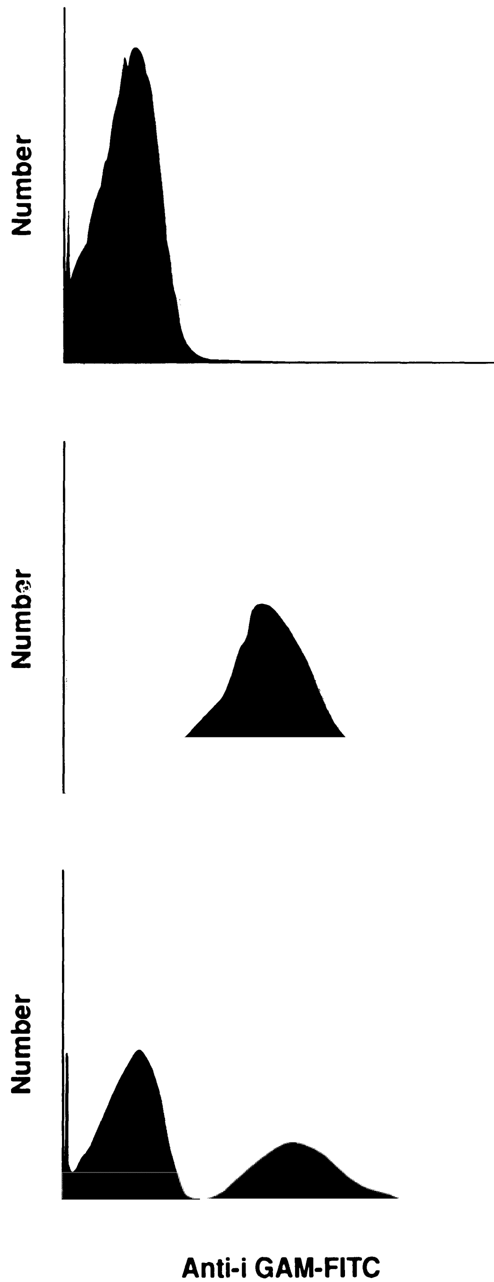


Figure 4. Fluorescent single parameter histograms of RBC suspensions stained with monoclonal anti-i followed by secondary labelling with FITC-goat anti-mouse (1:5 dilution, Becton Dickinson). FL1 = log green fluorescence. Top: Adult RBC suspension. Center: Cord RBC suspension. Bottom: 50% artificial mixture adult/cord RBC.

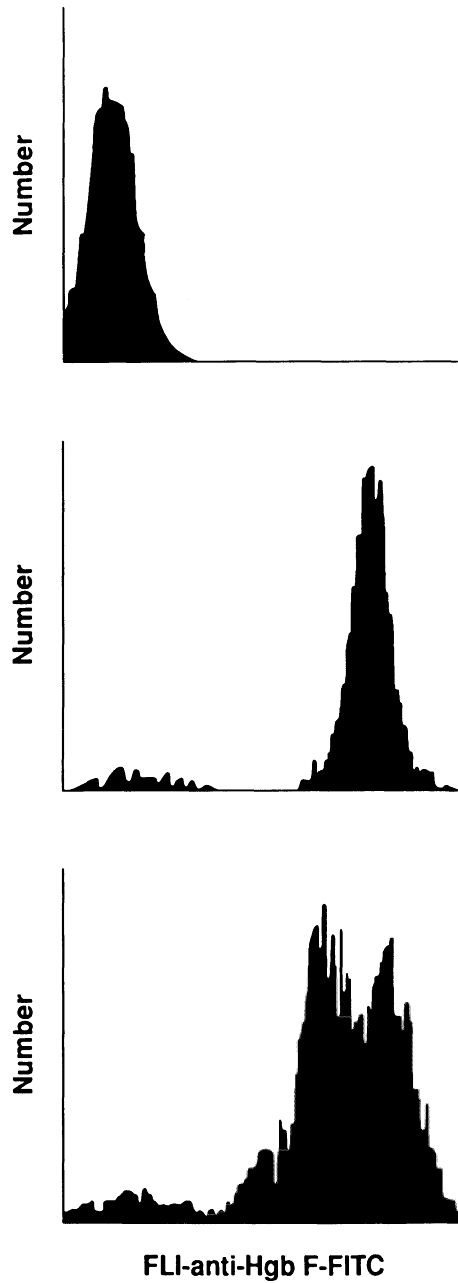


Figure 5. Fluorescent single parameter histograms of RBC suspensions stained with monoclonal anti-Hgb F [84]. FL1 = log green fluorescence. Top: Adult RBC suspension. Center: Cord RBC suspension. Bottom: 50% adult/cord artificial cell mixture.

time could allow such an assay a higher degree of reproducibility than the manual "Cold Standard" Kleihauer-Betke stain. The question then arises as to which fetal cell marker would be the appropriate indicator in a flow cytometric assay. Several surface antigens have been used successfully, including anti-D in the Rh negative mother [82,83). In Figure 3 a typical histogram and the corresponding regression function of artificial Rh positive/negative cell mixtures is shown. The ability to detect as little as 0.25% fetal cells makes this particular antibody combination of utility in all Rh negative women.

To broaden the potential scope of this type of assay, we evaluated in our laboratory the potential of a different fetal RBC marker: i-antigen. Using a monoclonal anti-i antibody a bimodal histogram distribution similar to D/anti-D could be obtained (Figure 4). However, the increased sensitivity gained by flow cytometric analysis was negated by i-antigen present in varying amounts in normal adult RBC populations (2-10%). Therefore, this assay may lack the specificity required to be used as a screening tool in fetal-maternal hemorrhage evaluations.

Several laboratories have used monoclonal antibodies to Hgb F in an attempt to detect fetal RBC's [84,85]. In Figure 5 typical results for a 50%/50% artificial cord/adult RBC mixture are shown. While the antibody specificity certainly is sufficient for slide preparations, cells in solution show technical problems and artifacts in regards to permeabilization of the cell membrane as well as antibody staining that are hard to overcome.

The use of Hgb F as a marker is particularly attractive for the determination of so-called F-reticulocytes. These are reticulocytes with high levels of Hgb F and can be found in conditions of stimulated Hgb F production. In β-globulin disorders such as sickle cell disease and β-thalassemia two recent studies described stimulation of fetal globin gene expression by hydroxyurea and butyrate respectively [86,87]. Enumeration of F-reticulocytes would be of interest in the therapeutic monitoring of such treatment modalities [88].

Flow cytometry has found extensive use in studies on platelet activation [89-93]. Platelet activation may also be of importance for transfusion medicine, particularly for the quality assurance of stored platelet products. Platelet storage lesion has recently been studied by several laboratories employing monoclonal antibodies to a variety of activation antigens seen on platelets, such as GMP-140 (CD62) [94-97]. While the percentage of platelets positive for activation markers significantly changes upon activation, there is currently no recommendation to include these assays routinely in Quality Assurance programs for platelet products.

Assays related to enumeration of cells

In order to avoid alloimmunization by HLA antigens in multiply transfused individuals, many efforts are undertaken to remove leukocytes from RBC and platelet transfusions [98,99]. To monitor the effectiveness of these procedures requires accurate counting techniques at very low concentrations of leukocytes, in the order of 0.0005×10^9/L and less. The problem is that most current, routinely used technologies are equipped to deal with leukocyte counts in the order of

$0.1\text{-}0.01\times10^9$/L. The best results presently available from studies using flow cytometry indicate an improvement of several logs over existing hematology analyzers [100-102]. An old standby in hematology, the improved Neubauer counting chamber or variants thereof, also has been suggested for ultra low leukocyte counts. Unfortunately, although published results with the Nageotte chamber claim a sensitivity of 0.00001×10^9/L, these results are based on "counting" or rather observing a single cell in the entire counting chamber [103]. It is a well known fact that chamber counts of samples with low WBC result in poor reproducibility because the inaccuracy increases with decreasing counts, often to quite unacceptable levels. With modern, automated hematology analyzers the coefficient of variation (CV) for WBC counts in the normal range ($4\text{-}11\times10^9$/L) is well below 5%. However, this CV increases significantly when WBC counts drop below 1.0×10^9/L. When data are obtained by manual chamber counts, that have a much smaller sampling size, the CV typically becomes several orders larger. One would have to doubt the clinical value of data with CV's of 50% and greater.

If the chamber count then does not represent a technique that is satisfactory from a point of reproducibility and accuracy, would the use of flow cytometry based methods offer a solution? Not necessarily, since the same sample size restrictions apply to both flow cytometers and electronic cell counters. Several recent papers have indicated a sensitivity of flow cytometry based techniques to detect WBC at concentrations of 0.00025 to 0.00005×10^9/L [104,105]. This is 2-3 logs beyond the threshold for routine hematology analyzers, but one should keep in perspective that in order to identify a total of 100 WBC, it takes 0.25-2 mL of fluid with WBC at these concentrations to be processed. At flow rates usually applied in flow cytometry it will take impractically long runs to count even this modest number of WBC. One solution in such situations might be a reduction of the total number of cells counted, but this results in a significant decrease in reproducibility. Another possibility is to concentrate, whenever possible, the cells of interest in a smaller volume [104]. However, with every manipulation of the sample the likelihood of errors also increases. An adequate routine method for accurate counts of WBC or other blood cells in very low concentrations is, in our opinion, currently not available.

Traditionally, radioactive isotopes such ^{51}Cr and ^{111}In have been used to determine red cell survival and red cell mass in patients. Using biotinylated red blood cells Zimran et al. described an elegant approach to studying in vivo survival of RBC without the use of radioactive isotopes [106]. Similarly, Cavill et al. and Read et al. have described non-radioactive methods for determining red cell mass in humans. In both methods the flow cytometer replaces the gamma counter as the analyzer [107,108].

An exciting new tool for identifying abnormal blood cells by chromosome specific DNA markers may become available for clinical use. In this technique blood or bone marrow cells are incubated with a fluorescence labelled DNA probe, under conditions for in situ hybridization (fluorescence in situ hybridiza-

tion: FISH) [109]. The method can be used on slides as well as on cells in suspension, making it possible to use flow cytometry. Detection of abnormal cells at an incidence of 1 per 1,000 or less has been reported [110]. Non-flow cytometry based polymerase chain reaction (PCR) dependent tests have also been used, and have the potential of detecting even fewer cells. However, much of this work was done on patients with relatively uncommon gene translocations, such as t(8;14) and t(4;11) and in the translocation often found in CML:t(9;22) [111,112]. A restriction for the application of PCR techniques is that these can only be used in those patients with an identifiable chromosomal marker such as mentioned above. As a final caveat we would like to emphasize that, although it is attractive to develop extremely sensitive detection techniques for minimal residual disease, their clinical usefulness is uncertain and may differ from disease to disease [113].

Identification of hematopoietic progenitor cells, capable of long-term repopulation of bone marrow, by CD34 antibody has been of great importance for "stem cell" harvests, particularly from the peripheral blood [114-116]. Human hematopoietic cells with expression of CD34 in the absence of CD38 have been found to be non-lineage committed progenitor cells, with extensive self renewal capacity. A recent paper has described two subsets of progenitor cells: one that is $CD34^+$, $CD38^-$, $HLA\text{-}DR^+$ and is capable of differentiation into all hematopoietic lineages, and a more primitive population of $CD34^+$, $CD38^-$, $HLA\text{-}DR^-$ cells that can differentiate into all hematopoietic lineages **and** into stromal cells that can support hematopoiesis [117].

From a transfusion medicine point of view CD34 identification has made it possible to monitor harvested bone marrow or peripheral blood cells for "stem cell" content. It can therefore serve as a quality control means of harvested cells that are intended for transplant purposes. It is also possible to use CD34 labelling for enrichment of "stem cell" content of harvested blood or bone marrow cells [118]. This can be applied in situations when the bone marrow contains unwanted cells, such as T lymphocytes in unrelated bone marrow transplants, or when there is the risk of the presence of leukemic, lymphoma or other tumour cells that could contaminate the transplant [118,119]. $CD34^+$, $CD38^-$ enrichment can also be used to select a population of cells suitable for gene insertion, because of their self renewal capacity [120].

If material intended for transplantation is stored, it will be necessary to measure cell survival to ensure a favourable outcome of the transplant procedure. A rapid method for measuring the proportion of dead cells is based on the exclusion of DNase by live cells [121]. Addition of cell surface markers to this assay may enable calculation of cytotoxicity resulting in cell death in individual subsets of mixed cell populations.

Conclusion

Flow cytometry has become an important technique for researchers as well as for clinicians. The technique offers rapid, multi-parameter analysis of cells in suspension, and can be applied to many assays in transfusion medicine. In recent years we have witnessed many improvements in flow cytometry instrumentation, and the development of many new antibodies and more sophisticated reagents. This will undoubtedly further stimulate the use of flow cytometry in transfusion medicine.

Acknowledgements

The authors would like to thank Dominique Blanchard from Centre Régional de Transfusion Sanguine, Nantes, France for the kind gift of anti-i monoclonal antibody and John Moulds from Gamma Biologics, Houston, Texas for polyclonal anti-i antibody. Delio Pascual of the Loma Linda University Medical Center Blood Bank has been indispensable in obtaining the appropriate red cell combinations for our studies. The experimental work done on fetal red blood cell detection described in this paper was supported in part by a research grant from TOA Medical Electronics Co., Ltd., Kobe, Japan.

References

1. Laerum OD, Bjerknes R (eds). Flow cytometry in hematology. London: Academic Press, 1992 (Analytical Cytometry Series).
2. Darzynkiewicz Z, Crissman HA (eds). Flow cytometry. San Diego: Academic Press, 1990 (Methods in cell biology; vol. 33).
3. Coon JS, Weinstein RS (eds). Diagnostic flow cytometry. Baltimore: Williams and Wilkins, 1991 (Techniques in Diagnostic Pathology).
4. Shapiro HM. Practical flow cytometry. 2nd ed. New York: Alan R. Liss 1988.
5. National Committee for Clinical Laboratory Standards. Clinical applications of flow cytometry: Quality assurance and immunophenotyping of peripheral blood lymphocytes; Tentative Guideline. Villanova, PA, 1992 (NCCLS Document H42-T).
6. Godfrey K. Simple linear regression in medical research. In: Bailar III JC, Mosteller F (eds). Medical use of statistics. 2nd ed. Boston: NEJM Books 1992:201-32.
7. Galen S, Gambino R. Beyond normality: The predictive value and efficiency of medical diagnoses. New York: John Wiley and Sons, 1975.
8. Swets JA. Measuring the accuracy of diagnostics systems. Science 1988;240:1285-93.
9. Maffetone MA. The economics of flow cytometry. In: Flow cytometry and image analysis in clinical medicine. Chicago, 1990 (AMA Biotechnology and Medicine Conference Series).
10. De Bruin HG, de Leur-Ebeling I, Aaij C. Quantitative determination of the number of FITC-molecules bound per cell in immunofluorescence flow cytometry. Vox Sang 1983;45:373-7.
11. McHugh TM, Reid ME, Stites DP, Chase ES, Casavant CH. Detection of the human erythrocyte surface antigen Gerbich by flow cytometry using human antibodies and phycoerythrin for extreme immunofluorescence sensitivity. Vox Sang 1987;53: 231-4.

12. Nance S, Garratty G. Correlates between in vivo hemolysis and the amount of RBC-bound IgG measured by flow cytofluorometry. (Abstract). Blood 1984;64(suppl 1): 64;88a.
13. Van der Meulen FW, de Bruin HG, Goosen CM, et al. Quantitative aspects of the destruction of red cells sensitized with IgG1 autoantibodies: An application of flow cytofluorometry. Brit J Haematol 1980;46:47-56.
14. Gebel HM, Lebeck LK. Crossmatch procedures used in organ transplantation. In: Rodey G (ed). Laboratory testing in organ transplantation. Philadelphia: WB Saunders, 1991:603-20 (Clinics in laboratory medicine; vol. 11).
15. Cook DJ, Terasaki PI, Iwaki Y, Terashita Gy, Lau M. An approach to reducing early kidney transplant failure by flow cytometry crossmatching. Clin Transpl 1987;1: 253-6.
16. Bray RA, Lebeck LK, Gebel HM. the flow cytometry crossmatch. Dual color analysis of T cell and B cell reactivities. Transplantation 1989;48:834-40.
17. Lazda BA, Pollak R, Mozes MF, Jonasson O. The relationship between flow cytometer crossmatch results and subsequent rejection episodes in cadaver renal allograft recipients. Transplantation 1988;45:562-5.
18. Talbot D, Stratton A, Givan AL, Shenton BK, Proud G, Taylor RM. DR typing in less than three hours using a flow cytometer. Transplant Proc 1988;20:382-3.
19. Albrecht J, Muller HAG. HLA-B27 typing by use of flow cytofluorometry. Clin Chem 1987;33:1619-23.
20. Prod MH, Schwartz L, Piggot S, Wever S, Cerva C, Gebel HM. Screening of HLA-B27 by flow cytometry. (Abstract) Human Immunol 1992;34:89.
21. Kuijpers RW, Dooren MC, von dem Borne AEGKr, Ouwehand WH. Detection of human monocyte-reactive alloantibodies by flow cytometry after selective downmodulation of the Fc receptor I. Blood 1991;78:2150-6.
22. Hanson CA. Applications of flow cytometry in diagnostic pathology. Am J Clin Pathol 1989;91(suppl 1):S27-31.
23. Robinson JP, Duque RE, Boxer LA, Ward PA, Hudson JL. Measurement of anti-neutrophil antibodies by flow cytometry: Simultaneous detection of antibodies against monocytes and lymphocytes. Diag Clin Immunol 1987;5:163-70.
24. Veys PA, Gutteridge CN, Macey M, Ord J, Newland AC. Detection of granulocyte antibodies using flow cytometric analysis of leukocyte immunofluorescence. Vox Sang 1989;56:42-7.
25. Lalezari P, Radel E. Neutrophil-specific antigens: Immunology and clinical significance. Semin Hematol 1974;11:281-90.
26. Klaassen RJ, Goldschmeding R, Vlekke AB, Rozendaal R, von dem Borne AEGKr. Differentiation between neutrophil-bound antibodies and immune complexes. Brit J Haematol 1991;77:398-402.
27. Kelton JG, Murphy WG, Lucarelli A, et al. A prospective comparison of four techniques for measuring platelet-associated IgG. Brit J Haematol 1989;71:97-105.
28. Ault KA. Flow cytometric measurement of platelet associated immunoglobulin. Pathol Immunopathol Res 1988;7:395-405.
29. Rosenfeld CS, Nichols G, Bodensteiner DC. Flow cytometric measurement of platelet antibodies. Am J Clin Pathol 1987;87:518-22.
30. Varon D, Karpatkin S. A monoclonal anti-platelet antibody with decreased activity for autoimmune thrombocytopenic platelets. Proc Natl Acad Sci USA 1983;80:6992-5.
31. Lynch DM, Howe SE. Antigenic determinants in idiopathic thrombocytopenic purpura. Brit J Haematol 1986;63:301-8.
32. Sinha RK, Kelton JG. Current controversies concerning the measurement of platelet-associated IgG. Transf Med Rev 1990;4:121-35.
33. Visentin GP, Wolfmeyer K, Newman PJ, Aster RH. Detection of drug-dependent, platelet-reactive antibodies by antigen-capture ELISA and flow cytometry. Transfusion 1990;30:694-700.

34. Worfolk LA, MacPherson BR. The detection of platelet alloantibodies by flow cytometry. Transfusion 1991;31:340-4.
35. Von dem Borne AEGKr, Decary F. ICSH/ISBT Working Party on platelet serology. Nomenclature of platelet-specific antigens. Vox Sang 1990;58:176.
36. Aster RH. The immunologic thrombocytopenias. In: Kunicki TJ, George JN (eds). Platelet immunobiology. Philadelphia: JB Lippincott, 1989:387-435.
37. Gale HB, Henry K. Measuring percents by flow cytometry to calculate absolute lymphocyte subset counts for HIV^+ specimens. Cytometry 1992;13:175-81.
38. Landay A, Ohlsson-Wilhelm BM, Giorgi JV. Application of flow cytometry to the study of HIV infection. AIDS 1990;4:479-97.
39. Lebeck LK, Lewis J. HIV infection: Immunobiology and laboratory diagnosis. Clin Lab Sci 1992;5:28-30.
40. USD Health and Human Services, Public Health Service CDC, Public Health Practice Program Office, Division of Laboratory Services. Guidelines for the performance of $CD4^+$ T cell determination in persons with human immunodeficiency virus infection. "The morbidity and mortality weekly report" (MMWR) 1992;41:RR-8.
41. Seeds MC, Bass DA. Measurements of granulocyte oxidative metabolism. In: Laerum OD, Bjerknes R (eds). Flow cytometry in hematology. London: Academic Press, 1992:132-42 (Analytica Cytology Series).
42. Vindeløv L, Christensen IJ. An integrated set of methods for routine flow cytometric analysis. In: Darzynkiewicz Z, Crissman HA (eds). Flow cytometry. San Diego: Academic Press, 1990:127-37 (Methods in cell biology; vol. 33).
43. Hedley DW. Flow cytometry using paraffin-embedded tissue: Five years on. Cytometry 1989;10:229-41.
44. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 1984;133:1710-5.
45. Kurki P, Ogata K, Tan EM. Monoclonal antibodies to proliferating cell nuclear antigen (PCNA)/cyclin as probes for proliferating cells by immunofluorescence microscopy and flow cytometry. J Immunol Methods 1988;109:49-59.
46. Crissman HA, Steinkamp JA. Detection of bromodeoxyuridine-labeled cells by differential fluorescence analysis of DNA fluorochromes. In: Darzynkiewicz Z, Crissman HA (eds). Flow cytometry. San Diego: Academic Press, 1990:199-216 (Methods in cell biology; vol. 33).
47. Andreeff M. Biological characterization and therapy monitoring of leukemia. In: Laerum OD, Bjerknes R (eds). Flow cytometry in hematology. London: Academic Press, 1992:232-66 (Analytical Cytometry Series).
48. Cornelisse CJ, van de Velde CJ, Caspers RJ, Moolenaar AJ, Hermans J. DNA ploidy and survival in breast cancer patients. Cytometry 1987;8:225-34.
49. Koss LG, Czerniak B, Herz F, Wersto RP. Flow cytometric measurements of DNA and other cell components in human tumors. A critical appraisal. Hum Pathol 1989; 20:528-48.
50. Suarez C, Miller DR, Steinherz PG, Melamed MM, Andreeff M. DNA and RNA determination in 111 cases of childhood acute lymphoblastic leukaemia (ALL) by flow cytometry: Correlation of FAB classification with DNA stemline and proliferation. Brit J Haematol 1985;60:677-86.
51. Davis BH, Bigelow NC. Flow cytometric reticulocyte quantification using thiazole orange provides clinically useful reticulocyte maturity index. Arch Pathol Lab Med 1989;113:684-9.
52. Houwen B. Reticulocyte maturation. Blood Cells 1992;18:167-86.
53. Davies AV, Cavill J, Bentley N, Fegan CD, Poynton CH, Whittaker JA. Evaluation of erythropoiesis after bone marrow tranplantation: Quatitative reticulocyte counting. Brit J Haematol 1992;81:12-7.

54. Pappas J, Houwen B, Bedros AA, Mast BJ, Kubose KK, Clement L. Reticulocyte maturation index is an early indicator of bone marrow recovery after cytotoxic chemotherapy. (Abstract). Blood 1992;80(suppl 1):456a.
55. Kienast J, Schmitz G. Flow cytometric analysis of thiazole orange uptake by platelets: A diagnostic aid in the evaluation of thrombocytopenic disorders. Blood 1990;75:116-21.
56. Ault KA, Rinder HM, Mitchell J, Carmody MB, Vary CPH, Hillman RS. The significance of platelets with increased RNA content (reticulated platelets). A measure of the rate of thrombopoiesis. Am J Clin Pathol 1992;98:637-46.
57. Muhlpfordt H, Berger J. characterization and grouping of *Trypanasoma brucei brucei, T.B. gambiense* and *T.B. rhodesiense* by quantitative DNA-cytofluorometry and discriminant analysis. Trop Med Parasitol 1989;40:1-8.
58. Van Vianen PHP, Thaithong S, Reinders PP, et al. Automated flow cytometric analysis of drug susceptibility of malaria parasites. Am J Trop Med Hyg 1990;43:602-7.
59. Mons B, Janse CJ. Detection and analysis of parasitic infections. In: Laerum OD, Bjerknes R (eds). Flow cytometry in hematology. London: Academic Press, 1992: 197-212 (Analytical Cytometry Series).
60. Foon KA, Todd RF III. Immunologic classification of leukemia and lymphoma. Blood 1986;68:1-31.
61. Greer JP, Macon WR, List AF, McCurley TL. Non-Hodgkin's lymphomas. In: Lee GR, Bithell TC, Foerster J, Athens JW, Lukes JN (eds). Wintrobe's clinical hematology. 9th edition. Philadelphia: Lea and Febiger 1993:2085.
62. Slaper-Cortenbach IC, Admiraal LG, Kerr JM, van Leeuwen EF, von dem Borne ARGKr, Tetteroo PA. Flow-cytometric detection of terminal deoxynucleotidyl transferase and other intracellular antigens in combination wth membrane antigens in acute lymphatic leukemias. Blood 1988;72:1639-44.
63. Crist WM, Grossi CE, Pullen DJ, Cooper MD. Immunologic markers in childhood acute lymphocytic leukemia. Semin Oncol 1985;12:105-21.
64. Knapp W, Dorken B, Rieber P, Schmidt RE, Stein H, von dem Borne AEGKr. CD antigens 1989. Blood 1989;74:1448-50.
65. Duque RE, Everett ET, Iturraspe J. Flow cytometric analysis of acute leukemias. Clin Immunol Newsletter 1990;10:43-50.
66. Lichtman MA, Henderson ES. Acute myelogenous leukemia. In: Lee GR, Bithell TC, Foerster J, Athens JW, Lukes JN (eds). Wintrobe's clinical hematology. 9th edition. Philadelphia: Lea and Febiger 1993:252.
67. Bray RA, Landay AL. Identification and functional characterization of mononuclear cells by flow cytometry. Arch Pathol Lab Med 1989;113:579-90.
68. Pirker R, Wallner J, Geissler K, et al. MDR 1 gene expression and treatment outcome in acute myeloid leukemia. J Nat Cancer Inst 1991;83:708-12.
69. Krishan A, Sauerteig A, Stein JH. Comparison of three commercially available antibodies for flow cytometric monitoring of P-glycoprotein expression in tumor cells. Cytometry 1991;12:731-42.
70. Whitford P, George WD, Campbell AM. Flow cytometric analysis of tumour infiltrating lymphocyte activation and tumour cell MHC class I and II expression in breast cancer patients. Cancer Lett 1992;61:157-64.
71. Rosenberg SA, Lotze MT, Yang JC, et al. Experience with the use of high-dose interleukin-2 in the treatment of 652 cancer patients. Ann Surg 1989;210:474-84; discussion 484-5.
72. Ferrari G, Rossini S, Nobili N, et al. Transfer of the ADA gene into human ADA-deficient T lymphocytes reconstitutes specific immune functions. Blood 1992;80: 1120-4.
73. Anstee DJ. Blood group-active surface molecules of the human red blood cell. Vox Sang 1990;58:1-20.

74. Van Bockstaele DR, Berneman ZN, Muylle L, Cole-Dergent J, Peetermans ME. Flow cytometric analysis of erythrocytic D antigen density profile. Vox Sang 1986; 51:40-6.
75. Arndt P, Garratty G. Variation of C antigen strength on r'r RBCs demonstrated by flow cytometry. (Abstract). Transfusion 1987;27:514.
76. Oien L, Nance S, Arndt P, Garratty G. Determination of zygosity using flow cytometric analysis of red cell antigen strength. Transfusion 1988;28:541-4.
77. Langlois RG, Bigbee WL, Jensen RH. Flow cytometric characterization of normal and variant cells with monoclonal antibodies specific for glycophorin A. J Immunol 1985;134:4009-17.
78. Lublin DM, Coyne KE. Phospholipid-anchored and transmembrane versions of either decay-accelerating factor or membrane cofactor protein show equal efficiency in protection from complement-mediated cell damage. J Exp Med 1991;174:35-44.
79. Lublin DM, Thompson ES, Green AM, Levene C, Telen MJ. Dr(a-)polymorphism of decay accelerating factor. Biochemical, functional and molecular characterization and production of allele-specific transfectants. J Clin Invest 1991;87:1945-52.
80. Cupp JE, Leary JF, Cernichiari E, Wood JCS, Doherty RA. Rare-event analysis methods for detection of fetal red blood cells in maternal blood. Cytometry 1984;5:138-44.
81. Medearis AL, Hensleigh PA, Parks DR, Herzenberg LA. Detection of fetal erythrocytes in maternal blood post partum with the fluorescence-activated cell sorter. Am J Obstet Gynecol 1984;148:290-5.
82. Nance S, Nelson J, O'Neill P, Lam H, Garratty G. Quantitation of fetal-maternal hemorrhage by flow cytometry, a simple and accurate method. (Abstract). Transfusion 1986;26:571.
83. Wong C, Sassetti RJ, McLeod BC. Flow cytometric determination of mixed red blood cell populations in transfused patients. Labmedica 1989;Aug/Sept:15-21.
84. Chen JC, Marcheai J, Poulik MD. Determination of hemoglobin F^+ (HBF^+) cells by flow cytometry (FC). FASEB 1989;3:268.
85. Patton WN, Nicholson GS, Sawers AH, Franklin IM, Ala FA, Simpson AW. Assessment of fetal-maternal haemorrhage in mothers with hereditary persistence of fetal haemoglobin. Am J Clin Pathol 1990;43:728-31.
86. Rodgers GP, Dover GJ, Uyesaka N, Noguchi CT, Schechter AN, Nienhuis AW. Augmentation by erythropoietin of the fetal-hemoglobin response to hydroxyurea in sickle cell disease. N Engl J Med 1993;328:73-80.
87. Perrine SP, Ginder GD, Faller DV, et al. A short term trial of butyrate to stimulate fetal-globin-gene expression in the β-globin disorders. N Engl J Med 1993;328:81-6.
88. Nagel RL, Vichinsky E, Shah M, et al. F reticulocyte response in sickle cell anemia treated with recombinant human erythropoietin: A double blind study. Blood 1993;81:9-14.
89. George JN, Pickett EB, Saucerman S, et al. Platelet surface glycoproteins: Studies on resting and activated platelets and platelet membrane microparticles in normal subjects, and observations in patients during adult respiratory distress syndrome and cardiac surgery. J Clin Invest 1986;78:340-8.
90. Abrams CS, Ellison N, Budzynski AZ, Shattil SJ. Direct detection of activated platelets and platelet-derived microparticles in humans. Blood 1990;75:128-38.
91. McEver RP. The clinical significance of platelet membrane glycoproteins. Hematol Oncol Clin North Am 1990;4:87-105.
92. Ginsberg MH, Frelinger AL, Lam SC, et al. Analysis of platelet aggregation disorders based on flow cytometric analysis of membrane glycoprotein IIb-IIIa with conformation-specific monoclonal antibodies. Blood 1990;76:2017-23.
93. Abrams C, Shattil SJ. Immunological detection of activated platelets in clinical disorders. Thromb Haem 1991;65:467-73.

94. Solberg C. Storage of human red blood cells and platelets. Some aspects concerning the factors leading to storage lesion characterized as morphological changes and vesiculation. Mini review based on a doctoral thesis. Ups J Med Sci 1988;93:201-14.
95. Bode AP. Platelet activation may explain the storage lesion in platelet concentrates. Blood Cells 1990;16:109-26.
96. Chernoff A, Snyder EL. The cellular and molecular basis of the platelet storage lesion: A symposium summary. Transfusion 1992;32:386-90.
97. Bode AP, Norris HT. The use of inhibitors of platelet activation or protease activity in platelet concentrates stored for transfusion. Blood Cells 1992;18:361-80.
98. Fisher M, Chapman JR, Ting A, Morris PJ. Alloimmunisation to HLA antigens following transfusion with leukocyte-poor and purified platelet suspensions. Vox Sang 1985;49:331-5.
99. Snyder EL. clinical use of white cell-poor blood components. Transfusion 1989;29: 568-71.
100. Bodensteiner DC. A flow cytometric technique to accurately measure post-filtration white blood cell counts. Transfusion 1989;29:651-3.
101. Dzik WH, Ragosta A, Cusack WF. Flow cytometric method for counting very low numbers of leukocytes in platelet products. Vox Sang 1990;59:153-9.
102. Friedman LI, Sadoff BJ, Stromberg RR. White cell counting in RBCs and platelets: How few can we count? Transfusion 1990;39:387-9.
103. Masse M, Naegelen C, Pellegrini N, Segier JM, Marpaux N, Beaujean F. Validation of a simple method to count very low white cell concentrations in filtered red cells or platelets. Transfusion 1992;32:565-71.
104. Takahashi TA, Hosada M, Sekiguchi S. Cytospin method: New method for the determination of residual leukocytes in leukocyte depleted platelet concentrates. Jpn J Transfus Med 1989;35:497-503.
105. AI EJM, Visser SCE, Prins HK, Pietersz RNI, Reesink HW, Huisman JG. A flow cytometric method for determination of white cell subpopulations in filtered red cells. Transfusion 1991;31:835-42.
106. Zimran A, Forman L, Suzuki T, Dale GL, Beutler E. In vivo aging of red cell enzymes: Study of biotinylated red blood cells in rabbits. Am J Hematol 1990;33: 249-54.
107. Hudson IR, Cavill IA, Cooke A, et al. Biotin labeling of red cells in the measurement of red cell volume in preterm infants. Pediatr Res 1990;28:199-202.
108. Read EJ, Cardine LL, Yu MY. Flow cytometric detection of human red cells labeled with a fluorescent membrane label: Potential application to in vivo survival studies. Transfusion 1991;31:502-8.
109. Trask B, Pinkel D. Fluorescence in situ hybridization with DNA probes. In: Darzynkiewicz Z, Crissman HA (eds). Flow cytometry. San Diego: Academic Press, 1990: 383-400 (Methods in cell biology; vol. 33).
110. Gray JW, Pinkel D, Trask B, et al. Application of flow karyotyping and fluorescence "in situ" hybridization to detection of chromosome aberations. In: Karry JP, Coffey DS, Gardner W (eds). Prognostic cytometry and cytopathology of prostate cancer. New York: Elservier 1989:304-13.
111. Guo JQ, Wang JYJ, Arlinghaus RB. Detection of BCR-ABL proteins in blood cells of benign phase chronic myelogenous leukemia patients. Cancer Res 1991;51:3048-51.
112. Kawasaki ES, Clark SS, Coyne MY, et al. Diagnosis of chronic myeloid and acute lymphocytic leukemias by detection of leukemia-specific mRNA sequences amplified in vitro. Proc Natl Acad Sci USA 1988;85:5698-702.
113. Wiernik PH. Hematologic malignancies. In: Spivak JL, Bell WR, Ness PM, Quesenberry PJ, Wiernik PH (eds). Year book of hematology 1993. St. Louis: Mosby Year Book, 1992;331&396.

114. Briddell RA, Brondy VC, Bruno E, Brandt JE, Srour EF, Hoffman R. Further phenotypic characterization and isolation of human hematopoietic progenitor cells using a monoclonal antibody to c-kit recpetor. Blood 1992;79:3159-67.
115. Baum CM, Weissman IL, Tsukamoto AS, Buckle AM, Peault B. The islation of a candidate human hematopoietic stem-cell population. Proc Natl Acad Sci USA 1992; 89:2804-8.
116. Morse EE, Dainiak N, Sorba S, King C, Gaffney J, Ascencao JL. Peripheral blood stem cell transfusion for marrow replacement. Ann Clin Lab Sci 1992;22:221-5.
117. Huang S, Terstappen LWMM. Formation of haematopoietic microenvironment and haematopoietic stem cells from single human bone marrow stem cells. Nature 1992; 360:745-9.
118. Hami LS, Shpall EJ, Johnston CS, Berenson R, Jones RB. The effect of PIXY=321 on 4-hydroperoxycyclophosphamide purged and $CD34^+$ positively selected bone marrow. Prog Clin Biol Res 1992;377:321-5.
119. Thomas TE, Lansdorp PM. Purification of CD34 positive cells from human bone marrow using high gradient magnetic separation. Prog Clin Biol Res 1992;377:537-44.
120. Dick JE, Magli MC, Huszar D, Philips RA, Bernstein A. Introduction of a selectable gene into primitive stem cells capable of long term reconstitution of the hematopoietic system of W/Wv mice. Cell 1985;42:71-9.
121. Frankfurt OS. Flow cytometric measurement of cell viability using DNase exclusion. In: Darzynkiewicz Z, Crissman HA (eds). Flow cytometry. San Diego: Academic Press, 1990:13-8 (Methods in cell biology; vol. 33).

NEW APPLICATIONS OF MOLECULAR DIAGNOSTICS IN LABORATORY TEST SYSTEMS

L.R. Overby

Molecular diagnostic test systems refer to techniques and laboratory protocols for detecting, identifying and amplifying nucleic acid sequences. By differentiating one sequence from another, even if they differ only by a single nucleotide base change, a wide range of clinically useful and scientifically valuable diagnostic information may be obtained. In the field of human diseases molecular diagnostics are applicable to infectious organisms, genetic and hereditary diseases, cell and tissue typing, and to genetic mutations, recombinants, duplications or rearrangements associated with various forms of cancer. Genomic matching between individuals has become practical and is used widely for parentage testing and for evidence in forensic and crime situations. In the future, individual DNA "fingerprints" may be deposited in data banks as sources of unique individual identification.

Molecular diagnostics first became practical long before the evolution of the biotechnology era. Gillespie and Spiegelman in 1965, found that single stranded DNA was irreversibly bound to cellulose nitrate filter membranes and could be hybridized specifically to radioactive RNA strands of opposite polarity [1]. The hybridization was easily detected by photo exposure or scintillation counting of the intact membranes. Current widely used blotting techniques have evolved from these original discoveries.

Electrophoretic separation of DNA on sizing gels after restriction enzyme cleavage has opened a wide range of applications based on restriction fragment length polymorphism (RFLP). These patterns are unique for each organism and serve as identity patterns for parentage testing in humans and for biological forensics evidence. Initially relative large quantities of nucleic acids (nanograms to micrograms) were required for analysis. However, the discovery of biochemical amplification via polymerase chain reaction (PCR) has vastly expanded the usefulness of molecular diagnostics [2]. In theory, a single segment of a DNA molecule in which some base sequence information is known can be amplified into billions of copies for analysis, cloning or sequencing.

This report does not include complete technical details for molecular diagnostic procedures. The various test systems are referenced and mentioned briefly and their relevance to future utility in laboratory medicine is reviewed. When world wide standards are generally accepted blood bank laboratories may employ

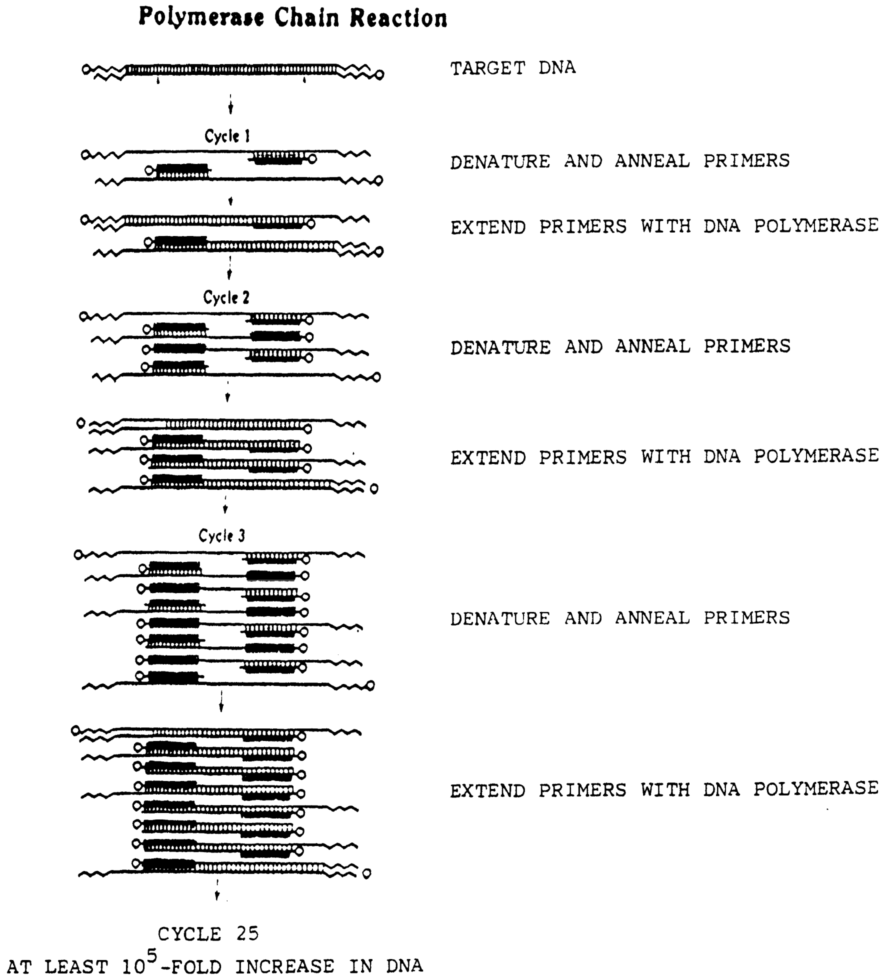


Figure 1. Schematic representation of exponential amplification by polymerase chain reaction. Heating denatures the double stranded DNA target to give two single stranded templates. Site-specific primers are annealed to each strand, flanking the target segment to be amplified. Thermostable tac-DNA polymerase then catalyzes synthesis of two pieces of double-stranded DNA, identical to the original target. The newly synthesized products can then serve as templates for the denaturing, annealing, and extensions in another cycle. In practice, 25 such cycles will normally generate at least a 100,000-fold increase in DNA.

molecular diagnostics in the fields of infectious disease diagnosis and screening, HLA typing and transplantation analyses, parentage testing, and tumour and genetic disease testing.

Biochemical amplification of nucleic acids

The PCR, first reported in 1985, has been refined further to improve laboratory utility [3-5]. As a result, this in vitro method for amplifying defined segments of DNA has become a valuable tool in basic molecular biology research and in clinical diagnostic testing. The cyclic procedure is illustrated in Figure 1. PCR involves melting DNA to convert double strand to single strand, annealing pairs of oligonucleotide primers (generally about 20 bases) that flank the segment to be amplified, and then extending the target sequence on each strand using DNA polymerase. Repetition of the cycle many times provides an exponential accumulation of replicate copies of the original DNA segment between the primers, sufficient to be detected easily on Southern blots after separation on sizing gels. The key to PCR efficiency is the use of tac-DNA polymerase, a thermostable enzyme, so that DNA in the reaction mixture can be melted at elevated temperature without inactivating the enzyme [6]. RNA can also serve as a target for PCR amplification if it is first copied into cDNA by reverse transcription. The ability to amplify as well as modify specific sequences of DNA starting with, in theory, only a single molecule has opened many new areas of molecular biology for experimental investigation.

The success of PCR has led to several other gene amplifying systems particularly applicable to diagnostic test systems [7]. The ligase chain reaction (LCR) uses two sets of complementary oligonucleotide probes that can hybridize adjacently on a complementary target strand [8]. As illustrated in Figure 2, a large molar excess of probes is used to hybridize to denatured target. Adjacently hybridized probes are joined by the action of thermostable DNA ligase. The products of these reactions are the ligated probes which are functionally equivalent to the initial target segment. The process is cycled repeatedly resulting in exponential growth of the number of target equivalents. LCR is projected to be especially useful in detecting genetic defects and infectious microorganisms. The repair chain reaction (RCR) is similar to LCR, except that the 3' and 5' ends of the two probes are separated by one or a few basis after the hybridization step [9]. The gap is filled in by DNA polymerase and then ligated, as in LCR.

A number of other nucleic acid amplifying technologies are under development to meet the needs for increased sensitivity in diagnostic test systems. Self sustained sequence replication (3SR) follows the replication cycle of typical retroviruses [10]. RNA is converted to cDNA and double stranded DNA with reverse transcriptase. Multiple copies of the target RNA with opposite polarity are then generated with T7 RNA polymerase by transcription off the double stranded DNA. Continuous recycling can amplify an RNA target 10 million-fold in an hour. Thermocycling is not required for 3SR. It may be especially useful in tracking gene action via detecting and quantifying mRNA.

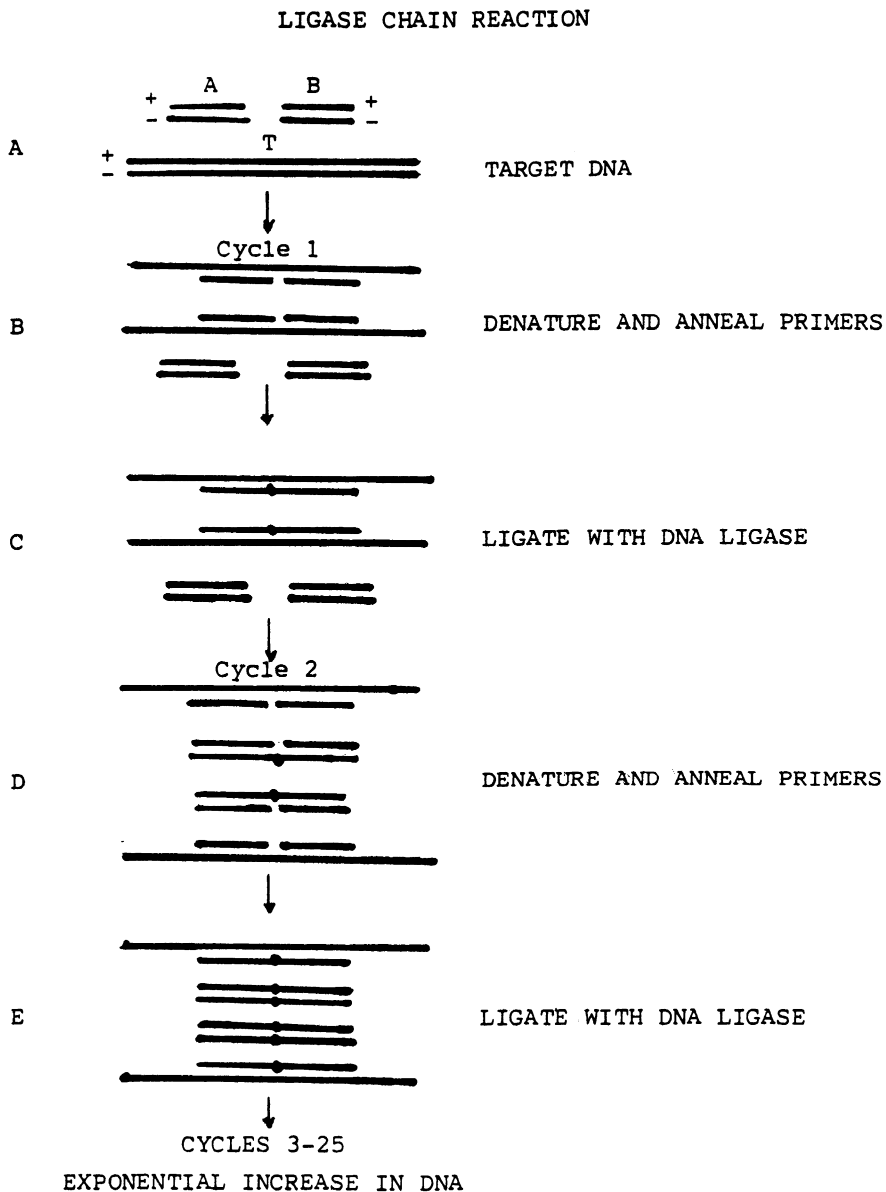


Figure 2. Schematic representation of exponential amplification by ligase chain reaction. A. Two sets of complementary oligonucleotide probes (A and B) are provided to hybridize adjacently on the target strands (T). B. The mixture is denatured at high temperature and annealed to form hybrids with the two probes juxtaposed on each target strand. C. The adjacently hybridized probes are joined by the action of thermostable DNA ligase. D and E. The products of the reaction are functionally equivalent to the original target. Repeated cycles of denaturing, annealing, and ligating results in exponential growth in target equivalents.

The above biochemical reactions geometrically amplify target nucleic acid sequences to achieve increased detectability. Similar high sensitivities have been achieved through exponential amplification of the RNA probe involved in a hybrid complex [11]. This procedure uses RNA probe sequences complementary to the target analyte inserted into the natural template for Qβ replicase. The specific target-RNA probe hybrid is rigorously purified and the RNA probe released to serve as a template for the replicase. A one billion-fold amplification was achieved in a 30 minute reaction. These results demonstrated feasibility for employing replicable RNA probes in molecular bioassays.

The same reason that biochemical amplifications have exquisite sensitivity also makes all of the techniques susceptible to amplification of trace quantities of previous amplification products or laboratory contaminants. These sources of false positive reactions currently limit use only in laboratories that practice rigorous contamination control and use improved automated instrumentation and biochemical protocols to minimize contamination problems associated with amplification reactions [5,12,13].

DNA probes for infectious diseases

Synthetic or cloned single stranded oligonucleotide probes are conveniently used to identify the specific nucleic acids of infectious organisms. Typical standard procedures include immobilizing on filter membranes nucleic acids extracted directly from a culture or clinical specimen or from a biochemical amplification reaction. Synthetic oligonucleotide probes complementary to the targeted sequences and labelled with radioisotopes or other reporter molecules are then hybridized with the immobilized nucleic acids. Hybridizations are conveniently detected by autoradiography for radiolabelled probes, and colour development for enzyme labelled probes [14]. In many cases liquid hybridization followed by separation of the analyte-probe hybrid is preferred over dot blot procedures [15]. Hybrids formed in solution are detected by a variety of means, including selective adsorption on particulates, membranes, or magnetic particles. On a research basis, PCR followed by observing the amplified segment on electrophoretic sizing gels is applicable to most infectious organisms for which specific primers are available.

Infectious diseases are normally diagnosed by isolation of the organism in culture or by immunoassay serologic profiles of antigens and antibodies. Methods for direct detection of bacterial or viral genomes with specific probes currently are not sensitive enough to be equivalent to culture or serology, but in many cases probe assays are valuable supplementary diagnostics, especially when coupled to PCR or other target amplification procedures. When culture is slow, difficult, or impossible probe diagnostics are valuable tools in the diagnostic laboratory. Many infectious diseases are amenable to gene diagnosis if methods substantially equivalent to culture evolve. Infections with the blood-borne hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) are currently diagnosed by immunological methodologies. The

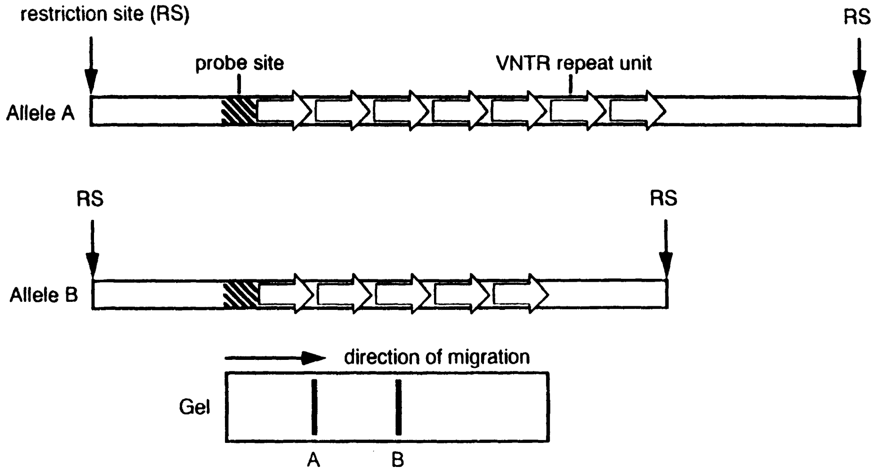


Figure 3. Highly variable regions of human DNA result from a variable number of tandem repeat (VNTR) sequences (arrows) between fixed restriction enzyme sites (RS). Illustrated are two alleles with different length restriction fragments depending upon the number of repeated sequences found between the restriction sites. The two bands are separatable on a sizing gel and observed in a Southern blot.

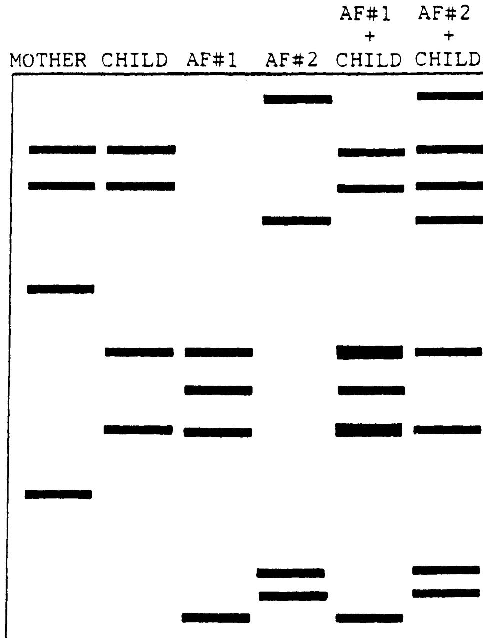


Figure 4. Profiles of DNA typing in a hypothetical paternity test. Depicted is an inclusion for alleged father #1 (AF#1) and an exclusion of alleged father #2 (AF#2).

same tests are used to screen blood donations. Supplemental testing of seropositive blood donors for the viral genomes can be valuable for confirming the immunoassay, for linking the infections with the course of disease, and for monitoring therapeutic efficacy. Hepatitis C patients with acute symptoms, resolving disease, or with chronic disease will usually have the same spectrum of viral antibodies, and the disease states cannot be distinguished serologically. However, tests for viral RNA and PCR show that the RNA is transient in acute, self-limited disease, but persists in patients who progress to chronicity [16].

There are many opportunities for probe diagnostics for infectious organisms: cytomegalovirus, herpes simplex virus, Epstein-Barr virus, bacteria and other single cell organisms (especially respiratory infections), sexually transmitted diseases (Neisseria gonorrhoea and Chlamydia trachomatis), and gastrointestinal diseases (Campylobacter and Rotavirus). Currently the sensitivity and specificity of almost all molecular diagnostic methods are inadequate for supplanting culture and serology for diagnosing these infections. The complicated and long protocols for biochemical amplifications also limit widescale use.

Table 1. Biochemical methods for DNA amplification.

Method		**Product**	**Enzymes**
Polymerase chain reaction	PCR	Target	DNA polymerase
Ligase chain reaction	LCR	Probe	DNA ligase
Self systained sequence replication	3SR	Target	Reverse transcriptase + DNA polymerase
QB probe replication	QBR	Probe	QB replicase
Repair chain reaction	RCR	Target	DNA polymerase + DNA ligase

Table 2. Reverse dot blot typing of HLA class II DQ, DP and DR loci using PCR amplification and immobilized probes*.

Locus	**Variabele regions**	**Number of probes**	**Primer pairs**	**Alleles identified**
DQ1A	4	10	1	7
DPB1	6	16	1	15
DQB1	6	16	2	18
DRB1	12	26	3	32

* Summarized from Erlich, et al. [17].

HLA typing

The HLA class II genes encode α and β peptide chains which are glycosylated and form cell surface heterodimers on antigen-presenting cells. The genes are located on the short arm of chromosome 6. The sequences are highly polymorphic, localized in the second exon of the genes. Detailed sequencing of the variable regions of the class II genes has led to a practical approach to HLA typing.

Erlich and colleagues have utilized a panel of oligonucleotide probes to detect and classify class II HLA-DR, -DQ, and -DP alleles [17]. The loci are initially amplified by PCR with conserved locus-specific primers and the PCR product tested with an allele-specific probe panel in either a dot blot or a "reverse" dot blot format. In the dot blot format the amplified product is immobilized and tested separately with each biotin-labelled probe. In the reverse format the panel of probes is immobilized as separate dots on a nylon membrane, and then hybridized with the biotin-labelled PCR product. The novel patterns of reactions with allele-specific probes serve to specify the HLA individual type. Currently four class II loci have been typed as summarized in Table 2.

The major advantages of sequence based HLA genotyping over conventional serotyping are: 1) the nature and location of the polymorphism is identified; 2) the exact sequence differences in two alleles are identified; and 3) new alleles can be identified as population genetics are expanded. The procedural difficulties and the need for a large library of specific probes and primers, currently the order of 100, are major disadvantages for general use of genetic HLA typing.

Parentage and forensics testing

Variations in DNA sequences are unique to each individual. When the primary base sequence is known differences may be detected with hybridization probes that will bind through base pairing if there are no mismatches in the sequences. A more practical way of distinguishing two DNA molecules is based on polymorphism reflected in the restriction fragment length polymorphism (RFLP). Restriction enzymes cut human DNA into millions of small fragments that give a unique individualized pattern upon sizing in electrophoretic gels and Southern blotting. In 1985, Jeffreys and colleagues reported minisatellite arrays in non-coding regions of human DNA that exhibit polymorphism due to a hypervariable number of tandem repeat units of short 15-75 base pairs (VNTR) [18,19]. The loci are extremely polymorphic with potentially hundreds of alleles at a single locus. Figure 3 illustrates VNTR in which two alleles have a different number of repeats between restriction enzyme sites, giving rise to two bands detected by a specific probe in a Southern blot. Figure 4 illustrates a hypothetical test for paternity in which two proves were used to identify bands corresponding to four alleles. The VNTR patterns of the mother-child-father can exclude parentage with certainty. If there is no exclusion, statistical calculations are performed to determine the probability of biological parentage. The probability of chance matching of VNTR DNA "fingerprints" has been controversial. How-

ever, as the population data bases have expanded recent calculations suggest that chance matching of three to five locus patterns is extremely rare, and may be unique [20].

Table 3 compares DNA typing with other widely used serological methods used for parentage testing [21]. The cumulative power of exclusion of an alleged parent was calculated for each test system. The exclusionary power of DNA typing far exceeds that of standard serological systems for parentage testing.

Table 3. Comparison of the cumulative power of exclusion in typical parentage testing systems.*

Test system	Probability of exclusion
DNA typing	99.5% - 99.9%
HLA serotyping	87%
Red cell enzymes + serum proteins	85% - 88%
Red cell surface antigens	61% - 72%
HLA serotyping + red cell surface antigens	98%

* The cumulative power of exclusion is a calculated value for the likelihood of an alleged parent being excluded by genetic matching with each test system [21].

DNA fingerprinting using VNTR and HLA typing has become an important tool in forensics. Matching a suspect's DNA with DNA found in biological specimens at the scene of the crime can provide evidence for guilt or innocence, and a large amount of case law is being generated in several countries. There has been controversy in both the technical and legal professions, but generally the fundamental technology is not challenged, but rather the proficiency of the testing laboratory. In addition, data banking of individual DNA profiles for identification purposes brings with it a host of ethical and civil liberties issues.

Genetic testing and cancer

It has been estimated that 3500 hereditary diseases are caused by modification of only one gene [22]. The specific molecular defect is known for about 200 of them. Genetic diseases are typically diagnosed by phenotype or by gene product analyses for modified proteins [23,24]. It is now possible to detect many genetic defects using allele-specific probes, combined with platelet amplification if required. The genetic determinants are well established for sickle-cell anemia, thalassemia, phenylketonuria, α-1-antitrypsin deficiency, Duchenne/Becker muscular dystrophy, x-chromosome-dependent mental deficiency, Huntington's chorea, and cystic fibrosis. Gene diagnosis can be important in prenatal counseling and in accurately discriminating diseases with widely differing consequences.

Specific mutations, deletions, duplications, recombinations, translocation or rearrangements of cellular DNA are known to be associated with various forms

of cancer. The techniques of molecular diagnostics are clinically useful to identify the nuclear changes and to type the specific cancer cell. Some forms of cancer appear to be caused by an over expression of genes that normally regulate cell growth, "oncogenes" [25,26]. In these cells the oncogene mRNA levels are elevated, which can aid diagnosis. Monitoring changes in oncogene mRNA levels may in turn aid prognosis. Analyses of DNA extracted from cancer cells or in situ hybridizations with fluorescent probes are widely used in large oncology centers as diagnostic aids and in guiding therapeutic decisions.

Summary

Molecular diagnostics are available for a wide variety of applications in the clinical laboratory: infectious diseases, genetic and hereditary diseases, cancer mutations, recombinations and rearrangements, HLA typing, parentage testing, forensics applications, and data banking of DNA fingerprints. These and other uses are expected to continue to grow, but will be limited until current protocols and techniques are replaced with simplified and automated procedures. Current testing protocols require highly trained technical staff and several hours or days for definitive results. Compared to routine serologic or biologic assays molecular diagnostics are relatively expensive.

A major advantage of molecular diagnostics is a high degree of specificity. However, compared to serologic assays, direct molecular assays are generally less sensitive in terms of analyte concentration. Several biochemical enzyme systems permit geometric amplification of the target nucleic acid prior to testing, resulting in an overall exceptional analyte detection sensitivity for molecular diagnostics.

Finally, molecular diagnostics have been used mainly in basic research. For routine use in clinical laboratories it will be important for the scientific community to set guidelines and standards plus some form of certification of laboratories on both a national and international basis. This will contribute to growth of molecular diagnostics both in the clinical and the medical-legal setting.

References

1. Gillespie D, Spiegelman S. A quantitative assay for DNA hybrids with DNA immobilized on a membrane. J Mol Biol 1965;12:829-42.
2. Saike RK, Scharf S, Faloona F, et al. Enzymatic amplification of β-globulin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 1985;230:1350-4.
3. Erlich HA, Gelfand DH, Saiki RK. Specific DNA amplification. Nature 1988;331: 461-2.
4. Mullis KB, Faloona FA. Specific synthesis of DNA in vitro via a polymerase catalyzed chain reaction. Methods Enzyol 1987;155:335-50.
5. Erlich HA, Gelfand D, Sninsky JJ. Recent advances in the polymerase chain reaction. Science 1991;252:1643-50.
6. Saiki RK, Gelfand DH, Stoffel S, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988;239:487-91.

7. Birkenmeyer LG, Mushahwar IK. DNA probe amplification methods. J Virol Meth 1991;35:117-26.
8. Backman K. Method for detecting a target nucleic acid sequence. European Patent Office – 320,308. Priority, December 11, 1987.
9. Backman K, Carrino JJ, Bond SB, Laffler TG. Improved method of amplifying target nucleic acids applicable to both polymerase and ligase reactions. European Patent Office – 439.182A. Priority, January 26, 1990.
10. Guatelli JC, Whitfield KM, Kwoh DY, Barringer KJ, Richman DD, Gingeras TR. Isothermal in vitro amplification of nucleic acids by a multienzyme reaction modeled after retrovirus replication. Proc Natl Acad Sci USA 1990;87:1874-8.
11. Lizardi PM, Guerra CE, Lomeli H, Tussie-Luna I, Kramer FR. Exponential amplification of recombinant-RNA hybridization probes. Biotechnology 1988;61:1197-202.
12. Mifflin TE. Control of contamination associated with PCR and other amplification reactions. Clin Chem News 1992;18 No 8:8-15.
13. Kwok S, Higuchi R. Avoiding false positives with PCR. Nature 1985;318:69-73.
14. Landini MP, Trevisani B, Guan MX, Ripalti A, Lazzarotto T, LaPlaca M. A simple and rapid procedure for the direct detection of cytomegalovirus in urine samples. J Clin Lab Analysis 1990;4:161-4.
15. Gillespie DH. The state of the art of clinical gene diagnosis. J Clin Lab Analysis 1987;1:42-51.
16. Schlauder GG, Leverenz GJ, Mattsson L, Weiland O, Mushahwar IK. Detection of hepatitis C viral RNA by the polymerase chain reaction in serum of patients with post-transfusion non-A, non-B hepatitis. J Virol Med 1992;37:189-200.
17. Erhrlich H, Bugawan T, Begovich AB, et al. HLA-DR, DQ and DP typing using PCR amplification and immobilized probes. Eur J Immunogenetics 1991;18:33-55.
18. Jeffreys AJ, Wilson V, Thein SL. Hypervariable minisatellite regions in human DNA. Nature 1985;314:67-73.
19. Jeffreys AJ, Wilson V, Thein SL. Individual specific fingerprints of human DNA. Nature 1985;316:76-80.
20. Risch NJ, Devlin B. On the probability of matching DNA fingerprints. Science 1992; 255:717-20.
21. Walker RH (ed). Inclusion probabilities in parentage testing. International Conference on Parentage Testing. American Association of Blood Banks, Arlington VA 1993.
22. Stanbury JB, Wyngaarden JB, Fredrickson DS, Goldstein JL, Brown MS (eds). The metabolic basis of inherited disease (5th ed). McGraw-Hill, New York 1983:39-59.
23. Ellis R (ed). Inborn errors of metabolic diseases. Elsevier/North Holland, New York, Amsterdam, 1980.
24. Davies KE (ed). Human genetic diseases. A practical approach. IRL Press, Oxford, Washington 1986.
25. Land H, Parada LF, Weinberg RA. Cellular oncogenes and multistep carcinogenesis. Science 1983;222:771-5.
26. Nau MM, Brooks BJ, Battey J, et al. L-myc, a new myc-related gene amplified and expressed in human small lung cancer. Nature 1985;318:69-73.

DISCUSSION

L. de Leij, P. Rebulla

L. de Leij (Groningen, NL): Dr. Huisman, I was intrigued about the false-positive results, which you got with the sera you had from patients with Sjögren syndrome. You promised us to tell something about the possibilities, that the false positive results were due to some antibodies which might be present in the serum of those patients or persons. But you have not come to that point in your presentation. May be you can do it now.

J.G. Huisman (Amsterdam, NL): What I tried to demonstrate was that p24 only reactors do have antibodies reacting with an epitope(s) which are exposed on the p24 protein, as soon as the protein is immobilized on a solid phase, irrespective it is an ELISA plate or a Western blot strip. For that reason we think that there should be a common epitope that is recognized by all of these sera. We were able to analyse these sera in the so-called PEPSCAN-analysis. In this technique overlapping nonapeptides are synthesized starting with the first amino-acid of the protein to amino-acid 9 and then from amino-acid 2 to amino-acid 10 etc.

What we observed is that a number of sera recognized a sequence, two of these sera even very strong, which has a homology to Herpes simplex virus type 1 – envelope protein. We also performed the experiment the other way around and looked in sera of proven HSV infected individuals, but negative for HIV, for antibodies to p24 of HIV, and indeed we found some sera reacting with p24 and they also react with the same sequence in the PEPSCAN analysis. Thus, for a significant number of p24 reactors we have identified the agent that could give rise to a non-specific antibody response.[1]

T.H. The (Groningen, NL): Dr. Chatila, I admired your presentation but as perhaps an oldfashioned immunologist the definition of the superantigen would be that it is no antigen for me.

1. Langedijk JPM, Vos WFHM, van Doornum GJJ, Rotman HAJ, Meloen RH, Huisman JG. Identification of cross-reaction epitopes recognized by HIV-1 false positive sera. AIDS 1992;6:1547-8.

T.A. Chatila (Boston, MA, USA): Like any new term, the term superantigen evolved from a peculiar background during '88, '89 as it was picking up. It was the term first quoted by Philip Amard in Denver. We had to get up to it. It has advantages and disadvantages. We really are not dealing with an antigen in the sense that these toxins, these products are activating T-cells and then particular Vβ product antigens; when the peptide antigens bound MHC, it interacts not only with Vβ sequence but also with Vα and the D and G sequences. I think it will have to be left to the historical legacy as it is. I guess if the person understands just what the D means in particular.

T.H. The: With respect to the question you posed to us about graft-versus-host in viruses in the framework of again these superantigens, I could not keep that question.

T.A. Chatila: I will repeat that. You can have two unrelated individuals, perfectly matched for MHC, that have the same DR, DQ, DP, MHC plasma molecules. Yet, if we assume that humans have retroviruses that have integrated in the genome now just like the mice, that may be the case, that may not be the case, but we cannot dismiss it. These retroviruses in mice make proteins which are superantigenic, come to the surface, combine the RBC and stimulate their own T-cells in a way like bispecific manner. When a human is born or a mouse for that matter is born with those proteins, the reactive T-cell is deleted to the finals. But if you have 30 or 40 viruses and each human being has a different virus or a combination of viruses integrated? What happens if I have a virus, a virus that your immune system has never seen before, but you may have some T-cells that would react to my viruses. So, those two individuals who were completely matched for MHC, are not matched for their virus integrants. Suppose we are going to identify the individual, I give you a graft for example. If I have the wrong virus integrant, your T-cell and my T-cell may come to react and you have a graft-versus-host disease. It is a parallel in the MHC system. That is the complexity of it.

T.H. The: In clinical practice it is well known, that certain viral infections upregulate transplant rejections as well as graft-versus-host and one of these are cytomegalovirus infections. Persistent blood born viral infections and monocytes, antigen presenting cells are involved in that. Is that a bit of help to your question?

T.A. Chatila: It might, some agents that can induce what infections can increase the graft-versus-host disease. Experimental animals, that are kept in a environment free of germs, have very much less percentage onset of graft-versus-host disease in transplant. In the case of cytomegalovirus it is possible that CMV can draft for a superantigen but you know it is still to be invested.

S. Stienstra (Nijmegen, NL): Dr. Huisman, you presented two slides of a patient W and you had on the X-axis a point O. During the presentation I thought you said that every two days you had a sample of this patient, but I only saw a few crosses along the lines of the slide.

J.G. Huisman: Yes indeed, there were two days intervals of this patient. We have analyses of ELISA, HIV-p24 antigen and HIV-RNA of each interval, resulting in the profile which I have shown. But I did not put all the data in the figure.

S. Stienstra: Yes, I was asking this because between day –9 and 0, you had no data indicated and I was wondering why.

J.G. Huisman: Well, I have all the data, but I did not include them all, because the message remains the same. The point that I would like to make was that there is a slight difference in sensitivity between the third and the second generation antibody test, in favour to the third generation test. Presumably two weeks difference. The sensitivity between the third generation antibody test and HIV antigen test in detecting HIV infection is probably a few days. We could not make a more explicit statement because the moment of primary infection is unknown. The difference in sensitivity between the HIV antigen test and the HIV-RNA detection is again a few days.

S. Stienstra: Dr. Overby and dr. Huisman, there is now coming a new simple way to perform PCR reaction from a Swiss company. They are even going to try this test in a blood bank screening situation in The Netherlands. At the protein level Pharmacia tried to make a combination of parameters, but they did not come into existence, because of the fact that the sensitivity was a problem. PCR is quite another technique and you might imagine that with more probes you could make a safety test for blood banking. It does not matter whatever is positive, it might be HIV, HCV or HIV-1 or whatever you want, you can do it in one test and get a positive or negative result for safer transfusion. How do you think about that?

L.R. Overby (Alamo, CA, USA): I think that doing a one step, one tube type test would be possible, but it would not eliminate false positive results. The blood bank laboratory will have a major responsibility in terms of training personnel and having well contained laboratories in which to do PCR. I think it is possible to do a one step, one tube PCR test, but we have a long way to go to make it more practical for everyone.

J.G. Huisman: Well, I will give you my opinion about that issue. It is of course very attractive to have all the primers for the different retroviruses, or whatever you want to test, in one testtube. But I think it is quite impossible to control all the amplification reactions. We have already experienced with this methodology although in a more simple format in trying to amplify one retroviral sequence

together with the amplification of a marker gene like albumin, the latter as a check for the presence of DNA. What we observed was that all combinations can occur, indicating that it is very difficult to control the different kinetics of each amplification set. I think the most difficult problem is the quality of the template (DNA or RNA) in a multi PCR. Sometimes you have to optimize for a particular primer set, which is not applicable for another primer pair. I think this is a very difficult problem.

S. Stienstra: So, that is another disillusion perhaps for combination kits. Do you have also a general opinion on using PCR in screening?

L.R. Overby: Well, a bias rather than an opinion. I do not see PCR replacing serological screening in the blood bank. Once the serology is well implemented I do not believe, with the background we have now, that we would want to transfuse the blood of an HCV or HIV seropositive donor, even if it was PCR negative.

M. van Geffen (Bilthoven, NL): Dr. Huisman, I have been tempted to raise a question. You showed us some very high-teck methodological, sequential techniques in the elucidation of the false positives of HIV and possible also the positive serological individuals. What is the relevance of these confirmatory testings of false positives and possibly also the false negatives in the background, seeing that there are still window plasma pools used for factor VIII concentrates and other blood products.

J.G. Huisman: I will give you my answer as a scientist not as a politician. What we like to understand was the nature of the atypical reactions, although it seems to be a minor problem. Our knowledge might help manufacturers to change the methodology and/or to improve their reagents. We have now identified some cross-reacting epitopes within the HCV genome homologous to p24 of HIV-1. Maybe it is worthwhile to exclude these sequences from the recombinant p24 protein, this to avoid false positive reactions in the HIV-serology. This could also avoid traumatic councilling of these donors.

M. Van Geffen: And what is your comment on certain window plasma and to plasma pools in this ever more sensitive testing of the plasma tools.

J.G. Huisman: The open window phase study is a very old subject. It started three years ago with rumours, that in a San Francisco cohort study, PCR positive signals were observed in high risk individuals 42 months before their seroconversion, which is in fact a fascinating observation. If this is true we should re-evaluate our whole screening strategy. Fortunately, it turns out that PCR sample to sample contamination was the reason for this result. From our own studies in the Amsterdam cohort it turns out that PCR positive results appear within three months after primary infection. This is also confirmed by others. If we extrapo-

late this observation of the open window phase to the low risk population, like blood donors, then a good antibody test in combination with an HIV-antigen test is a good approach to identify every infected individual.

J. Semple: Dr. Huisman, are there probes available in your PCR technology that are specific for other regions, such as the envelope gene of the HIV genome. Can you look at subtypes of the virus in some of these HIV positive patients?

J.G. Huisman: Yes. There are several reasons to introduce the PCR to analyse envelope sequences, One important reason is that we know that in the pathogenesis of AIDS more HIV strains are involved such as latent or slow replicating viruses and virulent strains. We know that these biological different strains of HIV, differ significantly in the envelope region. You can imagine that envelope proteins from a virulent strain have a higher affinity to the receptor of the target cell. Using PCR it is possible to perform a kind of "serotyping" to identify the phenotype of the virus that is circulating in the host.

J. Semple: In terms of probes in the envelope region, for example, you can correlate those to anybody specificities. So presumably, if there is a good correlation and there is a new variant that arises, you should be able to pick that out or screen with the PCR.

J.G. Huisman: This is correct, we know for sure, using PCR that some envelope regions are expressed to which no antibodies are produced, but this subject is too complicated to discuss.

A.C.J.M. Holdrinet (Breda, NL): Dr. Overby, I understood that HCV RNA is the most sensitive indicator for infectivity and you showed some information, where it disappeared for some time and came back. Do you believe in that PCR window in which the patient is contagious? Is that demonstrated?

L.R. Overby: Yes, there are examples of HCV RNA appearing, disappearing and then reappearing. I did not have a slide, but there are a great deal of data showing HCV RNA very early after HCV infection, long before any evidence for ALT elevations or seroconversions. The RNA may disappear and then reappear later. As dr. Huisman mentioned, retrospective studies are done on specimens in the freezer, taken every few weeks or every few months. This makes it very difficult to get a consistent time profile. I think viremia is present throughout the course of infection, and the patient is potentially contagious even though viremia is below the PCR detection threshold.

C.Th. Smit Sibinga (Groningen, NL): A comment and a question. Dr. Houwen, I would like to mention the study of the BEST working party of the ISBT. In a comparative study to count very low white cell residuals in standardized products we came to the conclusion, that the flow cytometry is really not the way to

go for routine application. We should shift away from that again and come back to counting chamber technology in a standardized fashion. So, I think here the fashion ability of the machine and the technology really blinded us in the very beginning and we came back to reality. In the very last part on the quantitative aspect you mentioned the application, which is rapidly gaining interest in helping us to establish the quality in terms of what types of cells are being collected in specifically peripheral stem cell collections. Flow cytometry at least is a more easy and more fast technique than the culturing technology to know whether you have really harvested proliferative cells. The same applies to the identification of the various types of white cells in a lymphocyte collection as a start to know whether you have the right cells for the propagation and interleukin stimulation. I think there is a real application of flow cytometry.

B. Houwen (Loma Linda, Ca, USA): I do not think enumeration of stem cells as a possible application of flow cytometry technology was missing from my presentation: It was indicated in one of my tables on the quantitative aspects of flow cytometry. However, because of time constraints I did not elaborate on this application. The discovery of CD34 as a marker for highly immature hematological precursor cells serves as an illustration as to how technology evolves. In the early days of bone marrow transplantation nucleated cells were counted in the harvested material, either in a counting chamber or electronically, while sometimes bone marrow as well as peripheral blood progenitor cells were cultured in vitro to assess the numbers of progenitor cells present. Currently labelling of cells with antibodies directed against CD34 provides an excellent method for the quality assurance of bone marrow and peripheral blood stem cell harvests.

C.Th. Smit Sibinga: Thank you. Dr. Chatila, in the second part of your presentation you came to the point, where following the signal transducing receptor stimulation the production of cytokines is generated which may relate to graft-versus-host. Could it be that in the near future you might come to a cytokine PCR as an early diagnostic tool to come to GvHD; even prevention and follow up of treatment. Could that be foreseen?

T.A. Chatila: Using PCR for cytokine quantitations now can be done in many laboratories still on an experimental basis. I would say yes, it can be done. I think issues relating to sensitivities and specificities are something to be kept in mind. Cytokines have now come up as a therapy for graft-versus-host disease and as an inhibitor of TNF function.

IV. CLINICAL IMMUNOLOGY AND BLOOD TRANSFUSION

PROSPECTS AND LIMITATIONS OF ADOPTIVE IMMUNOTHERAPY AND GENE THERAPY

M. Cottler-Fox

Introduction

While the concept that tumours might express antigens which could be used as targets for their destruction was discussed early in this century [1], it was not until almost 50 years later that experimental support for the idea appeared in studies on rats and mice [2,3]. Evidence that human cancers could also invoke humoral and cell-mediated immune responses was finally provided in the 1960s [4,5]). By the early 1980s several factors had combined to encourage new approaches to the immune manipulation of cancer. First, progress had been made in understanding the cellular immune response to antigens presented in conjunction with major histocompatibility complex (MHC) molecules. Second, advances in molecular biology had made it possible to obtain large quantities of cytokines, the molecules which regulate the immune system. Third, apheresis and cell culture techniques had improved, making it possible to collect large numbers of autologous lymphocytes and expand them in culture. This paper will review the results of adoptive immunotherapy to date as a means of exploring its limitations, and then focus on current areas of research designed to improve or expand its clinical utility, including gene therapy.

IL-2 and lymphokine activated killer (LAK) cells

The concept of adoptive immunotherapy as initially applied by Rosenberg and colleagues meant the infusion of autologous lymphocytes after in vitro stimulation with IL-2 [6]. These LAK cells, generated by incubation of lymphocytes in IL-2, were non-B, non-T cells capable of lysing fresh tumour cells in a non-MHC restricted fashion [7,8]. Extensive studies in animal models demonstrated that IL-2 or IL-2 plus LAK cells could mediate the regression of established lung and liver metastases in a dose related fashion, with the combination being generally more effective than IL-2 alone [9]. It was also shown that continued exposure to IL-2 allowed activated cells to continue to expand and maintain anti-tumour activity in vivo [10]. During IL-2 infusion LAK cell precursors in the blood decreased, only to increase substantially after it was stopped. These basic observations led to a protocol in humans with advanced cancers beginning

in November 1984 in which IL-2 was administered, LAK cells were collected by repeated aphereses, and the LAK cells were then infused followed by bolus IL-2. Later, high dose IL-2 alone was added to the protocol without LAK cells. A total of 320 patients were treated, of which 20 achieved a complete remission (14 with LAK/IL-2, 6 with IL-2 alone), with 9 remaining continuously free of disease from 13 to 75 months as of March 1991. A number of patients also achieved a partial response (29 with LAK/IL-2 and 15 with IL-2 alone) [11]. While these results in patients with advanced renal carcinoma, melanoma, colorectal carcinoma and non-Hodgkin's lymphoma were encouraging, it was obvious that further improvement was needed. It was particularly worrisome that bolus high dose IL-2 was associated with significant side effects, chief among which were hypotension requiring pressors and a capillary leak syndrome leading to fluid and colloid accumulation in visceral organs and soft tissues. Anemia, thrombocytopenia and sepsis were also significant problems [12,13].

Tumour-filtrating lymphocytes (TIL)

The search for a cell of the immune system with improved anti-tumour effect led to the description in 1986 of tumour-infiltrating lymphocytes [14]. TIL are those lymphocytes which can be grown from single-cell suspensions of tumours by culture in IL-2. As with other classic T cells, TIL are MHC restricted, and also recognize cancer-associated antigens [15]. Secretion of interferon gamma by murine CD8+ TIL cocultured with tumour correlates well with in vivo anti-tumour effect [16]. Human TIL can be either CD4+ or CD8+ T cells. A phase II trial was initiated using TIL with high dose IL-2 in patients with advanced melanoma [17]. In this trial response rates as high as 50% were seen in patients (who had previously failed to respond to IL-2) given TIL/IL-2 plus cyclophosphamide to inhibit possible natural host "suppressor" responses to the immune therapy. However, small numbers of patients were treated, few complete responses were seen, and the toxicity of IL-2 was still significant.

Improving LAK and TIL therapy

A number of potential improvements are currently being investigated in efforts to improve adoptive immunotherapy of cancer. LAK cells are potent mediators of antibody dependent cellular cytotoxicity, and it may therefore be possible to improve their killing ability by combining them with monoclonal antibodies specific to a given tumour or to a general class of tumour. Recently, genes encoding antigens recognized by MHC-restricted tumour-specific T cell clones have been identified [16,18], suggesting the possibility of generating cytotoxic T cell clones (CTL) in vitro as a form of tumour therapy. Alternatively, antigen presenting cells may be exposed to tumour-specific antigen in vitro and then used to boost LAK or TIL cell function either in vitro or, possibly, in vivo. Cytokines other than IL-2 are also capable of stimulating immune activity, and it

is possible that exposure of LAK or TIL in vitro or in vivo to these cytokines may improve their anti-tumour effects [19,20].

Isolation of more effective anti-tumour cells may be possible if cells are collected from tumour-draining lymph nodes after pretreatment of the host with cytokines [11] or after increasing immunogenicity of tumour cells by genetically altering them to express foreign genes [21]. Finally, the synergy of adoptive immunotherapy with radiation or chemotherapy remains to be investigated.

Broadening the concept of adoptive immunotherapy

Although a concept originally developed with reference to cancer adoptive immunotherapy has been redefined more recently as the transfer of educated immunocompetent cells into a host with the goal of mediating a desired immune response [22]. Thus, viral infections associated with lack of an adequate host immune response become likely target for adoptive immunotherapy. The first such effort, against cytomegalovirus (CMV), is based on two pieces of data: first, that adoptive transfer of CMV-specific T cells into immunosuppressed mice protects against viral challenge and is effective against established pneumonia; and second, that human CMV-infected patients with clinical disease usually have impaired virus-specific T cell responses. Results from 3 CMV seropositive marrow transplant patients who received CMV-specific CD8+ CTL clones generated and expanded in vitro from the MHC identical marrow donor have been reported to date [23]. No toxicity was seen, the CTL persisted for at least one month post-infusion, and no patient developed CMV viremia or pneumonia. While larger numbers of patients will need to be treated before a definitive evaluation of this therapeutic intervention can be made, successful reconstitution of CMV immunity in marrow transplant recipients suggests that similar results may also be possible in solid organ transplant and in patients with HIV disease. Indeed, a protocol looking at genetically modified CD8+ HIV-specific T cells for HIV seropositive patients undergoing allogeneic marrow transplant is in progress in Seattle [24], while Koenig and colleagues at the NIH are using autologous HIV-specific CTL clones expanded in vitro.

Just as the combination of physical cell separation methods with monoclonal antibodies has given us the ability to isolate and expand CTL, it is also possible to prepare populations of helper and suppressor T cells. In this way it may eventually be possible to use adoptive immunotherapy as an approach to therapy of human autoimmune diseases.

Gene therapy

Gene therapy was originally envisioned as a procedure for treating genetic diseases and malignancy. However, the earliest gene transfer protocol actually evolved as an offshoot of adoptive immunotherapy, as gene marking seemed likely to be a good method for tracking TILs [11]. Lack of side effects or pathology attributable to the gene transfer established that it was possible to transfer a

gene safely into human cells using a retroviral vector. This success, and the ability to collect large numbers of lymphocytes by apheresis with expansion in vitro led to protocols involving the transfer of genes to stimulate immunity against or otherwise cause destruction of tumour cells. The first such protocol involved transfer of the gene for tumour necrosis factor (TNF) into TIL in the belief that local secretion of this cytokine by cells which had trafficked to the tumour would enhance destruction. The genes for TNF and IL-2 have also been transferred into tumour cells injected subcutaneously, in an effort to stimulate specific immune response, resulting either in tumour destruction at other sites of disease or in collection of more effective TIL from lymph nodes draining the site of injection. It is anticipated that it will take until 1993 to accrue the 50 patients required for data analysis of this protocol, but to date there have at least not been untoward effects of the therapy [11].

The first human gene therapy trial for correction of a genetic defect involves treatment of adenosine deaminase (ADA) deficiency by transfer of the ADA gene into patients' T cells, the cells most affected by the disorder. ADA deficiency leads to high levels of 2'-deoxyadenosine in the circulation which is toxic to both B and T cells and thus results in severe combined immunodeficiency. ADA deficiency can be treated by marrow transplantation or by injections of polyethylene glycol conjugated to bovine ADA (PEG-ADA). However, only about one third of such patients have marrow donors and PEG-ADA is painful to receive, expensive, and not completely effective.

In the NIH ADA deficiency trial, two patients receiving PEG-ADA also receive infusions of their own cells transduced with a retroviral vector expressing human ADA and neomycin as a selectable marker. Immune function in both patients has improved significantly by all markers studied, including isohemagglutinin titers, skin tests for antigen sensitivity, and cytotoxic T cell assays. The ADA level in circulating cells has increased and lymphocyte counts have gone from below normal with only PEG-ADA into the normal range. Interestingly, a large number of corrected T cells persisted in the first patient for over six months after cell infusions were stopped for a period of observation. No toxicity other than mild fever responsive to acetaminophen has been noted in this trial [25]. A similar protocol utilizing marrow-derived hemopoietic precursors is currently underway in Italy, and a protocol for ADA correction of peripheral blood-derived hemopoietic stem cells at NIH will start soon.

The role of transfusion medicine in adoptive immunotherapy and gene therapy

An increased understanding of hemopoiesis and cytokine growth factors have combined with cell culture and cell separation science to create novel new blood components for innovative uses [26]. Transfusion medicine services, sources of expertise in sterile cell collection, separation, storage and infusion, will continue to be partners in developing adoptive immunotherapy and gene therapy. Participation in the development of the next generation of blood components, which

will be patient-specific rather than generic, is an opportunity no member of the transfusion medicine community will want to miss.

References

1. Himmelweit B (ed). The collected papers of Paul Ehrlich. Oxford, Pergamon Press, 1957.
2. Foley EJ. Antigenic properties of methylcholanthrene-induced tumours in mice of the same strain origin. Cancer Res 1953;13:835-7.
3. Prehn R, Main D. Immunity to methylcholanthrene-induced sarcomas.J Nat Cancer Inst 1957;18:769-78.
4. Hellstrom I, Hellstrom KE, Pierce GE, Yang JPS. Cellular and humoral immunity to different types of human neoplasms. Nature 1968;220:1352-4.
5. Herberman H. Cell-mediated immunity to tumour cells. Adv Cancer Res 1974;19: 207-63.
6. Lotze MT, Line BR, Mathisen DJ, et al. The in vivo distribution of autologous human and murine lymphoid cells grown in T cell growth factor (TCGF): Implications for the adoptive immunotherapy of tumors. J Immunol 1980;125:1487-93.
7. Grimm EA, Ramsey KM, Mazumder A, et al. Lymphokine-activated killer cell phenomenon. II. Precursor phenotype is serologically distinct from peripheral T lymphocytes, memory cytotoxic thymus-derived lymphocytes, and natural killer cells. J Exp Med 1983;157:884-97.
8. Roberts K, Lotze MT, Rosenberg SA. Separation and functional studies of the human lymphokine-activated killer cell. Cancer Res 1987;47:4366-71.
9. Lafreniere R, Rosenberg SA. Adoptive immunotherapy of murine hepatic metastases with lymphokine-activated killer cells and recombinant IL-2 can mediate the regression of both immunogenic and non-immunogenic sarcomas and an adenocarcinoma. J Immunol 1985;135:4273-80.
10. Ettinghausen SE, Lipford EH, Mule JJ, et al. Systemic administration of recombinant interleukin-2 stimulates in vivo lymphoid cell proliferation in tissues. J Immunol 1985;135:1488-99.
11. Rosenberg SA. The immunotherapy and gene therapy of cancer. J Clin Oncol 1992; 10:180-99.
12. Lotze MT, Matory YL, Rayner AA. Clinical side effects and toxicity of interleukin-2 in patients with cancer. Cancer 1986;58:2764-72.
13. Ettinghausen SE, Moore JG, White DE, et al. Hematologic effects of immunotherapy with lymphokine-activated killer cells and recombinant interleukin-2 in cancer patients. Blood 1987;69:1654-60.
14. Rosenberg SA, Spiess P, Lafreniere R. A new approach to the adoptive immunotherapy of cancer with tumor infiltrating lymphocytes. Science 1986;233:1318-21.
15. Hom SS, Topalian SL, Simoni ST, et al. Common expression of melanoma tumor-associated antigens recognized by human tumor-infiltrating lymphocytes: Analysis by HLA restriction. J Immunother 1991;10:153-64.
16. Barth RJ, Mule JJ, Spiess PJ, et al. Interferon gamma and tumor necrosis factor have a role in tumor regression mediated by murine CD8+ tumor-infiltrating lymphocytes. J Exp Med 1991;173:647-58.
17. Rosenberg SA, Packard BS, Aebersold PM, et al. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma, special report. N Engl J Med 1988;319:1676-80.
18. Darrow TL, Slingluff CL, Seigler HF. The role of class I antigens in recognition of melanoma cells by tumor-specific cytotoxic T lymphocytes: Evidence for shared tumor antigens. J Immunol 1989;142:3329-35.

19. Kawakam Y, Rosenberg SA, Lotze MT. Interleukin-4 promotes the growth of tumor-infiltrating lymphocytes cytotoxic for human autologous melanoma. J Exp Med 1988;168:2183-91.
20. Mule JJ, McIntosh JK, Jablons DM, et al. Antitumor activity of recombinant interleukin-6 in mice. J Exp Med 1990;171:629-36.
21. Rosenberg SA, Aebersold P, Cornetta K, et al. Gene transfer into humans – immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. N Engl J Med 1990;323:570-8.
22. Greenberg P, Goodrich J, Riddell S. Adoptive immunotherapy of human cytomegalovirus infection: Potential role in protection from disease progression. Transplant Proc 1991;23:97-101.
23. Riddell SR, Watanabe KS, Goodrich JM, Li CR, Agha ME, Greenberg PD. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. Science 1992;257:238-41.
24. Riddell SR, Greenberg PD, Overell RW, et al. Phase I study of cellular adoptive immunotherapy using genetically modified CD8+ HIV-specific T cells for HIV seropositive patients undergoing allogeneic bone marrow transplant. Hum Gene Ther 1992;3:319-38.
25. Miller AD. Human gene therapy comes of age. Nature 1992;357:455-60.
26. McCullough J. A new generation of blood products. Transfusion 1992;32:299-301.

CLINICAL STRATEGIES FOR THE PREVENTION OF PLATELET ALLOIMMUNIZATION

E.J. Lee

The use of platelets for transfusion has increased dramatically over the past 20 years since the use of chemotherapy has become a routine treatment for patients with cancer. Within the most recent decade, the application of bone marrow transplantation to many malignancies not previously treated with this modality, and the identification and production of large quantities of human cytokines have resulted in larger numbers of patients receiving higher doses of chemotherapy. Although the cytokines currently used are potent stimulants of granulocytopoiesis and erythropoiesis, none yet discovered has been shown to stimulate production of platelets. A recent study that evaluated patterns in blood transfusion in the United States between 1982 and 1988 found that the number of red blood cell units transfused has remained the same. Over the same interval, there was a 50% increase in the number of units of platelets transfused. Indeed, the use of platelets obtained from a single donor by apheresis has grown even more rapidly, increasing from 16.8% of all platelet transfusions in 1982 to 24.6% in 1987 [1]. It seems likely that the usage of platelets will continue to increase.

The most significant clinical problem in platelet transfusion therapy is that sometimes transfused platelets do not raise the patient's platelet count. The most important cause of refractoriness to platelet transfusions is alloimmunization. Although other clinical factors such as coagulopathy and splenomegaly have been shown to be associated with poor responses to platelet transfusions [2], alloimmunization remains the most important potentially preventable cause of refractoriness. The purpose of this review is to assess whether alloimmunization can be prevented by a strategy that can be broadly applied and is cost effective.

Relatively few large series have been published that report the natural history of alloimmunization. At the University of Maryland Cancer Center, the records of 397 patients with acute myeloid leukemia (AML) who survived a minimum of four weeks were reviewed. Of these adults, 174 (44%) developed lymphocytotoxic antibody at some time during their course [3]. As suggested by an earlier study from our institution [4], these data demonstrate that a large fraction of patients with AML never become alloimmunized. Other smaller studies also report a frequency of alloimmunization between 30-60% in patients with AML [5,6]. Thus, it seems likely that 44% is a good approximation of the true incidence of alloimmunization in patients with AML. In contrast, the small number

of patients with aplastic anemia reported in the literature have a very high rate of alloimmunization [5], probably because they were not receiving concomitant myelo- and immunosuppressive treatment. Very little data have been published about the frequency of alloimmunization in patients with acute lymphocytic leukemia (ALL). Of 68 patients with ALL treated at the University of Maryland between 1975 and 1986, 12 (18%) became alloimmunized at some point during their course [3]. As this is the largest series reported in the literature, there is considerably less confidence about the true incidence of alloimmunization in patients with acute lymphocytic leukemia. Nonetheless, it seems likely that alloimmunization occurs less frequently in this group of patients compared to those with AML possibly because of the use of corticosteroids during induction chemotherapy. It is very possible that steroids further impair the immune response of the transfusion recipient.

For patients with solid tumours, the frequency with which alloimmunization occurs is unknown. Patients with solid tumours treated with standard doses of chemotherapy are a heterogenous group that infrequently require large numbers of blood products. Thus, as a large scale problem has not been perceived, serial data have not been carefully collected on this group of patients. Within the group of patients undergoing either autologous or allogeneic bone marrow transplantation, refractoriness to platelet transfusions occurs with a high frequency. The mechanisms by which refractoriness occurs in this patient population may be different in patients who do not undergo bone marrow transplantation and may include autoantibody formation. Again, large series have not been published in which the frequency of alloimmunization has been carefully reported. As bone marrow transplantation is usually not the initial therapy for most patients, other factors to be considered in these patients include prior transfusion exposure, underlying disease, and prior treatment. Therefore, the inclusion of patients with diagnoses other than AML in a trial testing a method of decreasing the frequency of alloimmunization renders interpretation of that trial more difficult.

Alloimmunization to platelets represents the immune response of the transfusion recipient to donor HLA antigens and is reflected by antibody directed at HLA class I antigens. However, exposure to HLA class I antigens, which are not expressed on human platelets, is necessary for sensitization to occur. The source of these necessary HLA class I antigens is thought to be the leukocytes that "contaminate" platelet and red blood cell transfusions. Among these contaminating mononuclear cells are the antigen presenting cells which serve as the most effective stimulus for antibody formation. Attempts to reduce the frequency of alloimmunization have, for the most part, focused on altering this interaction between donor leukocytes and recipient immune system. Three methods of preventing alloimmunization have been tested in significant numbers of patients. Most extensively studied has been leukocyte depletion to reduce the quantity of antigen/antigen presenting cells. A second method is reducing the number of different antigens to which the recipient is exposed by using only platelets obtained by apheresis from a single donor. The third method involves interfering with the function of the antigen presenting cells by irradiating the platelet pro-

duct with ultraviolet B (UV-B) light. As recently reviewed by Deeg, UV-B has been shown to abrogate the ability of mononuclear cells to serve as antigen presenting cells thus impairing the immune response of the recipient [8].

In order to provide convincing evidence of efficacy, the design of a clinical trial should give consideration to the following: 1) a prospective randomization with appropriate control arms using standard platelet products; 2) the patients participating in the trial should be newly diagnosed patients with AML because of the lack of data about the frequency of alloimmunization in patients with other diagnoses; 3) patients treated in such a trial should undergo identical chemotherapy for their disease; 4) the trial should include stratification for prior pregnancies and transfusions and should accrue enough patients to render the results statistically interpretable; 5) objective criteria for alloimmunization should be utilized, such as the development of lymphocytotoxic antibody, as well as objective criteria for platelet refractoriness; 6) patients who are immunized at presentation should be excluded; 7) all patients in such a trial should receive leukocyte-depleted red blood cell products; 8) there should be adequate quality control of the manipulation of platelet products; 9) the maneuver tested should be one that can be applied in all transfusion setting without markedly increasing the workload of the blood center.

Leukocyte depletion

Eight separate series or trials have been reported in which leukocyte depletion of blood products was done with the goal of reducing the rate of alloimmunization. Unfortunately, most of the trials reported contain significant flaws that detract from the strength of the conclusions. Two of these utilized historical controls. Brand and colleagues achieved leukocyte depletion by centrifugation producing a platelet product that contained less than 20×10^6 leukocytes per transfusion [9]. They noted that only 10% of their patients required HLA matched platelet transfusions and that an additional 21% developed lymphocytotoxic antibodies but did not require HLA matched platelet transfusion. In their earlier report, the authors compared this result to a control group in which 93% of patients ultimately became refractory to random donor platelets [10]. However, in addition to using historical controls, only 148 of 335 patients had AML. The remainder had acute lymphocytic leukemia, lymphoma, aplastic anemia or other diseases. Furthermore, the methods used to achieve leukocyte depletion in this series could not be applied easily in other settings. Nonetheless, this is a very large series with only 10% of patients becoming refractory to random donor platelets. These data strongly suggest that a reduction in the frequency of alloimmunization may be achievable with leukocyte depletion.

Saarinen and colleagues reported on 26 pediatric patients with a variety of diagnoses [11]. Only 6 had AML and 15 of the 26 underwent bone marrow transplantation. Of these 26 children, 5 received HLA matched platelets, although none were considered refractory. Lymphocytotoxic antibodies were not routinely studied. In contrast, in a group of patients used as a historical control,

13 required HLA matched platelets and 11 were considered to be refractory. The difficulties in interpreting the results of this paper are evident. The inclusion of a multitude of diagnoses, the lack of serologic confirmation, the use of an historical control and the broad range of treatments dilute the conclusion that leukocyte depletion provided great benefit to these patients.

Two additional trials provide data from which it is difficult to draw conclusions. In a prospectively randomized trial, Murphy and colleagues reported on 86 patients with acute leukemia [12]. Patients were randomized to one of 3 arms: either a control group receiving platelets from single donors obtained by apheresis; a leukocyte poor group receiving leukocyte-depleted single donor platelets; or an HLA matched group in which patients received platelets obtained by apheresis from donors matched for at least 3 of the 4 HLA A and B antigens. Both the leukocyte poor group and the HLA matched groups received filtered red blood cell transfusions, but not the "control" arm. Only 61 of the 86 patients were evaluable due to granulocyte transfusions or death. Of these 61 patients, 18 became alloimmunized with the majority of these being in the control arm (15 or 31) compared to 0 of 11 for the HLA matched arm and 3 of 19 for the leukocyte poor arm. The authors conclude that either method of manipulating platelets was superior to the control arm, but it should be noted that platelet recovery was assessed 20 hours after transfusion, rather than within one hour. Furthermore, leukocyte depletion in this trial was achieved by an additional centrifugation step or the use of the dual stage technique with the IBM 2997 cell separator, resulting in "doses" of white cells of either 220×10^6 white cells per transfusion or 90×10^6 white cells per transfusion respectively. These levels of leukocyte depletion are not in keeping with current technology using filters to remove leukocytes. Furthermore, the small number of patients in each arm of this trial renders the interpretation more difficult.

A randomized trial reported by Oksanen, et al. compared patients receiving standard platelet concentrates that contained standard doses of white blood cells in the range of 100×10^6 per unit to patients receiving filtered platelet concentrates that contained less than 1×10^6 white blood cells per unit [13]. However this study, which had a total of 31 patients overall, resulted in only 1 patient becoming alloimmunized although an additional 5 (evenly split between the two arms) developed alloimmunization with transient HLA antibodies. The title of this paper suggests that leukocyte depletion prevents alloimmunization, but statistically, 1 of 15 is not different from 0 of 16. In addition, in this relatively small trial, a number of patients with ALL or who were undergoing bone marrow transplantation were included.

The remaining four clinical trials provide data that are more difficult to dismiss. At the University of Maryland Cancer Center, Schiffer et al. used centrifugation to achieve leukocyte depletion [14]. All 98 patients on this trial had AML and all were treated with the same regimen. However, only 56 of the 98 patients entered were considered evaluable due to early death, granulocyte transfusions, or alloimmunization upon entry on study. Of the evaluable patients, 31 received standard platelet products whereas 25 patients received

leukocyte depleted products. In this study, each leukocyte-depleted platelet transfusion contained approximately 72×10^6 white blood cells per transfusion compared to 390×10^6 white blood cells per transfusion in the control group. Overall, 13 of 31 (47%) of the control patients became alloimmunized, similar to the historical observations made at this Center. In the patients receiving leukocyte-depleted platelet products, only 5 of 25 became alloimmunized. This difference, however, was not statistically significantly different ($p=.071$) and the frequency with which HLA-matched platelet transfusions were necessary was not different (6 of 31 in the control group compared to 4 of 25 patients in the leukocyte-depleted group). Although a trend is suggested by this paper, these results do not establish that leukocyte depletion reduces the frequency of alloimmunization.

With the advent of blood product filters, more substantial reductions in the number of WBC are possible. Three additional trials have since been published in the literature. Sniecinski and colleagues reported on 56 patients with a variety of diagnoses who received either standard platelet and red cell products or filtered blood products. Of the 40 considered evaluable, 23 had AML, 10 had ALL, 2 had aplastic anemia and 5 had other diagnoses. The platelet products transfused by these investigators after filtration, contained 6×10^6 white blood cells per transfusion. Of the 20 patients in the control arm, 10 (50%) developed alloimmunization, whereas, only 3 of the 20 patients in the filtered group became alloimmunized. Despite small numbers of patients, decisions to not analyze patients receiving less than 5 transfusions, and the inclusion of multiple different diagnoses, these data nonetheless suggest that a significant reduction in alloimmunization resulted from leukocyte depletion.

In a randomized study reported by Andreu and colleagues, 112 patients received either standard blood products or filtered blood products [16]. Of the 112 patients, only 69 were evaluable, but again a large number of different diagnoses were included (AML 36, ALL 19, CML 7, refractory anemia 3, lymphoma 4). Although all patients were previously untreated, this study was conducted at 4 different sites, 1 of which used only single donor platelets obtained by apheresis, whereas the other centers utilized random donor platelet concentrates. Treatment regimens varied from center to center. In this study, the dose of white cells for each platelet transfusion in the leukocyte depletion arm was 47×10^6 white cells. The results of this study showed that 11 of 35 patients in the control arm (31%) became alloimmunized, whereas, 4 of 34 patients in the leukocyte-depleted arm (12%) became immunized. Although the problems inherent in this study are obvious, nonetheless, a statistically significant difference was noted.

Van Marwijk Kooy and colleagues, conducted a prospectively randomized trial in which leukocyte depletion achieved by filtration was compared to leukocyte depletion by centrifugation [17]. The patients in the centrifugation arm received a dose of white cells of approximately 35×10^6 per transfusion, whereas the group that received platelets by that were filtered received less than 5×10^6 white blood cells per transfusion. The 68 patients in this protocol were previously untreated and all had acute leukemia. However, these investigators ex-

cluded patients with recent transfusions (n=2) and females with a history of pregnancy (n=13). All patients received identical chemotherapy. In the centrifugation group 11 of 26 (42%) patients developed HLA antibodies compared to 2 of 27 patients (7%) in the filtration group. Of the 26 patients in the centrifugation group, 12 became refractory (46%), compared to 3 of the 27 patients (11%) of the filtered group. This study is the most persuasive yet published, however, the level of leukocyte depletion achieved in the centrifugation ("control") arm in this study gave white blood cell doses similar to that achieved by Andreu and colleagues in their filtration arm, yet the rate of alloimmunization in van Marwijk Kooy's trial was 42% compared to 12% in Andreu's trial. It is reasonable to expect that similar levels of leukocyte depletion should result in a similar reduction in the rate of alloimmunization.

Of the above papers, only the reports from Schiffer and van Marwijk Kooy studied the effects of leukocyte depletion in patients with AML. The paper by Schiffer did not find a significant difference, but did not achieve the level of leukocyte depletion currently achievable. However, van Marwijk Kooy's series does not include recently transfused patients or patients with a history of pregnancy. Furthermore, this study contained only 53 evaluable patients. It therefore seems appropriate to suggest that changes in standard practice be based on reproducible experiences in larger numbers of patients.

Single donor platelets

Two randomized trials have been reported that suggest that it is possible to reduce the frequency of alloimmunization by the use of platelets obtained by apheresis from single donors. Sintnicolaas and colleagues reported on a trial in which they gave single donor platelets to 34 patients, most of whom had AML [18]. Patients received either single donor platelets or a control arm in which multiple random donor platelet units were given. Alloimmunization was not different between the two groups, although lymphocytotoxic antibody was measured after the first 2 transfusions only. However, the response to the first 2 platelet transfusions measured by platelet recovery decreased in patients receiving random donor platelets but remained the same in patients receiving single donor platelets. This relatively small study does not provide sufficient information on serology or interpret response to platelet transfusion according to defined criteria for refractoriness, and is therefore not persuasive.

Gmür et al. reported a prospectively randomized study in which patients received either a control arm consisting of random donor platelet concentrates or single donor platelets obtained by apheresis [19]. All 54 patients entered on this study had acute leukemia (47 AML, 7 ALL). In the patients receiving random donor platelets, 15 of 27 (56%) developed lymphocytotoxic antibody and 14 (52%) became refractory. Of the patients receiving the single donor platelets, 4 developed lymphocytotoxic antibody and 4 (15%) became refractory. These differences were highly statistically significantly different. Although this carefully conducted study suggests that the use of single donor platelets reduces the

frequency of alloimmunization, this study contained a relatively small number of patients within each arm.

Gmür and colleagues subsequently conducted a randomized trial in which patients with leukemia received single donor platelets that were within a standard or leukocyte-depleted apheresis product [20]. There was no difference in the rate of alloimmunization or refractoriness between the 2 arms. However, as noted by the investigators, the degree of leukocyte depletion achieved was relatively modest. Also, of interest is that 32% of the 56 patients studied became alloimmunized.

This method of reducing the frequency of alloimmunization would markedly increase the workload in blood centers where the major requirement is for red blood cells, and the relative benefit of different methods of reducing the frequency of alloimmunization has yet to be established. Therefore, it would be premature to adopt a policy of using only single donor platelets at this time.

Ultraviolet-B irradiation

A report has been published by Sherman and colleagues in which patients with leukemia received paired transfusion which were either unmodified or UV-B irradiated [21]. The results of these transfusions, as measured by platelet recovery, were similar. No comments were made in this report about the rate of alloimmunization. In abstract form, Menitove and colleagues reported on a trial in progress in which 32 patients received either UV-B irradiated platelet concentrates or control products [22]. They noted that 17% of patients receiving UV-B-irradiated platelets became alloimmunized compared to 36% of control patients. Although these results are preliminary, and a final report on this trial has yet to be published, these results were intriguing because of the ease with which UV-B irradiation can be accomplished. The use of UV-B irradiation however, requires special plastic blood bags transparent to UV-B and a standardized machine for applying the dose of light.

Summary

There are good theoretical and preclinical reasons to think that leukocyte depletion, single donor platelets or UV-B irradiation may reduce the frequency with which alloimmunization occurs. Although each study is flawed in one or more ways, the large body of data supporting leukocyte depletion suggests that it will be possible to reduce the rate of alloimmunization. Filters to remove white blood cells from platelets are convenient and reasonably easy to use. Single donor platelets would, if found to be the optimal method, considerably increase the workload of most blood centers. UV-B could be easily applied but requires a machine to deliver the light, and plastics that are transparent to UV-B.

Currently, a trial funded by the National Heart, Lung and Blood Institute (NIH, Bethesda, MD) called the trial to reduce alloimmunization to platelets (TRAP study) is being conducted. Patients are randomized to a control arm con-

sisting of pools of random donor platelet concentrates, or to one of the above three maneuvers that may reduce the frequency of alloimmunization. The intent is to accrue more than 500 patients with AML. This study, due to be completed in 1994, will provide the clearest indications yet as to the optimal method of giving platelet transfusions to minimize the frequency of alloimmunization.

References

1. Surgenor DMN, Wallace EL, Hao SHS, Chapman RH. Collection and transfusion of blood in the United States, 1982-1988. N Engl J Med 1990;322:1646-51.
2. Bishop JF, McGrath K, Wolf MM, et al. Clinical factors influencing the efficacy of pooled platelet transfusions. Blood 1988;71:383.
3. Lee EJ, Schiffer CA. Serial measurement of lymphocytotoxic antibody and response to nonmatched platelet transfusions in alloimmunized patients. Blood 1987;70:1727 -9.
4. Dutcher JP, Schiffer CA, Aisner J, Wiernik PH. Long-term follow-up of patients with leukemia receiving platelet transfusions: Identification of a large group of patients who do not become alloimmunized. Blood 1981;58:1008-11.
5. Holohan TV, Terasaki PI, Deisseroth AB. Suppression of transfusion-related alloimmunization in intensively treated cancer patients. Blood 1981;58:122-8.
6. Howard JE, Perkins HA. The natural history of alloimmunization to platelets. Transfusion 1978;18:496-503.
7. Meryman HT. Transfusion-induced alloimmunization and immunosuppression and the effects of leukocyte depletion. Transfusion Med Rev 1989;3:180-93.
8. Deeg HJ. Transfusions with a tan - Prevention of allosensitization by ultraviolet irradiation. Transfusion 1989;29:450-5.
9. Brand A, Claas FHJ, Voogt PJ, Waser MNJM, Eernisse JG. Alloimmunization after leukocyte-depleted multiple random donor platelet transfusions. Vox Sang 1988;54: 160-6.
10. Eernisse JG, Brand A. Prevention of platelet refractoriness due to HLA antibodies by administration of leukocyte-poor blood components. Exp Hematol 1981;9:77-83.
11. Saarinen UM, Kekomaki R, Siimes MA, Myllyla G. Effective prophylaxis against platelet refractoriness in multitransfused patients by use of leukocyte-free blood components. Blood 1990;75:512-7.
12. Murphy MF, Metcalfe P, Thomas H, et al. Use of leukocyte-poor blood components and HLA-matched-platelet donors to prevent HLA alloimmunization. Brit J Haematol 1986;62:529-34.
13. Oksanen K, Kekomaki R, Ruutu T, Koskimies S, Myllyla G. Prevention of alloimmunization in patients with acute leukemia by use of white cell-reduced blood components - A randomized trial. Transfusion 1991;31:588-94.
14. Schiffer CA, Dutcher JP, Aisner J, Hogge D, Wiernik PH, Reilly JP. A randomized trial of leukocyte-depleted platelet transfusion to modify alloimmunization in patients with leukemia. Blood 1983;62:815-20.
15. Sniecinski I, O'Donnell MR, Nowicki B, Hill LR. Prevention of refractoriness and HLA alloimmunization using filtered blood products. Blood 1988;71:1402-7.
16. Andreu G, Dewailly J, Leberre C, et al. Prevention of HLA immunization with leukocyte-poor packed red cells and platelet concentrates obtained by filtration. Blood 1988;72:964-9.
17. Van Marwijk-Kooy M, van Prooijen HC, Moes M, Bosma-Stants I, Akkerman JWN. Use of leukocyte-depleted platelet concentrates for the prevention of refractoriness and primary HLA alloimmunization: A prospective, randomized trial. Blood 1991; 77:201-5.

18. Sintnicolaas K, Sizoo W, Haije WG, et al. Delayed alloimmunization by random single donor platelet transfusions. Lancet 1981;i:750-4.
19. Gmür J, von Felten A, Osterwalder B, et al. Delayed alloimmunization using random single donor platelet transfusion: A prospective study in thrombocytopenic patients with acute leukemia. Blood 1983;62:473-9.
20. Gmür J, Burger J, Sauter C, Oelz O, Frick PG. Alloimmunization by leukocyte-rich or leukocyte-poor random single donor platelets. In: Rock G (ed). Apheresis. Wiley-Liss, New York, Progress in Clinical and Biological Research 1990;337:45-8.
21. Sherman L, Menitove J, Kagen LR, et al. Ultraviolet-B irradiation of platelets: A preliminary trial of efficacy. Transfusion 1992;32:402-7.
22. Menitove JE, Kagen LR, Aster RH, et al. Alloimmunization is decreased in patients receiving UV-B irradiated platelet concentrates and leukocyte-depleted red cells. **Blood 1990;16(suppl 1):404d(abstract).**

IMMUNE MEDIATED TRANSFUSION REACTIONS: BYSTANDER IMMUNE CYTOLYSIS – A FREQUENTLY OVERLOOKED MECHANISM OF HEMOLYSIS?

B.A. van Dijk

Immune mediated transfusion reactions mostly involve febrile non-hemolytic transfusion reactions (FNHTR), allergic reactions, hemolytic transfusion reactions (HTR) and delayed hemolytic transfusion reactions (DHTR).

FNHTR's are caused by donor leukocytes and can be prevented by making the donor blood leukocyte poor through filtration. In the Netherlands many blood banks remove the buffy-coat routinely from donor blood, so FNHTR's occur less frequently anyhow.

Allergic reactions occur very frequently and are usually mild. They are caused by antibodies against plasma proteins. Prevention can easily be accomplished by administration of antihistaminic drugs to the patient or by a single washing procedure of the red cell concentrate.

HTR's occur when existing and detectable blood group antibodies, anti-A/-B as well as irregular antibodies, are overlooked because of clerical errors. Severe, even life threatening, hemolysis occurs. Of course this kind of transfusion reaction is preventable and should never take place.

DHTR's are caused by the presence of slumbering, but at the moment of transfusion not yet detectable, irregular antibodies that the patient made earlier in his life because of transfusion or pregnancy. Generally 3-7 days after transfusion the antibody titer, stimulated by the recent transfusion, starts to rise and destruction of donor red cells ensues. This type of transfusion reaction can not be prevented.

Clinical symptoms of DHTR may include fever, unexplained anemia and jaundice, evident approximately 5-10 days after transfusion. Sometimes hemoglobinuria, acute renal failure and disseminated intravascular coagulation may occur. The two most notorious irregular antibodies that cause DHTR are anti-Jka and anti-E [1].

True DHTR with evident clinical symptoms occurs in about 1:10,000 units transfused [2]. More often however – 1:1,500 units transfused – DHTR is only a laboratory diagnosis [2]: The antibody and positive direct antiglobulin test (DAT) are found in the course of crossmatching more units of blood for a patient who was transfused one week earlier. The patient however does not show any clinical symptoms of hemolysis. Such cases of laboratory DHTR are now called delayed serologic transfusion reactions (DSTR).

A large series of DHTR/DSTRs was presented by Ness et al. from the John Hopkins Hospital, Baltimore [2]. As expected, they found initially positive DAT's due to anti-IgG with or without anti-C3d. In the long term however – 25 to 200 days posttransfusion – they found persistently positive DAT's in many of their patients, unlike what traditionally is expected.

Most likely, the eluted allo-antibodies found in the Johns Hopkins study on the long term were actually auto-antibodies mimicking allo-antibodies, which has been described often [3].

More evidence that blood transfusions can induce auto-immunity was recently published by Kaminski et al. [4]. He observed significant levels of auto-cytotoxicity in multitransfused patients when performing cytotoxic T-lymphocyte precursor frequency analyses.

The phenomenon of allo- and auto-immunization occurring concurrently, is more common than one thinks. In our hospital we frequently encounter DSTR's – that is: without clinical hemolysis – that turn on the production of auto-antibodies and a persistently positive DAT. Sheila Worlledge [5] has stated that even alloimmunized patients who are given compatible blood, may subsequently develop a positive DAT due to auto-antibody production.

Another possibility was postulated by Salama and Müller-Eckhardt from Germany [6]. They also found long-term positive DAT's in DHTR, but they eluted predominantly allo-antibodies, even 100 days posttransfusion. Since the duration in which allo-antibodies could be eluted, in some cases even exceeded the life span of transfused red cells, Salama and Müller-Eckhardt proposed that the antibodies in hemolytic reactions attach to autologous as well as allogeneic cells. This results in complement activation and accelerated destruction of not only donor red cells, but transiently and partially also of autologous cells, in that way acting as "innocent bystanders". There are several reports that support this idea, the most striking case being that of Polesky and Bove in 1964 [7]. In the course of a Cr51 survival study of a patient's autologous red cells – which study appeared to be normal – an apparently incompatible Jka-positive unit of blood initiated a DHTR and disturbed the normal autologous survival curve: the autologous Jka-negative red cells of the patient showed a 28% hemolysis!

Petz [8] defined hemolysis of "bystander" cells as immune hemolysis of cells that are negative for the antigen against which the relevant antibody is directed. He suggested that bystander hemolysis may be more common than has previously been recognized, and compared the phenomenon in the following clinical settings.

Some patients with paroxysmal nocturnal hemoglubinuria (PNH) experience auto-hemolysis following transfusion of apparently compatible blood. Dacie [9] suggested that this was brought about by transfusion of plasma along with allogeneic red cells, and might be due to an antigen-antibody reaction involving leukocyte antibodies. Sirchia [10] supposed an interaction between transfused leukocytes and specific antibodies in the patient's serum.

In patients with sickle cell anemia several cases of DHTR are described in which the patient's hematocrit after the onset of hemolysis dropped to a significantly lower level than was present prior to transfusion [8,11-13]. This suggests that some of the patient's own red blood cells had been hemolyzed in addition to the transfused RBC's.

Although in the immune response to drugs that cause hemolysis the patient's red cells are not completely "innocent" bystanders – since the drugs themselves seem to alter the red cell membrane [14] – the resultant antibodies are not directed against unmodified intrinsic red cell antigens, so that the term "bystander" is still appropriate.

Escaping malaria parasites from infected red cells cause intravascular bursting and hemolysis. However, only 2% of the erythrocytes are parasitized where 60% of the cells are destroyed [15]. Possibly immune complexes of malarial antigen with antibody causes a complement-mediated immune hemolysis [16].

Bone marrow transplantation (BMT) and the passenger lymphocyte syndrome. Following minor ABO-incompatible BMT – e.g. patient group A, donor group O – delayed hemolysis can occur about 10 days after transplantation. Clinically this is seen as an increased need for red cell transfusion or even severe intravascular hemolysis. Anti-A and anti-B are detected in the serum and red cell eluates in all cases. The causative ABO-antibodies have been shown to be produced by socalled "passenger" lymphocytes, which are infused with the stem cells as part of the donor marrow.

The same phenomenon was earlier recognized in kidney transplantation. In both BMT and kidney transplantation the use of cyclosporin for GvHD-prophylaxis is prerequisite for the development of the syndrome.

Recently Petz et al. [17] observed in several consecutive patients excessive hemolysis after minor ABO-incompatible BMT with marrow from matched, but unrelated donors. Except for hemolysis requiring hemodialysis in some patients, the transfusion requirements for group O erythrocytes exceeded in all patients the expected baseline requirement by an average of 1.5 liter in the first three weeks after BMT. The degree of hemolysis could not be explained through destruction of the autologous ABO-incompatible red cells by the anti-A or anti-B antibodies produced by donor passenger lymphocytes. Petz suggested that in these patients large volumes of transfused group O erythrocytes were hemolyzed in addition to the patient's own red cells: innocent bystander hemolysis "pur sang"!

In Nijmegen we performed 36 allogeneic BMT's during the last 12 months, 10 of which with marrow from unrelated donors (Table 1). The average RBC requirement in the first 3 weeks after BMT was considerably lower than the patients in Petz's study: his patients used 26 units of blood in case of minor ABO-incompatibility, against 7.5 units in our patients (Table 2). In both Petz's and our patients cyclosporin was used for GvHD-prophylaxis. Of course the number of patients studied are low: Petz three, we two patients.

Table 1. Number of BMT's, 8/91 – 8/92, Nijmegen.

ABO-compatibility	Sibling-donor	Unrelated donor
Compatible	22	5
Major incompatible	–	3
Minor incompatible	4	2
Total	26	10

Table 2. Number of RBC's transfused during first 3 weeks after BMT, 8/91 – 8/92, Nijmegen.

ABO-compatibility	Sibling-donor	Unrelated donor
Compatible	5.2	7.2
Major incompatible	–	8.7
Minor incompatible	6.3	7.5

A good explanation for the difference in blood usage between Petz's and our patients is the fact that in our transplant unit T-cell depleted donor marrow is infused, which consequently – due to the counter flow centrifugation technique – also is B-lymphocyte poor.

Serologically we never encounter a positive DAT or eluate due to anti-A or anti-B in our minor ABO-incompatible BMT's, nor do we see strongly elevated anti-A or anti-B levels in the patient's serum.

However, Petz's study is quite convincing and stimulating. The idea of "innocent bystander hemolysis" is very intriguing.

Petz [8] has given some additional possible explanations for the bystander hemolysis of donor blood in his minor ABO-incompatible, matched unrelated BMT's.

1. It has been demonstrated, also in minor ABO-incompatible BMT's, that group O cells can absorb A and B antigens from plasma, especially in patients who are secretors [8,18]. The amount of absorbed A and B antigens in Petz's patients could have been enough to cause hemolysis by the anti-A/-B produced by the passenger lymphocytes in the donor bone marrow.
2. Two Japanese studies [19,20] have shown that glycosyl-transferase – responsible for the synthesis of ABO-specific antigens – in BMT-patients do not change after BMT, although the blood groups of the patients converted to that of their bone marrow donors. So it is possible that when passenger lymphoyctes produce strongly reactive anti-A or anti-B after minor ABO-incompatible BMT, these antibodies may cause hemolysis of red cells typing as group O.

Unfortunately Petz did not perform survival studies of transfused group O red cells. A shortened survival would have given still more proof of accelerated destruction of donor blood. Since in Nijmegen we routinely monitor the disappearance of autologous erythrocytes and the appearance of donor red cells after allogeneic BMT by means of an in-house developed fluorescence technique using fluorescent microspheres [21], we also used this technique in some 5 BMT-patients to see whether infused donor RBC's have a shortened – or possibly prolonged – survival.

In all 5 patients – one of which had a minor ABO-incompatibility with his bone marrow donor – the overall survival of one unit group O RBC's was not extremely shortened (range 95-125 days) and showed the normal slope as is seen in non-hematological surgery patients (Bär BAM and de Man AJM; to be published).

The idea of innocent bystander cytolysis is not new. We all know it as a concept from the syndrome of posttransfusion purpura, in which HPA-1a antigen in the donor's plasma may be transfused with HPA-1a positive platelets and absorbed onto the patient's HLA-1a negative platelets. The platelets are then lysed when the patient produces anti-HPA-1a [22].

The concept of innocent bystander hemolysis of red cells however – as published by Petz [8] – is new for many of us and is, in my opinion, reason to reconsider some established theories.

References

1. Taswell HF, Pineda AA, Moore SB. Hemolytic transfusion reactions: Frequency and clinical and laboratory aspects. In: Bell CA (ed). A seminar on immune-mediated red-cell destruction. Washington, DC, American Association of Blood Banks 1981: 71-92.
2. Ness PM, Shirey RS, Thoman SK, Buck SA. The differentiation of delayed serologic and delayed hemolytic transfusion reactions: Incidence, long-term serologic findings, and clinical significance. Transfusion 1990;30:688-93.
3. Shirey RS, Ness PM. New concepts of delayed hemolytic transfusion reactions. In: Nance SJ (ed). Clinical and basic science aspects of immunohematology. Arlington, VA, American Association of Blood Banks, 1991:179-97.
4. Kaminsky ER, Hows JM, Goldman JM, Batchelor JR. Lymphocytes from multi-transfused patients exhibit cytotoxicity against autologous cells. Brit J Haematol 1992;81:23-6.
5. Worlledge SM. The interpretation of a positive direct antiglobulin test. Brit J Haematol 1978;39:157-62.
6. Salama A, Müller-Eckhardt C. Delayed hemolytic transfusion reactions: Evidence for complement activation involving allogeneic and autologous red cells. Transfusion 1984;24:188-93.
7. Polesky HF, Bove JR. A fatal hemolytic transfusion reaction with acute autohemolysis. Transfusion 1964;4:285-92.
8. Petz LD. The expanding boundaries of transfusion medicine. In: Nance SJ (ed). Clinical and basic science aspects of immunohematology. Arlington, VA, American Association of Blood Banks 1991:73-113.
9. Dacie JV. The haemolytic anaemias. New York: Grune and Stratton, Inc. 1967:1231-7.

10. Sirchia F, Ferrone S, Mercuriali F. Leukocyte antigen-antibody reaction and lysis of paroxysmal nocturnal hemoglobinuria erythrocytes. Blood 1970;36:334-6.
11. Petz LD, Garratty G. Acquired immune hemolytic anemias. New York, Churchill-Livingstone 1980:318-20.
12. Chaplin H, Zarkowsky HS. Combined sickle cell disease and auto-immune hemolytic anemia. Arch Intern Med 1981;141:1091-3.
13. Milner PF, Squires JE, Larison PJ, et al. Posttransfusion crisis in sickle cell anemia: Role of delayed hemolytic reactions to transfusion. South Med J 1985;78:1462-9.
14. Habibi B. Drug-induced red blood cell autoantibodies codeveloped with drug specific antibodies causing haemolytic anaemias. Brit J Haematol 1985;61:139-43.
15. Neva FA, Sheagren JN, Shulman NR, Canfield GJ. Malaria: Host-defense mechanisms and complications. Ann Intern Med 1970;73:295-306.
16. Woodruff AW, Andsdell VE, Pettitt LE. Cause of anaemia in malaria. Lancet 1979;i: 1055-7.
17. Gajewski JL, Petz LD, Calhoun L, et al. Hemolysis of transfused group O red blood cells in minor ABO-incompatible unrelated-donor bone marrow transplants in patients receiving cyclosporine without posttransplant methotrexate. Blood 1992;79: 3076-85.
18. Crookston MC, Tilley CA. A and B and Lewis antigens in plasma. In: Human blood groups. 5th International Convocation of Immunologics. Basel, Karger 1977:246-56.
19. Yoshida A, Schmidt GM, Blume KG, Beutler E. Plasma blood group glycosyltransferase activities after bone-marrow transplantation. Blood 1980;55:699-701.
20. Matsue K, Yasue S, Matsuda T, et al. Plasma glycosyltransferase activity after ABO-incompatible bone marrow transplantation and development of an inhibitor for glycosyltransferase activity. Exp Hematol 1989;17:827-31.
21. De Man AJM, Foolen WJA, van Dijk BA, Kunst VAJM, de Witte TM. A fluorescent microsphere method for the investigation of erythrocyte chimaerism after allogeneic bone marrow transplantation using antigenic differences. Vox Sang 1988;55:37-41.
22. Shulman NR, Reid DM, Jones CE, et al. Evidence that adsorption of alloantigen-antibody complexes is the basis for posttransfusion purpura. Blood 1990;76(Suppl 1): 390a (abstract).

BLOOD TRANSFUSION AND CANCER: MODULATION OR TOLERANCE?[1]

J.G.A. Houbiers[2,3], L.M.G. van de Watering[2], C.J.H. van de Velde[3], A. Brand[2]

Blood transfusion and cancer

The concept of immunosurveillance has evoked a wide spread interest in the role of the immune system in the natural history of cancer. Clinical data from immunodeficient [1] and immunosuppressed [2] patients show an increased incidence of malignancy. Blood transfusions (BT) have diverse immunomodulating effects. For instance, pre-transplant random-donor BT diminish the incidence of renal allograft rejection [3,4] and can give rise to leukocyte antibodies [5]. Several essential factors and clues for the mechanisms of this BT induced immunosuppression in allograft transplantation have recently been elucidated, but the precise mechanism(s) remains unclear. Combining these observed phenomena Gantt raised the question whether immunomodulating effects of peri-operative blood transfusion might adversely affect the prognosis of cancer patients [6]. Surgical resection of tumours often requires blood transfusion. In case of a curative operation, the primary tumour is removed, but nevertheless a percentage of the patients will develop distant metastases [7]. Minimal residual disease in the form of undetectable micrometastases and/or tumour cells spilled in the operation region or into the circulation during surgery might be explanations for these observations. However, not all patients having a similar cancer stage show recurrence of the tumour. Probably minimal residual disease not always results in cancer recurrence. It is suggested that besides tumour characteristics and genetics the immune defense might play a role in the outgrowth of micrometastases. Before addressing possible mechanisms the relationship between blood transfusion and cancer prognosis has to be clarified on a clinical level (the horizontal arrow in Figure 1).

1. This work was partially supported by the Praeventiefonds and the Macropa Foundation.
2. Department of Immunohaematology & Blood Bank, University Hospital Leiden, The Netherlands.
3. Department of Surgery, University Hospital Leiden, The Netherlands.

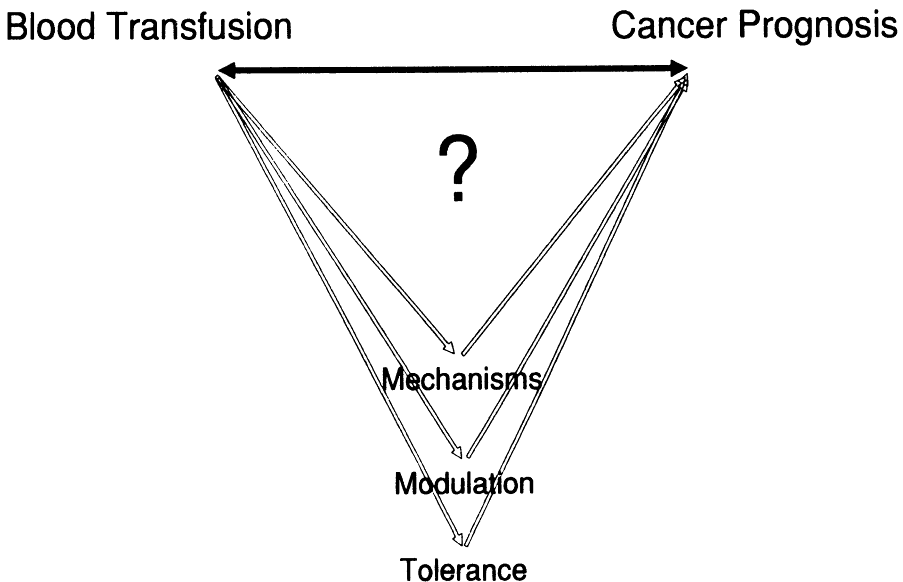


Figure 1. Triangular model representing the relationship between peri-operative blood transfusions and cancer prognosis in respect to intermediate mechanisms.

Table 1. Overview of retrospective studies comparing cancer prognosis of transfused versus non-transfused cancer patients.

Site of primary tumour	Sign.[1] adverse	Cox[2] sign.	No sign.[1,3] effect	Cox[2] no sign.
Colorectal	16/–1	7	17	14
Breast	3		8	
Lung	3		5	
Kidney	2		2	
Prostate	4		0	
Stomach	2		2	
Cervix	1		3	
Vulva	1		0	
Head and neck	2		1	
Larynx	1		0	
Soft tissue	1		0	
Bone	1		0	

1. Number of studies reporting a (non) significant effect of blood transfusion on cancer prognosis (5/10 year survival and/or recurrence free survival). One study (–1) reports a statistical significant beneficial effect of BT.
2. Number of studies in which multivariate analysis revealed blood transfusion a significant independent factor of cancer prognosis.
3. Over 70% of these negative studies have too small study population to discriminate even a 20% difference between the two study groups.

Retrospective studies

The first study investigating Gantt's question was published by Burrows and Tartter in 1982 [8]. Colorectal cancer patients receiving peri-operative blood transfusions showed a decreased 5 year survival rate compared to non-transfused patients (50% versus 83%). The two study-groups in this retrospective study were similar in respect to other factors relevant for cancer prognosis. In the following decade over 70 retrospective studies have been published, reporting statistical significant adverse effects, neutral effects, but hardly any advantageous effects of blood transfusion on prognosis of soft-tissue sarcomas, colon, rectal, breast, lung, kidney, prostate, vulva, uterine cervix, head and neck and stomach cancer. A summary is given in Table 1, while for reviews references 9, 10 and 11 are recommended.

Many authors checked whether blood transfusion was an independent factor for cancer prognosis using multivariate statistical analysis which adjusts for other important co-variates (e.g. Cox regression analysis [12]). In retrospective studies – lacking randomized study groups – blood transfusion might be a surrogate marker (confounding factor) for relevant prognostic factors like cancer stage, age, surgical technique, tumour size, differentiation grade of tumour, duration of operation and blood loss [7,10]. Voogt and coworkers [13] in our institution retrospectively analyzed BT effects in a group of 113 colon cancer patients (Dukes' cancer stage A, B and C1). Overall 5 year survival rates for transfused (n=86) and non transfused (n=27) patients were 48% and 74% respectively (p=0.007). The two study-groups were comparable with regard to age, sex, Dukes' stage, cancer localization and operation procedure. After controlling for these relevant co-variates, using Cox regression statistics, the transfusion status remained next to the Dukes' stage the most significant prognostic indicator (relative risk: 3.42; p=0.005). However, if in a retrospective study the independent influence of a prognostic factor is proven, still unknown related factors might actually cause the relationship: for these biassing factors, obviously, correcting statistics can not be applied.

Most retrospective studies (>35) analyzed patients with colorectal cancer [reviewed in 10 and 14]. It is difficult to compare these studies because of differences in patient-characteristics (cancer stage, average age), endpoints (cancer recurrence or overall survival), statistics (no control statistics, uni-, bi- or multivariate analyses) and blood products (red blood cell concentrate (RBC), whole blood, the use of FFP or plasma, blood additive solutions, plastic blood bags and storage time). The endpoint cancer recurrence measures BT-effects on the tumour while overall survival reflects all deleterious BT effects. Moreover, many authors have reported on studies lacking methodological perfection, have omitted essential statistical control analyses, have selected patients in a biassing way which resulted in not comparable study groups, have failed to analyze, or even report on other prognostic factors or have presented the data insufficiently. This results in inadmissible checking and recalculation of the presented results [10,14].

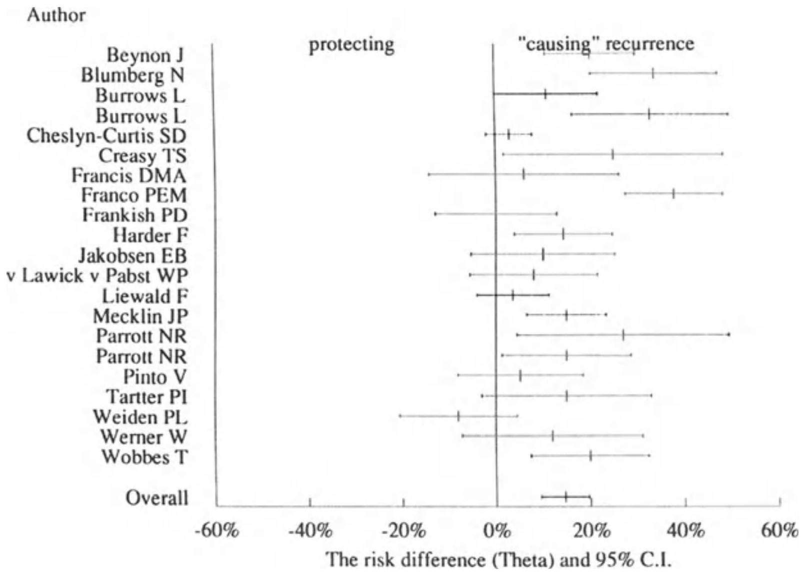


Figure 2A. Summarizing figure of meta-analysis of the correlation between blood transfusion and colorectal cancer prognosis: 5 year recurrence free survival (A) and 5 year survival (B). On the horizontal axis the risk difference (theta: 5 y survival rate of non-transfused minus 5 y survival rate of transfused) and the 95% confidence interval are depicted. Vertically the authors of the incorporated studies are lined.

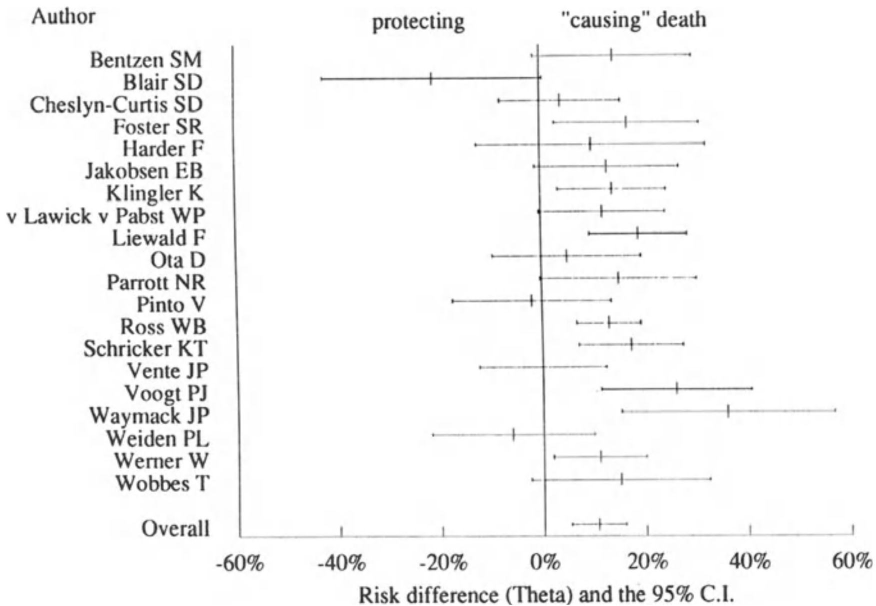


Figure 2B. Summarizing figure of meta-analysis of the correlation between blood transfusion and colorectal cancer prognosis: 5 year recurrence free survival (A) and 5 year survival (B). On the horizontal axis the risk difference (theta: 5 y survival rate of non-transfused minus 5 y survival rate of transfused) and the 95% confidence interval are depicted. Vertically the authors of the incorporated studies are lined.

Notwithstanding these imperfections, for colorectal cancer peri-operative blood transfusion seems an important factor determining long-term prognosis. To underpin this statement we performed a socalled meta-analysis [15-17]. A meta-analysis is a process of formally combining the quantitative results of separate studies in order to increase the statistical power of estimated effects. In this meta-analysis every study was treated as a sample (event). Of all available literature the papers describing original data, investigating the effects of homologous whole blood or red blood cell concentrate (RBC) on colorectal cancer prognosis and reporting 5 year (disease free) survival rates were selected. Thirty four of the 46 retrieved reports on blood transfusion and colorectal cancer fulfilled these criteria. The summarizing Figure 2 shows a highly statistical significant 10.6% decreased 5 year survival rate (2A) and a 14.3% lower 5 year recurrence free survival (2B) for transfused patients. Again, it must be concluded that the transfused patients experience more effects of blood transfusion than effects on cancer recurrence alone. In bi-variate analyses the differences in age between the transfused and non-transfused patients (66.8 versus 64.2 years) appeared to account for 4% of the 10.6% survival rate decrease. Other established prognostic factors (tumour localization, cancer stage and sex) were either equally distributed or had minimal influence on the survival rates. Age appeared not to correlate with cancer recurrence leaving, for the (limited number of) prognostic factors analyzed, blood transfusion as the only significant prognostic factor for disease free survival [14].

Most retrospective studies investigate the role of homologous red blood cells mixed with leukocytes (the buffy-coat free RBC still contains about 1×10^9 leukocytes) and in case of whole blood also containing plasma. Additional transfusions of plasma are often ignored in the retrospective data analysis. Some authors, though, focussed on putative effects of plasma transfusion [18-20]. Schricker et al. [20] observed a decrease in 5 year survival rate of 17.3% comparing non-transfused with transfused colorectal cancer patients. The detrimental effect of whole blood or RBC also showed a dose-effect relationship. In the Cox-regression analysis, applied to control these findings, the effect of BT could totally be explained by its close correlation to relevant prognostic factors (e.g. tumour localization and cancer stage). Unexpectedly, this analysis revealed transfusion of fresh frozen plasma (FFP) as an independent prognostic factor ($p<0.025$). Independent of red blood cell transfusion or the distribution of relevant prognostic factors, colon as well as rectum cancer patients who had received FFP showed a decreased prognosis compared to patients who were never transfused with FFP. The authors conclude that transfusion of FFP to cancer patients should be avoided. However, they did not observe survival differences between patients receiving RBC or whole blood only. Blumberg et al. [19,21] showed, employing multivariate statistics, that small numbers (<4 units) of RBC including as few as one unit of whole blood were associated with earlier cancer recurrence and death compared to transfused of equal numbers of RBC only. Whole blood contains plasma and about three times more leukocytes than RBC.

The effect of autologous blood transfusion on cancer prognosis has not been investigated in retrospective studies.

Clinical trials, experimental studies

In animal models the results of experimental studies have been conflicting. Several investigators could show a promoting effect, while others suggested an inhibitory effect of allogeneic blood transfusions on experimental tumour growth [reviews 9-11]. Differences in animal strains used as well as differences in experimental tumours, (preparation of) blood products, routes of inoculation and methods to assess tumour progression could account for these rather confusing data.

Multivariate statistics and meta-analysis can confirm correlations found in retrospective studies. However, a causal relationship between blood transfusion and decreased cancer prognosis can only be concluded from well designed clinical trials. Randomization for either or not receiving blood would be unethical. Therefore randomization is only feasible within the patient-population in which a blood transfusion is indicated for medical reasons. Comparative trials between different blood products are easy to design.

Our group decided to investigate the immunosuppressive effects of transfused leukocytes in colorectal cancer patients by means of a clinical trial [13]. This design was chosen because in a prospective study Persijn et al. showed that one to three by filtration leukocyte depleted ($<5\times10^6$ leukocytes/unit RBC) blood transfusions resulted in a significantly inferior kidney graft survival as compared to pre-transplant transfusion of only one unit of standard RBC (about 1×10^9 leukocytes/unit) [22]. This pivotal role of leukocytes transfused to suppress the allograft reaction was confirmed by other studies, clinical and experimental [23-25]. In the "Cancer Recurrence And Blood transfusion" (CRAB) project we investigated several basic aspects of (leukocyte containing) blood transfusions in colorectal cancer patients. In the CRAB clinical trial colorectal cancer patients operated upon with curative intent are randomized (and stratified per participating hospital) to receive, on medical indication, either leukocyte-depleted RBC or standard RBC [26]. Leukocyte-depleted blood is hypothesized to prevent the BT associated immunosuppression and thereby to result in better cancer survival compared to transfusion of standard RBC. During the 3.5 years of patient intake (until December 1990) the participating 21 Dutch and 3 German hospitals have entered 1148 patients. Of these 1148 patients 696 match the intake criteria (proven malignancy without metastatic spread), of which 66% received standard RBC or fresh leukocyte-depleted RBC. The results-parameters (recurrence free period, patient survival and (non) infectious post-operative complications) will be analyzed after a median follow-up of 2.5 and 5 years. Recently a Danish group started a similar clinical trial among colorectal cancer patients (comparing leukocyte depleted versus standard BT, [27]). Another Dutch study, initiated by the Rotterdam group, compared colorectal cancer patients receiving either predeposit autologous or standard homologous RBC in a multicentre clinical trial. Analysis at a median follow-up time of 2.5 years failed to show significant differences between the two trial-groups [28,29].

Modulation or tolerance?

In case clinical trials indeed show a causal relationship between transfusion of allogeneic blood/leukocytes and cancer prognosis the horizontal arrow of the triangular model in Figure 1 (clinical, causal relationship) has been proven. Of course, in a causal relationship blood transfusion must act by modulating some function which in turn has influence on cancer cell growth.

Peri-operative blood transfusions are given at the stage that a manifest solid tumour has been growing for years without effective destruction by the patients immune system by either selection of immune response resistent [30,31] or (locally) immune response suppressing tumour sublines [31] or induction of tumour specific tolerance [32]. After resection of the solid tumour with curative intent minimal residual disease might remain: by surgical procedures spilled or circulating tumour cells and/or pre-existing micro-metastases. A blood transfusion at this stage and in general must be "a shock to the well balanced homeostasis". Several mechanisms can be proposed for the observed effects of blood transfusion on cancer: 1) immunosuppressive effects on anti-tumour responses; 2) effects on the formation of thrombi, which might enhance metastatic properties; 3) effects on the tumour cells: changes in growth or metastatic capacities; 4) direct, or indirect effects on the micro-environment influencing the homing of the (spilled) tumour cells.

Speculating that the immune system (option 1) is the linking mechanism between blood transfusion and cancer prognosis, BT are presumed to suppress (left side) immune responses which are relevant for lysis of tumour cells (right side; Figure 1).

Blood transfusion – immune system

Immunosuppressive effects of blood transfusion have been well documented in transplantation medicine [3,4,34].

At the clinical level – blood transfusion is reported to influence 1) allograft survival, 2) possibly cancer prognosis, 3) possibly the recurrence of M. Crohn (decreased recurrence rate, [35,36]), and 4) post-operative infections (increased rate [27,37,38]). This later aspect has been confirmed in the CRAB-trial: transfused patients experienced more post-operative infections [39]. Transfusion of leukocyte depleted blood resulted in similar infection rates compared to standard blood. Since red blood cell transfusion was the major, statistically independent, prognostic factor for the chance of infection we hypothesize that red cells, cellular debris and other substances in stored blood must be responsible for this observed immunosuppression, which most probably is aspecific.

Although other authors in these proceedings (partly) cover blood transfusion effects, several aspects will be discussed briefly:

1. In humans the immunosuppressive effect on allo-responses seems a long lasting effect [4]; and
2. One unit of allogeneic blood appeared sufficient to increase renal allograft survival [4].

3. For the BT-effect in organ transplantation, transfusion of fresh leukocytes appeared essential [22,23].
4. Allograft donor specific blood transfusion seems superior to random donor BT in regard to transplant survival [40,41]; whereas
5. Some HLA-alleles of recipients as well as graft donors are associated with "high responsiveness" [42].
6. Lagaaij et al. [43] demonstrated the effect of HLA-DR antigen sharing between the blood transfusion donor and the graft recipient on subsequent graft survival. In two prospective trials matching of one HLA-DR allele between the third party blood (donor) and the patient significantly increased the outcome of kidney and heart transplantations after 5 and 1 year respectively. Leukocyte antibody formation followed a similar pattern: predominantly, HLA-DR mismatched blood is immunizing. This HLA-DR sharing phenomenon is also investigated in the prospective CRAB-HLA study. In several hospitals patients in the standard RBC arm and their blood donors are HLA-typed. Patients who received at least one unit of HLA-DR matching blood are compared to patients transfused with HLA-DR mismatched blood in respect to cancer prognosis, leukocyte antibody formation and virus-serology.

Several in vitro effects of BT – pointing at a decreased immune function have been observed:

1. Decreased natural killer cell and phagocytic activities [27,29,44].
2. Decreased delayed type of hypersensitivity response, measured as DNCB skin test [45].
3. Several effects on the T-cell compartment: decreased numbers of T-cell, reversed CD4/CD8 ratio, increased HLA-DR expression (T cell activation), increased T suppressor cell activity and decreased mitogenic responses [46-52].
4. A recent observation of Van Twuyver et al. give some elucidation of the mechanism of the BT effect. Patients showed a significant decrease in number of blood donor-HLA-specific cytotoxic T-lymphocytes (CTL precursor frequency) in case of transfusion with haplo-identical (at least sharing of one HLA-DR and one HLA-B allele) blood [53]. This phenomenon supports the mentioned clinically relevant HLA-DR sharing effects. The authors suggest that haplo-identical lymphocytes can survive more easily than completely mismatch lymphocytes in the blood recipient and that their subsequent continuing presence (chimerism) down regulates the immune response against the non-sharing HLA-antigens.

Immunosuppressive effects of blood transfusions, thus, are diverse but increasingly well documented. Although the underlying mechanisms remain unclear, different effects probably are caused by different mechanisms. For cancer prognosis the influence (left hand arrow in Figure 1) on the natural killer and T cell activity seems important [56].

Immune system – Cancer

Prove for the right hand arrow relationship (Fig. 1) has been gathered at the clinical and in vitro level.

At the clinical level – the reported increased incidence of cancer in patients with immunosuppressive therapy or with an immune deficiency 1,2], families with high incidences of breast cancer and melanomas appearing to have decreased natural killer cell activity [76], as well as beginning successes if immunotherapy of cancer (e.g. melanoma and renal cell carcinoma [54]) point at this relationship. In experimental murine models established tumours can be lysed by adoptive transfer of cytotoxic T-lymphocytes (CTL) [55].

At the in vitro level – many details about anti-tumour responses are known [56]. A tumour cell must bear a tumour specific antigen (TSA) or at least a tumour associated antigen (TAA, time point or quantitative discrimination) which the immune system is not tolerized for to induce an immune response. Since every (intra-)cellular protein is processed into peptides which are presented by HLA-molecules to T lymphocytes [57] all mutated structures of a tumour cell are potential TSA:

1. Differentiation antigens (TAA, e.g. CD19 in leukemia).
2. Retrogenetic antigens (TAA, e.g. αFP, CEA).
3. Virus-antigens (TSA, e.g. EBV, HTLV, HCV, HBV).
4. All mutated structures in a tumour cell (TSA, e.g. mutated oncogenes as RAS, p53).

In relation to these tumour antigens it has become clear that the oncogenic pathway influences the immunogenicity of tumours:

1. Virus-induced tumours (cervical carcinoma) are strongly immunogenic; the virus antigens (peptides) evoke a dominant antiviral, and thus anti-tumour response [58].
2. UV light-induced tumours (melanoma) are immunogenic by individual antigens because of random mutations [59].
3. Chemically-induced tumours are not/weakly or weakly immunogenic related to a low respectively high dose of the carcinogen. The immune response is polyclonal [60].
4. The socalled spontaneous tumours (the common human cancers) show weak or negligible immunogenicity [61].

The in vitro cloning – of tumour specific cytotoxic T-lymphocytes out of tumour infiltrating leukocytes (TIL) further proves the existence of anti-cancer immune responses [62,63]. These findings and the possibilities to induce human T lymphocytes responses in vitro against mutated RAS [64] or mutated p53 [65] bear the potential of immunotherapy of cancer directed at the basis of oncogenesis. In a murine model vaccination with peptides resulted in protection against the outgrowth of Human Papilloma Virus-induced tumours [66]. Various mechanisms exist, however, to escape from an immune response:

1. Loss of expression of HLA or adhesion molecules [30,67,68].

2. Selection of TSA-negative tumour-clones [69].
3. Immunosuppression by tumour cell products [32].
4. Induction of tolerance for the TSA [33]. Immune responses against tumour cells, thus, are well documented, but their clinical significance still has to be clarified.

Having described possible mechanistic "arrows" for both sides of the clinical relationship between blood transfusion and cancer the question arises of the synthesis: is the immunosuppression exerted by BT of influence on tumour cell growth? Opportunities for (specific) tolerance as well as for aspecific immunomodulation as interconnecting mechanism are conceivable.

Tolerance which per definition is specific for a particular antigen, can be induced by the tumour cells themself to escape from immune responses, but also BT can result in such specific immune unresponsiveness. HLA-DR sharing BT reduce allo-immune responses against non-sharing (HLA-)antigens [43]. Since these responses predominantly are T cell mediated [70] and directed against peptides [70] it might be possible that the tolerance extends to other peptides (antigens) presented by the allogeneic donor leukocytes as well. It was observed that after HLA-DR sharing BT anti-CMV responses were impaired [71]. CMV carriers have virions present in circulating leukocytes [72]. In this respect the BT induced tolerance in cancer patients might be comparable to the organ transplantation situation. One mechanism of peripheral tolerance induction results in a state of T cell unresponsiveness called "anergy" [73). Anergy is hypothesized to be induced by inappropriate antigen presentation: one, instead of two stimuli [74,75]. Tumour cells, not being "professional" antigen presenting cells, might give such an incomplete signalling to the immune system. Similarly, transfused leukocytes, among which are B lymphocytes and monocytes, might give incomplete antigen presenting of either processed antigen of recipient tumour cells or donor antigens. In the latter case it must be hypothesized that tumour cells and donor leukocytes have TSA epitopes in common. In stead of anergy T suppressor cells downregulating anti-tumour responses have to be considered as tolerance inducing mechanism. All these hypothesis are testable once it has been established which (anergic of suppressed) CTL responses against which TSA are present in a cancer patient. The tumour specific CTLs cloned out of melanoma TIL of which the TSA has been characterized [60] show opportunities for research of tolerance to tumour cells. As an illustration: transplantation of syngeneic, interleukin-2 (IL-2) producing, tumour cells resulted in tumour specific CTLs responses against the parental, poorly immunogenic, tumour line [33]. This points at impaired/absent generation or transition into an anergic state of CTLs directed at the parental tumour line which was overcome by local IL-2 production [75].

Aspecific immunosuppressive effects of peri-operative BT are another possible explanation for the deleterious prognosis of transfused patients. The disturbances caused by BT might prevent temporarily the induction of every (specific) immune response. In this stage growing micrometastases can pass a

threshold for effective immune attack and escaping tumour clones can emerge. Natural killer cell activities and phagocytic capacities are impaired after BT. Since these immune functions are the first line of defence against aberrant cells [29,56] pre-operatively spilled tumour cells might get a chance to settle and grow out.

In conclusion it is clear that the balances between the micro-environment, the tumour cells and the immune system are complex and delicate and thus probably easy to disturb. Understanding these interactions will give opportunities to interventions and to "engineered" immunotherapy of cancer. Blood transfusion of specific products or altered cells might even enforce immunosurveillance. At the clinical level, however, the relationship between blood transfusion and cancer first has to be demonstrated in order to know whether blood transfusion should be avoided or alternative (blood) products are applicable in cancer treatment.

References

1. Kersey JH, Spector BD, Good RA. Cancer in children with primary immunodeficiency diseases. J Pediat 1974;84:263-4.
2. Kinlen LJ, Sheil AGR, Peto J, Doll R. Collaborative United Kingdom-Australasian study of cancer in patients treated with immunosuppressive drugs. N Engl J Med 1979;2:1461-6.
3. Opelz G, Sengar DDS, Mickey MR, Terasaki PI. Effect of blood transfusions on subsequent kidney transplants. Transplant Proc 1973;5:253-9.
4. Persijn GG. HLA-matching and blood transfusion(s) in renal transplantation [dissertation]. Leiden, The Netherlands: Eurotransplant Foundation, Leiden University, 1985.
5. Brand A, Claas FHF, Voogt PJ, Wasser MNFM, Eernisse JG. Alloimmunization after leukocyte-depleted multiple random donor platelet transfusion. Vox Sang 1988;54: 160-6.
6. Gantt CL. Red Blood cells for cancer patient [letter]. Lancet 1981;ii:363.
7. Van de Velde CJH, Bloem R, Zwaveling A. Management of colorectal cancer. Eur J Cancer Clin Oncol 1986;22:339-44.
8. Burrows L, Tartter P. Effect of blood transfusions on colonic malignancy recurrence rate [letter]. Lancet 1982;ii:662.
9. Francis DMA. Relationship between blood transfusion and tumour behavior. Gr J Surg 1991;78:1420-8.
10. Blumberg N, Heal JM. Transfusion and host defenses against cancer recurrence and infection. Transfusion 1989;29:236-45.
11. Wu HS, Little AG. Perioperative blood transfusion and cancer recurrence. J Clin Oncol 1988;6:1348-54.
12. Cox DR. Regression models and life tables. J R Stat Soc [B] 1972;34:187-202.
13. Voogt PJ, van de Velde CJH, Brand A, et al. Peri-operative blood transfusion and cancer prognosis. Different effects of blood transfusion on prognosis of colon and breast cancer patients. Cancer 1987;59:836-43.
14. Houbiers JGA, Spierings E, Brand A, et al. Blood transfusion and colorectal cancer prognosis; a meta-analysis [manuscript in preparation].
15 Glass GV. Primary, secondary and meta-analysis of research. Edu Res 1976;5:3-8.
16. Sacks HS, Berrier J, Reitman D, Ancona-Berk VA, Chalmers TC. Meta-analyses of randomized controlled trials N Engl J Med 1987;316:450-5.

17. Andersen JW, Harington D. Meta-analyses need new publication standards [editorial]. J Clin Oncol 1992;10:878-80.
18. Marsh J, Donnan PT, Hamer-Hodges DW. Association between transfusion with plasma and the recurrence of colorectal carcinoma. Brit J Surg 1990;77:623-6.
19. Blumberg N, Heal JM, Murphy P, et al. Association between transfusion of whole blood and recurrence of cancer. Brit Med J 1986;293:530-3.
20. Schricker KTh, Hermaned P, Guggenmoos-Holzmann I, Neidhardt B, Resch Th, Freudenberger K. Verschlechterung des kolorektalen Karzinoms. Beiträge zur Infusionstherapie. Basel, Karger 1990;26:307-12.
21. Blumberg N, Heal J, Chuang Ch, Murphy P, Agarwal M. Further evidence supporting a cause and effect relationship between blood transfusion and earlier cancer recurrence. Ann Surg 1988;207:410-5.
22. Persijn GG, Cohen B, Lansbergen Q, van Rood JJ. Retrospective and prospective studies on the effect of blood transfusion in renal transplantation in the Netherlands. Transplantation 1979;28:396-401.
23. Rapaport FT, Dausset J. The possible role of leukocyte components in the production of the beneficial effects of blood transfusion in human transplantation. Transplant Proc 1983;15:952-5.
24. Maki TM, Okazaki H, Wood ML, Monaco AP. Suppressor cells in mice bearing intact skin allograft after blood transfusion. Transplant Proc 1983;15:952-5.
25. Wood P, Horsburgh T, Brent L. Specific unresponsiveness to skin allografts in mice. Transplantation 1981;31:8-14.
26. Repelaer van Driel OJ, van de Velde CJH. Blood transfusion and prognosis in surgical cancer patients [letter]. Eur J Cancer Clin Oncol 1988;24:1797.
27. Jensen LS, Andersen AJ, Christiansen PM, et al. Postoperative infection and natural killer cell function following blood transfusion in patients undergoing elective colorectal surgery. Brit J Surg 1992;79:513-6.
28. Busch ORC, Hop WCJ, Hoynck van Papendrecht MAW, Marquet RL, Jeekel J. Relationship between blood transfusion and recurrence of colorectal cancer: Results of a prospective randomized trial. Eur J Surg Oncol 1992;18(suppl 1):29.
29. Hoynck van Papendrecht MAW. Prevention of blood transfusions in oncologic surgery [dissertation]. Rotterdam, The Netherlands: Erasmus University, 1991.
30. Smith M, Marsh SGE, Bodmer JG, Gelsthorp K, Bodmer WF. Loss of HLA-A,B,C, allele products and lymphocyte function-associated antigen 3 in colorectal neoplasia. Proc Natl Acad Sci USA. 1989;86:5557-61.
31. Vinuela JE, Rodriguez R, Gil J, et al. Antigen shedding vs development of natural suppressor cells as mechanism of tumor escape in mice bearing Ehrlich tumor. Int J Cancer 1991;47:86-91.
32. Perdrizet GA, Ross SR, Stauss HJ, Singh S, Koeppen H, Schreiber H. Animals bearing malignant grafts reject normal grafts that express through gene transfer the same antigen. N Engl J Med 1990;171:1205-19.
33. Fearon ER, Pardoll DM, Itaya T, et al. Interleukin-2 production by tumour cells bypasses T helper function in the generation of anti-tumour response. Cell 1990;60: 397-40.
34. Lagaaij EL. Immunization and immune suppression by blood transfusions [dissertation]. Leiden University, The Netherlands 1990.
35. Williams JG, Hughes LE. Effect of perioperative blood transfusion on recurrence of Crohn's diseases. Lancet 1990;ii:131-2.
36. Steup WH, Brand A, Weterman IT, Zwinderman KH, Lamers CB, Gooszen HG. The effect of perioperative blood transfusion on recurrence after primary operation for Crohn's disease. Scan J Gastrenterol 1991;188(suppl):81-6.
37. Tartter PI, Quintero S, Barron DM. Perioperative blood transfusion associated with infectious complications after colorectal cancer operations. Am J Surg 1986;152: 479-82.

38. Wobbes Th, Bemelmans BLH, Kuypers JHCJ, Beerthuizen GIJM, Theeuwes AGM. Risk of postoperative septic complications after abdominal surgical treatment in relation to perioperative blood transfusion. Surg, Gyn & Obstet 1990;171:59-62.
39. Houbiers JGA, Arts IT, Hermans J, et al. Blood transfusion and postoperative infectious complications: Different blood component – different effects [manuscript in preparation].
40. Peugh WN, Wood KJ, Morris PJ. Genetic aspects of the blood transfusion effect. Transplantation 1988;46:438-43.
41. Opelz G. Current relevance of the transfusion effect in renal transplantation. Transplant Proc 1985;17:1015-22.
42. Hendriks GFJ, D'Amaro J, Persijn GG, et al. Excellent outcome after transplantation of renal allografts from HLA-DRw6 positive donors even in HLA-DR mismatches. Lancet 1983;ii:187-9.
43. Lagaaij EL, Henneman PH, Ruigrok M, et al. Effect of one-HLA-DR-antigen-matched and completely HLA-DR-mismatched blood transfusions on survival of heart and kidney allograft. N Engl J Med 1989;321:701-5.
44. Gascon P, Zoumbos NC, Young NS. Immunologic abnormalities in patients receiving multiple blood transfusions. Ann Intern Med 1984;100:173-7.
45. Schot JDL, Schuurman RKB. Blood transfusion suppresses cutaneous cell-mediated immunity. Clin Exp Immunol 1986;65:336-44.
46. Grady RW, Akbar AN, Giardina PJ, Hilgartner MW, de Sousa M. Disproportionate lymphoid cell subsets in thalassaemia major: The relative contributions of transfusion and splenectomy. Brit J Haemat 1985;59:713-24.
47. De Martino M, Rossi ME, Muccioli AT, Vullo C, Vierucci A. Altered T cell subsets in polytransfused beta-thalassemia patients: Correlation with sex and age at first transfusion. Vox Sang 1985;48:296-304.
48. Neri A, Brugiatelli M, Jaccolino P, Callea V, Rouco F. Natural killer cell activity and T subpopulations in thalassemia major. Acta Haemat 1984;71:263-9.
49. Matsumoto T, Sakagami K, Orita K. Role of donor specific blood transfusions in prolongation of kidney graft survival. Transplant Proc 1987;19:2264-7.
50. Quintiliani L, Buzzonetti A, DiGirolamo M, et al. Effects of blood transfusion on the immune responsiveness and survival of cancer patients: A prospective study. Transfusion 1991;31:713-8.
51. Sibrowski W, Wegner W, Kühnl P. Immunomodulatory activity of different blood products on the mitogen-induced human lymphocyte transformation. Transfusion Medicine 1992;2:215-21.
52. Munn CG, Markenson AL, Kapadia A, de Sousa M. Impaired T-cell mitogen responses in some patients with thalassemia intermedia. Thymus 1981;3:119-28.
53. Van Twuyver E, Mooijaart RJD, den Berge IJM, et al. Pretransplantation blood transfusion revisited. N Engl J Med 1991;325:1210-3.
54. Rosenberg SA, Lotze MT, Muul LM, et al. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. N Engl J Med 1987;316:889-97.
55. Kast WM, Offringa R, Peters PJ, et al. Eradication of adenovirus E1-induced tumours by E1A-specific cytotoxic T lymphocytes. Cell 1989;59:603-14.
56. Melief CJM. Tumor eradication by adoptive transfer of cytotoxic T lymphocytes. Adv Canc Res 1992;58:143-75.
57. Townsend D, Bodmer H. Antigen recognition by class I restricted T lymphocytes. Ann Rev Immunol 1989;7:601-24.
58. Melief CJM, Kast WM. Efficacy of cytotoxic T lymphocytes against virus-induced tumours. Cancer Cells 1990;2:116-20.
59. Van den Eynde B, Lethe B, van Pel A, de Plaen E, Boon T. The gene coding for a major tumour rejection antigen of tumour p815 is identical to the normal gene of syngeneic DBA/2 mice. J Exp Med 1991;173:1373-84.

60. Prehn RT, Prehn LM. The autoimmune nature of cancer. Cancer Res 1987;47:927-32.
61. Hewitt HB, Blake ER. Walder AS. Acritique of the evidence for active host defence against cancer, based on personal studies of 27 murine tumours of spontaneous origin. Brit J Cancer 1976;33:241-59.
62. Degiovanni G, Lahaye Th, Herin M, Hainaut Ph, Boon Th. Antigenic heterogeneity of a human melanoma tumor detected by autologous CTL clones. Eur J Immunol 1988;18:671-6.
63. Schrier P. personal communication.
64. Jung S, Schluesener J. Human T lymphocytes recognize a peptide of single point-mutated, oncogenic ras proteins. J Exp Med 1991;173:273-6.
65. Houbiers JGA, Nijman HW, van der Burg SH, et al. In-vitro induction of cytotoxic T lymphocytes responses against peptides of mutant and wild-type p53. [Submitted Eur J Immunol].
66. Feltkamp MCW, Smits HL, Vierboom MPM, et al. Vaccination with cytotoxic T cell epitope-containing peptide protects against a tumor induced by Human Papillomavirus type 16-transformed cells [submitted Eur J Immunol].
67. Hilders CJM, Houbiers JGA, Krul EJT, Fleuren GJ. The expression of human lymphocytic antigen in the pathway to cervical carcinoma. Am J Clin Pathol 1993;in press.
68. Houbiers JGA, Tollenaar RAEM, van der Burg SH, et al. Absence of correlation between loss of expression of HLA-A2 and mutated p53 in human colorectal tumours. [manuscript in preparation].
69. Boon T, van Pel A, de Plaen E, et al. Tum-transplantation antigens, point mutations, and antigenic peptides: A model for tumor - specific transplantation antigens. Cancer Cells 1989;1:25-8.
70. De Koster S. The role of peptides in allorecognition [dissertation]. Leiden, The Netherlands: Leiden University 1992.
71. Lagaaij EL. Personal communication.
72. Graan de-Hentzen YCE, Gratama JW, Hudde GC, et al. Prevention of primary cytomegalovirus infection in patients with hematologic malignancies by intensive white cell depletion of blood products. Transfusion 1989;29:757-60.
73. Morahan G, Allison J, Miller JFAP. Tolerance of class I histocompatibility antigens expressed extrathymically. Nature 1989;339:622-4.
74. Bretscher PA, Cohn M. A theory of self-nonself discrimination. Science 1970;169: 1042-9.
75. Jenkins MK. The role of cell division in the induction of clonal anergy. Immunol Today 1992;13:69-73.
76. Steinhauer E, Doyle A, Reed J, et al. Defective natural cytotoxicity in patients with cancer: Normal number of effector cells but decreased recycling capacity in patients with advanced disease. J Immunol 1982;129:2255-9.

BLOOD PRODUCT INDUCED IMMUNOMODULATION AND ITS CLINICAL CONSEQUENCES

H.G. Watson, Ch.A. Ludlam

Introduction

The introduction of concentrates of factor VIII has been described as the double edged sword with the obvious benefits in terms of quality of life, improved patient care and economic benefits of increased hemophiliac employment and decreased cost of health care [1] being off set by the unwanted serious side effects of concentrate use. Clinically the most significant of these are the development of factor VIII inhibitors and the high incidence of concentrate transmitted virus infection particularly with the hepatotropic viruses, hepatitis B [2] and C, [3] and also with human immunodeficiency virus. A more controversial area relates to the possible immuno-modulatory effects of factor VIII concentrates and particularly to the clinical significance of these.

Factor VIII concentrates may be made from the pooled plasma of more than 25,000 donors, intermediate purity concentrates containing only about 0.1% factor VIII and host of other plasma proteins, particularly fibrinogen, and fibronectin. When transfused these constitute an appreciable alloantigen load. It may be that specific alloantigens, viral antigens or large protein load may impair the immune system. This may be important in both HIV positive and HIV negative individuals as we will discuss later.

In the early 1980's the AIDS epidemic started in the hemophiliac population [4]. However, once serological testing for HIV became available, it was clear that not all immunological abnormalities in hemophiliacs were due to infection with HIV and that seronegative multi-transfused hemophiliacs, also demonstrated a variety of abnormalities suggestive of immunological perturbation [5]. Since then many studies have been performed to identify evidence of immunological dysfunction in hemophiliacs and to demonstrate in vitro the suppressive effects of factor concentrates and to isolate the responsible components. More recently clinical studies have focused on the possible role of different concentrates on the progression of HIV infection.

Several studies have shown significant abnormalities in T-cell subset distribution and in T4/T8 ratio. Interestingly more profound abnormalities are seen in individuals with hemophilia A treated with factor VIII than in individuals treated with factor IX which is prepared by a different method and was pre-

viously a purer product. Lymphocyte functional abnormalities have also been demonstrated, and diminished ex-vivo response to broad spectrum mitogens like phytohemagglutinin and concavalin-A have been described [6]. In view of the important role of natural killer cells in immuno-surveillance, Matheson's description of impaired response of natural killer cells to beta and gamma interferon in HIV negative individuals is worrying [7]. This may be due to eventual down regulation of interferon receptors as a result of continuous stimulation by foreign antigens possibly contained in concentrates.

Hypergammaglobulinemia reflecting persistent B-cell stimulation has also been documented in some but not all studies [5].

T-cell activation in HIV negative hemophiliacs has been demonstrated by several studies in which a variety of activation markers have been studied. Most recently Chellucci demonstrated increased expression of CD3 and DR markers in HIV negative as well as HIV positive individuals [8]. Other studies have shown increased serum levels of soluble interleukin receptors and beta 2 microglobulin in non-HIV infected individuals [5]. However, not all studies agree with these findings and some studies from large centres have failed to demonstrate evidence of B or T-cell activation. This may be explained by differences in methodology, different exposure to concentrates and possible differences in the progression of liver disease.

Studies showing evidence of T-cell activation are particularly interesting for several reasons. Firstly, in-vitro data suggests that infection by HIV of CD4 positive cells is enhanced if the cells are in an activated state [9], thus raising the possibility that concentrate use predisposes recipients to HIV infection. Secondly, activation of HIV infected cells may increase viral replication and therefore possibly adversely affect disease progression [10]. Thirdly, although in vivo studies may suggest T-cell activation the majority of in-vitro studies demonstrate inhibition of T-cell responses by factor VIII concentrates, although a few studies have shown that some concentrates are capable of providing an enhancing effect on mitogenesis. Inhibition of T-cell responses may of course have significant consequences in particular in relation to increased susceptibility to opportunistic infection and may have been important in the early stages of HIV infection in view of the suggested central role of cytotoxic T-cell response to initial exposure to HIV [11].

A variety of studies have shown that abnormalities of monocyte phagocytosis and down regulation of their Fc receptors can be demonstrated following in-vitro exposure to factor VIII concentrates, however, recent studies have demonstrated that monocyte phagocytic function is acutely inhibited after factor VIII infusion [12]. Inhibition of monocyte function may be important with regard to antigen handling capacity and it is note worthy that clinical sequelae probably related to monocyte dysfunction have been reported.

Comparison of T4/T8 ratios in different groups of transfused and non-transfused individuals demonstrates that in comparison to individuals multiply transfused with single donor products more marked abnormalities of T4 to T8 ratio are seen

in hemophiliacs usually due to a slight fall in T4 accompanied by a slight rise in T8 numbers.

An early study from Edinburgh [13], the first study of a large group of heavily transfused HIV sero-negative individuals, demonstrated decreased CD4 levels in 33% of hemophilia A patients, with less profound abnormalities being seen in patients with hemophilia B. As almost all subsequent studies have shown, the degree of these abnormalities was not related to disease severity or to the amount of concentrate used. They were also not associated with abnormalities of liver function tests or hepatitis B virus status. However, even if these abnormalities are in part due to liver disease this is not surprising, as no correlation is seen between ALT abnormalities and the degree of histological progression of HCV liver disease on biopsy specimens. A follow-up study in 1989 of 59 Edinburgh hemophiliacs showed similar results [5]. On this occasion, however, evidence of lymphocyte activation was sought and found with more than 50% of both hemophilia A and B patients having greater than 5% activated T-cells and 43 and 26% respectively, as opposed to 2% of controls having beta 2 microglobulin levels of greater than 2 mg/l.

Assessing cell mediated immunity by skin testing provides a good functional test of the immune system, depending on both monocyte and T-cell function in both afferent and efferent loops. Early studies on hemophiliacs performed using the neoantigen, dinitrochlorobenzene revealed that the degree of cutaneous anergy in patients with early HIV infection was similar to that seen in heavily treated HIV negative individuals [14]. Cutaneous anergy is a feature of progressing HIV infection, and later studies, performed using the multitest system which assesses recall to 7 commonly encountered antigens showed almost complete anergy in the majority of HIV infected patients 4 years post-infection [5]. Interestingly the impairment of response at this stage was less pronounced in seronegative individuals but as a group these were still abnormal compared to non-transfused controls. Of all the tests of immune dysfunction performed in hemophiliacs only testing of cell mediated immunity has repeatedly provided a direct correlation between the amount of factor VIII concentrate used and degree of depression of responses. Interestingly, correlation with concentrate usage is not seen in individuals with Christmas disease and it would therefore appear that the different method of preparing of factor IX concentrate removes the element(s) responsible for depression of cell mediated immunity. It will be interesting in future to see the results of comparison of cell mediated immunity in individuals changing from intermediate purity to high purity concentrates produced by different techniques of ion exchange chromatography or monoclonal antibody technology.

Although the majority of studies suggest T cell activation in hemophiliacs, most in vitro studies performed to date show evidence for dose dependant inhibition of T-cell and monocyte function by factor VIII concentrate. It is possible that some of the abnormalities seen in this group of patients is a reflection of coinciding

viral infection and liver damage. However in view of the difficulty in monitoring the progression of liver disease it is likely that long term studies of recipients of only virus inactivated products, will provide the answers to these questions.

In view of the large number of hemophiliacs infected with HIV (in the UK 39% of hemophilia A, 5% of hemophilia B patients) and the 100% incidence of hepatitis C virus infection in recipients of non-heat treated concentrates [3], it is crucial that the concentrates least likely to adversely affect the progression of these iatrogenic diseases are used. The availability of new high purity products produced by ion exchange chromatography, monoclonal antibody techniques and recombinant technology have opened up a new range of therapeutic options with possible benefits and disadvantages which require further study.

There is much controversy surrounding the choice of coagulation factor concentrates to be used, especially in HIV infected individuals, the issues being related to the increased costs of producing high purity concentrates, and the lack of substantial evidence for their beneficial effects, especially in view of the generally excellent profile of certain intermediate purity concentrates in terms of viral safety and absence of immunological sequelae in recipients [15]. The UK Haemophilia Centre Directors have now recommended the use of high purity concentrates of factor VIII in this group of patients [16]. However, the debate does not end at this point and further studies to compare the clinical effects of high purity products produced by different methods are now indicated.

The exact mechanism by which concentrates inhibit T-cell responses is not clear. Studies of interleukin-2 secretion in response to mitogenic stimulation in the presence of different factor VIII and factor IX concentrates have shown varying degrees of inhibition by different concentrates; in general less inhibition is found with high purity monoclonal antibody purified products [17]. Interestingly, different methods of viral inactivation produce no significant difference in the inhibitory effect of concentrates, as at one time it had been feared that heat treating concentrates might result in the production of harmful immunomodulating neoantigens and immunoglobulin aggregates. Recent studies have demonstrated 60 and 200kd molecules which may be responsible for these effects [18]. Other studies have suggested that the observation of decreased interleukin-2 secretion may be related to down modulation of interleukin-2 receptors [19], the expression of which are required for optimal interleukin-2 secretion. These studies also indicated down regulation of HLA class II antigen and CD71 expression suggesting that concentrates may inhibit a very early stage of lymphocyte activation.

Monocyte dysfunction has been demonstrated immediately following factor VIII infusion. Other studies have shown that in vitro monocyte phagocytic function and Fc receptor expression are depressed in the presence of factor VIII concentrates [12,20]. Further it has been demonstrated that although all purities of factor VIII have inhibitory effects of monocytes, monoclonal antibody purified concentrates appear to be significantly less suppressive [12]. As in the case of

inhibition of interleukin-2 secretion from lymphocytes the inhibitory effects on monocytes appear to be independent of virus inactivation methods. Gel filtration studies have shown that a high molecular weight fraction containing red cell antigen and antibody may be responsible for inducing monocyte dysfunction [20], again suggesting a possible advantage in using monoclonal antibody purified products, although an early monoclonal antibody produced product has been shown to demonstrate inhibitory effects similar to intermediate purity products possibly due to the presence of VIII anti-VIII complexes, the inhibitory effect which have previously been demonstrated [21].

What is the clinical importance of these observations?

Four main areas require brief review. These are, anti-factor VIII inhibitor formation, problems with infection, effects of concentrates on progression of HIV infection and some information on the progression of liver disease.

As early studies into the possible beneficial effects of high purity concentrates began matters became further complicated by the observation that patients receiving high purity or recombinant concentrates seemed to have a higher incidence of inhibitors, than patients treated with intermediate purity concentrate [22]. It was suggested that this may be due to slightly altered stereochemistry of the recombinant protein and possibly due to the absence of other inhibitory proteins from high purity products. However, a recent prospective study over 15 years has demonstrated that in individuals frequently tested for inhibitors the incidence may be as high as 33% [22]. Interestingly, amongst patients developing inhibitors, very few were of clinical significance, often being transient and at a low titre.

Are HIV negative hemophiliacs at greater risk of infection? Studies of patients undergoing surgery have shown that the incidence of post-operative wound infection in hemophiliacs is equal to that observed in non-hemophiliacs [24]. A study of patients post-arthroplasty, however, suggested a higher incidence of joint infection in both HIV-positive and negative patients. A study based on death certificates in the pre-AIDS era in the USA did show that an increased proportion of hemophiliacs succumbing to non-bleeding deaths were dying from infection, especially due to pneumonia [25]. There may, however, be other explanations such as immobility as a result of non-fatal bleeding episodes. Few other cases or examples of increased susceptibility to infection have been reported. The one outstanding report of apparent increased susceptibility to infection has come from Birmingham Childrens Hospital in the UK. 38% of a population of hemophilic boys exposed to an index case, developed pulmonary tuberculosis [26]. The observation that the incidence was similar to that seen in children who were severely immunocompromised as a result of receiving high dose chemotherapy for leukemia and solid tumours was felt to be very significant. Interestingly, the degree of immunosuppression appeared to be slightly less in hemophiliac children who handled the disease as a primary infection unlike the children with malignant disease many of whom developed miliary tubercu-

losis. The children who did develop TB had significantly higher use of blood products. Patients using factor VIII, factor IX and cryoprecipitate all succumbed to TB suggesting that clinically significant impairment of cell mediated immunity may result from the use of all three of these blood products. The development of oesophageal candidiasis in an HIV negative woman with Von Willebrands disease who had received concentrate on several occasions [27] has been reported as an example of clinically significant immunosuppression in the absence of any other predisposing illness.

The high prevalence of HIV infection in hemophiliacs has resulted in concern over the possible adverse affects of concentrates in HIV disease progression. Does concentrate and its purity affect progression?

Several large epidemiological studies suggest that the rate of progression to AIDS in hemophiliacs is equal to that seen in non-hemophiliacs [28]. Further Goederts studies show an equal rate of progression in mild, moderate and severe hemophiliacs and also no difference between patients with hemophilia A or B [29], suggesting that the use of concentrates of factor VIII and IX are not risk factors for more rapid progression of HIV infection.

Therefore on what grounds have physicians caring for hemophiliacs decided to change particularly HIV infected individuals to high purity products?

Evidence from in vitro studies suggests that cell activation plays a possible role in enhancing viral replication and also in increasing the susceptibility of cells to HIV infection. Studies investigating P24 antigen production by infected PBMCs have demonstrated enhancement of production in the presence of intermediate purity factor VIII concentrates. If this was to occur in vivo, then concentrates might well adversely affect the course of HIV infection.

Clinical studies have shown conflicting results as to the benefits of concentrates produced using monoclonal antibodies on disease progression.

Of the studies performed to date only those of Goldsmith [30] and de Biasi [31] have suggested a possible benefit for treatment of HIV infected hemophiliacs with high purity products.

In de Biasi's 2 year study 20 hemophiliacs were followed, 10 continuing on intermediate purity concentrate (Kryobulin), 10 changing to Hemofil M a monoclonal antibody produced product. The results demonstrated comparative stabilization of CD4 counts in the group changed to high purity, with the predictable fall seen in the group continued on intermediate purity concentrate. Further in the high purity group two regained cutaneous responses to recall antigens and none showed clinical evidence of disease progression. In Goldsmith's shorter study, 13 HIV positive hemophiliacs changed to high purity monoclonally purified concentrate showed significantly less decline of CD4 counts over a 15 month period than a matched group who continued on intermediate purity concentrate. Other larger studies have failed to demonstrate any of these benefits [32].

Finally does concentrate affect liver disease?

It is certainly noteable that the first two liver transplants carried out in the UK in hemophiliacs were on individuals with mild hemophilia and end stage liver disease. Based on these observations Makris studied the severity of liver disease in biopsy specimens of 60 hemophiliacs and found that significantly more individuals with mild hemophilia and therefore infrequent transfusion of clotting factor concentrates had severe liver disease [33]. The explanation of these findings must be speculative, however, if as has been suggested control of liver disease in hemophiliacs is partially due to the immunosuppressive effect of con-centrates then this may be a cause for concern when patients are changed from intermediate to high purity concentrate, as we move towards an era of high purity products.

Summary

Whilst there is little doubt that a variety of immunological abnormalities are seen in the immune system of HIV-negative hemophiliacs, there is little evidence that significant morbidity and mortality has arisen as a result of concentrate use per se. It is important that concentrates least likely to exacerbate the course of HIV infection are used, however at present the in vitro evidence in favour of high purity concentrates is stronger than that from clinical studies.

Studies into the immunological sequelae of transfusion of pooled products are important and should continue with long term follow-up of large numbers of patients being easily facilitated by the present set of regional hemophilia centres in Western Europe and North America. However it should be remembered that the incidence of hemophilia is constant worldwide and we must therefore not loose sight of the goal to provide safe, virus inactivated, efficacious concentrates for all hemophiliacs.

Acknowledgement

The authors thank Miss Audrey Trotter for her assistance in preparing the manuscript.

References

1. Pierce GF, Lusher JM, Brownstein AP, Goldsmith JC, Kessler CM. The use of purified clotting factor concentrates in hemophilia. Influence of viral safety, cost and supply on therapy. JAMA 1989;261:3434-8.
2. Stirling ML, Murray JA, MacKay P, Black SH, Peutherer JF, Ludlam CA. Incidence of infection with hepatitis B virus in 56 patients with haemophilia A. J Clin Pathol 1983;36:577-80.
3. Watson HG, Ludlam CA, Rebus S, Zhang LQ, Peutherer JF, Simmonds P. Use of several second generation serological assays to determine the true prevalence of hepatitis C virus infection in haemophiliacs treated with non-virus inactivated factor VIII and IX concentrates. Brit J Haematol 1992;80:514-8.

4. Centres for Disease Control. Pneumocystis carinii pneumonia among persons with hemophilia A. Morbidity and Mortality Weekly Report 1982;31:365-7.
5. Cuthbert RJG, Ludlam CA, Steel CM, Beatson D, Peutherer JF. Immunological alterations in HIV seronegative haemophiliacs: Relationships to blood product therapy. Brit J Haematol 1992;80:364-9.
6. Sullivan JL, Brewster FE, Brettler DB, et al. Hemophiliac immunodeficiency: Influence of exposure to factor VIII concentrate. LAV HTLV-III, and herpes viruses. J Pediatr 19??;108:504-10.
7. Matheson DS, Green BJ, Poon MC, Fritzler MJ, Hoar DI, Bowen TJ. Natural killer cell activity from hemophiliacs exhibits differential responses to various forms of interferon. Blood 1986;67:164-7.
8. Chellucci C, Hassan HJ, Gringeri A, et al. PCR analysis of HIV-1 sequences and differential immunological features in seronegative and seropositive haemophiliacs. Brit J Haematol 1992;81:558-67.
9. Gowda SD, Stein BS, Mohagheghpour N, Benike CJ, Engleman EG. Evidence that T cell activation is required for HIV-1 entry into CD4+ lymphocytes. J Immunol 1989;142:773-80.
10. Zagury D, Bernard J, Leonard R, et al. Long-term cultures of HTLV-III infected T cells: A model of cytopathology of T-cell depletion in AIDS. Science 1986;231:850-3.
11. Autran B, Letvin NL. HIV epitopes recognised by cytotoxic T lymphocytes. AIDS 1991;5(suppl 2):S145-50.
12. Pasi KJ, Hill FG. In vitro and in vivo inhibition of monocyte phagocytic function by factor VIII concentrates: Correlation with concentrate purity. Brit J Haematol 1990;76:88-93.
13. Carr R, Veitch SE, Edmond E, et al. Abnormalities of circulating lymphocyte subsets in haemophiliacs in an AIDS free population. Lancet 1984;i:1431-4.
14. Madhok R, Gracie A, Lowe GD, et al. Impaired cell mediated immunity in haemophilia in the absence of infection with human immunodeficiency virus. Brit Med J [Clin Res] 1986;293:978-80.
15. Evans JA, Pasi KJ, Hill FG. Consistently normal CD4, CD8 levels in haemophilic boys only treated with a virally safe factor VIII concentrate (BPL 8Y). Brit J Haematol 1991;79:457-61.
16. UK Regional Haemophilia Centre Directors Committee. Recommendations on choice of therapeutic products for the treatment of patients with haemophilia A, haemophilia B and von Willebrand's disease. Blood Coagulation and Fibrinolysis 1992;3:205-14.
17. Thorpe R, Dilger P, Dawson NJ, Barrowcliffe TW. Inhibition of interleukin-2 secretion by factor VIII concentrates: A possible cause of immunosuppression in haemophiliacs. Brit J Haematol 1989;71:387-91.
18. Wadhwa M, Dilger P, Tubbs J, Barrowcliffe TW, Mahon B, Thorpe R. Mechanisms of inhibition of T-cell IL-2 secretion by factor VIII concentrates. Brit J Haematol 1992;82:575-83.
19. Hay CR, McEvoy P, Duggan-Keen M. Inhibition of lymphocyte IL-2 receptor expression by factor VIII concentrate: A possible cause of immunosuppression in haemophiliacs. Brit J Haematol 1990;75:278-81.
20. Mannhalter JW, Ahmad R, Leible H, Gottlicher J, Wolf HM, Eible MM. Comparable modulation of human monocyte functions by commercial factor VIII concentrates of varying purity. Blood 1988;71:1662-8.
21. Mannhalter JW, Ahmad R, Eibl MM, Leibl H. Modulation of human monocyte functions by factor VIII anti-factor VIII complexes present in an affinity-purified factor VIII (letter). Blood 1990;75:810-1.
22. Schwartz RS, Abildgaard CF, Aledort LM, et al. Human recombinant DNA derived anti-hemophilic factor (FVIII) in the treatment of hemophilia A. N Engl J Med 1990; 323:1799-805.

23. Ehrenforth S, Kreuz W, Scharrer I, et al. Incidence of development of factor VIII and factor IX inhibitors in haemophiliacs. Lancet 1992;339:594-8.
24. Buehrer JL, Weber DJ, Meyer AA, et al. Wound infection rates after invasive procedures in HIV-1 seropositive versus HIV-1 seronegative hemophiliacs. Arch Surg 1990;211:492-8.
25. Aronson DL. Cause of death in hemophilia A patients in the United States from 1968 to 1979. Am J Hematol 1988;27:7-12.
26. Beddall AC, Hill FG, Geroge RH, Williams MD, Al-Rubei K. Unusually high incidence of tuberculosis among boys with haemophilia during an outbreak of the disease in hospital. J Clin Pathol 1985;38:1163-5.
27. Watson HG, Goulden NJ, Ludlam CA. Oesophageal candidiasis in an HIV-negative individual treated with factor VIII concentrate. Trombosis and Haemostasis 1993; (in press).
28. Eyster ME, Gail MH, Ballard JO, Al-Mondhiry H, Goedert JJ. Natural history of human immunodeficiency virus infections in haemophiliacs: Effects of T-cell subsets, platelet counts and age. Ann Int Med 1987;107:1-6.
29. Goedert JJ, Kessler CM, Aledort LM, et al. A prospective study of human immunodeficiency virus type 1 infection and the development of AIDS in subjects with haemophilia. N Engl J Med 1989;321:1141-8.
30. Goldsmith JM, Deutsche J, Tang M, Green D. CD4 cells in HIV-1 infected haemophiliacs: Effect of factor VIII concentrates. Trombosis and Haemostasis 1991;66: 415-9.
31. De Biasi R, Rocino A, Miraglia E, Mastullo L, Quirino AA. The impact of a very high purity factor VIII concentrate on the immune system of human immunodeficiency virus infected haemophiliacs: A randomised, prospective, two-year comparison with an intermediate purity concentrate. Blood 1991;78:1919-22.
32. Mannucci PM, Gringeri R, De Biasi R, Baudo F, Morfini M, Cigvarella N. Immune status of asymptomatic HIV-infected hemophiliacs: Randomised, prospective two year comparison of treatment with a high purity or an intermediate purity factor VIII concentrate. Trombosis and Haemostasis 1992;67:310-3.
33. Makris M, Preston FE, Simmons K, Triger DR, Underwood JCE. The natural history of chronic liver disease in haemophilia; more rapid progression relates to age and mild disease. Abstracts International Society of Haematology 1992; p. 73.

TRANSFUSION MEDICINE AND IMMUNOLOGICAL RESEARCH: PERSPECTIVE FOR THE FUTURE

T.H. The

The title of this presentation could erroneously suggest a knowledge about the future, but it is my privilege to pose the question: "Where do we stand now and where to go?"

Since we all have witnessed the enormous progress made in the field of immunology and blood transfusion it seems a paradox to state that transfusion medicine is still in its infancy. Professor Jon van Rood recently expressed this situation as "practise as a doctor without being registered or qualified". Indeed, many doctors who prescribe blood transfusions do not have the knowledge and the training to recognize and to deal with the clinical side-effects of blood transfusions, nor are they trained to treat these clinical complications in a correct way.

Blood transfusions may modulate the fundamental immunological regulation pathways of the host in a pluripotential way of action which are governed by many external and genetic factors. It is clear that all these variables clouded the field of investigation. We still do not know how to achieve the beneficial immunological blood transfusion effects and to avoid its deleterious, unwanted ones. However, results achieved in recent years in the clinical immunological field of organ transplantation are remarkable and may serve as an example of a characteristic multidisciplinary research.

What are the conditions for inducing tolerance by pre-transplantation blood transfusion? Results from clinical observations have shown a down-regulation of immunity to a subsequent organ graft if pre-transplant blood transfusion is given under the condition that blood donor and recipient share at least one HLA molecule. A single pre-transplant blood transfusion can significantly facilitate the acceptance of a subsequent transplanted kidney allograft. The studies of Lagaaij et al. [1] from the group of Van Rood have shown that the presence of a single "autologous" HLA class II antigen on the allogeneic cells of the transfusion donor determines whether or not blood transfusion will lead to immunization or to immune suppression. In a retrospective study renal allograft recipients showed an increased graft-survival if they received a pre-transplant blood transfusion with one DR antigen matched. Patients transfused with 1 DR antigen matched showed a 5 year graft survival of 81% compared to the group trans-

fused without DR matching and the group without any transfusion, showing 5 years graft survival rates of 57% and 45% respectively. This observation suggests that recognition of "self" molecules down-regulates immune responsiveness. Thus, for tolerance induction donor and recipient should share a common DR antigen of HLA class II.

It was recently shown by Van Twuyver et al. [2] that we can monitor this tolerizing effect of blood transfusion by measuring cytotoxic T lymphocyte precursor frequencies. A significant down-regulation was observed when donor and recipient share one DR antigen. This decline of cytotoxic T lymphocyte precursors could not be seen before a latency period of 4 weeks after transfusion and appeared to have a long-lasting effect for at least one year post-transfusion. These results suggest that tolerance induction is caused by a low grade chimerism of transfused donor cells in the recipient.

The cellular mechanisms for tolerance induction are still unknown but it is evident that similarity is required between donor and recipient. Recent observations by others and our group suggest that foreign antigens showing immunological cross-reactivity with HLA ("molecular mimicry") may likewise modify immune responsiveness. Fujinami et al. [3] described sequence homology and immunological cross-reactivity between the cytomegalovirus immediate early antigen-2 (CMV IE-2) and positions 53-57 of the HLA-DR β chain.

We have shown, in collaboration with Dr. Lagaaij, that virus-specific T cell responses against CMV antigens are down-regulated in individuals who have the haplotypes DRw13, Dw18, DQw6, DRw14, Dw9, DQw5; and DRw15 (DR2), DQw6. These haplotypes have in common that they share a 5 out of 6 amino acid sequence with the CMV IE-2 protein at positions 52-57 of HLA-DQ β. Roenhorst et al. of our group [unpublished data] showed that T cell memory responses against cell-associated CMV antigens are significantly down-regulated after blood transfusion. A remarkable heterogeneity and dichotomy of the effect can be seen. These cross-reactivities might form one explanation for the well-known depression of immune reactivity that can be observed both in vivo and in vitro in patients with CMV infection.

From these results it can be concluded that the host immune T cell repertoire is down-regulated when there is a similarity between HLA and the eliciting antigen. This immunological rule applies already for a similarity of a short stretch of 6 amino acids between the foreign antigen and self DR or DQ antigens in the β chain of HLA class II. Under these conditions the immune recognition of "self" molecules in foreign antigens could down-regulate specific immune responses.

The foregoing research illustrates that transfusion medicine offers fruitfull challenges for clinical immunology, perspectives for future research because it is possible to modulate and down-regulate specific immune responses of patients. We know some of the conditions to achieve tolerance by pre-transplant blood transfusion, we can monitor its effect and perhaps we know the mechanisms. This

BLOODTRANSFUSION-IMMUNOLOGY

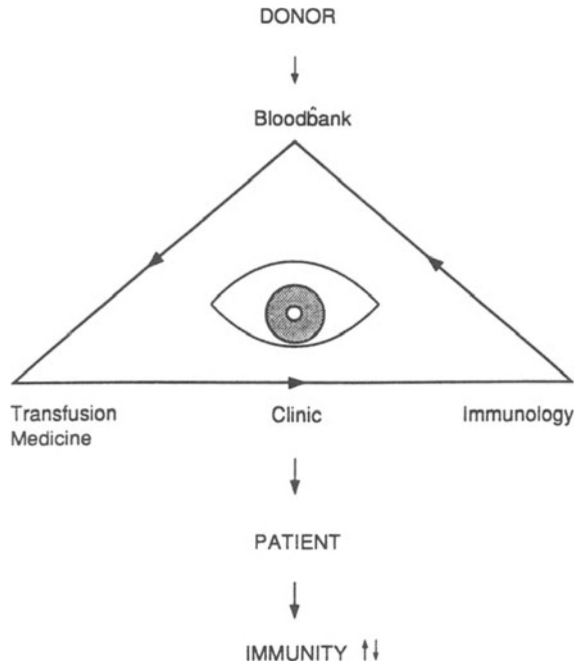


Figure 1. **Bloodtransfussion-immunology**

is an opening of a new, broad road for the treatment of immunological diseases. The challenge for the future is to adopt these immunomodulating therapeutic interventions in clinical practice. This future is not ours to say, neither can we look into this future but an important strategy for the achievement is a strong alliancy between the blood bank, transfusion medicine and immunology. Our belief in success is based on our admiration for the immune system. This may inspire us all to find new therapeutic strategies of blood and blood components for the treatment of our patients.

We may conclude that we still live in a stage of premature knowledge about the immunomodulating effects of allogeneic blood transfusions. Further progress seems to be dependent on the development of the "clinical interface" (Cash, 1992 [4]) and the maturity of transfusion medicine as a clinical discipline in a close interrelationship with clinical immunology and cell biology (Figure 1). Such multidisciplinary clinical and laboratory research may have the competence to meet the challenging goals of the future.

References

1. Lagaaij El, Henneman JPH, Ruigrok M, et al. Effect of one HLA-DR antigen matched and completely HLA-DR mis-matched blood transfusions on survival of heart and kidney allografts. N Engl J Med 1989;321:701-5.
2. Van Twuyver E, Mooijaart RSD, ten Berge IJM, de Waal LP. Pretransplantation blood transfusion revisited. N Engl J Med 1991;325:1210-3.
3. Fujinami RS, Nelson JA, Walker L, Odstone MBA. Sequence homology and immunologic cross-reactivity of human cytomegalovirus with HLA-DR β chain: A means for graft rejection and immunosuppression. J Virol 1988;61:100-5.
4. Cash JD. Transfusion medicine today and tomorrow: The facts and the fiction. In: Smit Sibinga CTh, Das PC, Cash JD (eds). Transfusion medicine: Facts and fiction. Boston/Dordrecht/London: Kluwer Academic Publishers 1992:191-4.

DISCUSSION

C.Th. Smit Sibinga, T.H. The

C.F. Högman (Uppsala, S): A comment on dr. Lee's very nice presentation. A problem, that we are facing, which was addressed by dr. Rebulla, is that we need a very even quality of our product. It can be questioned, whether we in fact could reach these goals sufficiently, at least with the presently available technique. Because as was very nicely shown in your presentation, one single failure can be sufficient to introduce the unwanted immunological effects. As dr. Rebulla showed even if we have a confidence limit of two standard deviations, it will not be a rare event, that we will have products which do not live up to the expectations. Do you have any comments?

E.J. Lee (Baltimore, MD, USA): I think the comment is extremely well taken. This constitutes one of the arguments for evaluating UV-B irradiation. The irradiation is more certain as opposed to depending on the filtration system. Fewer variables are involved with UV-B than with filtration in which flow rate and issues pertaining to bedside filtration may affect the outcome. Obviously, there are few clinical data on UV-B at the moment.

T. Warkentin (Hamilton, C): Dr. Lee, as you indicated platelet transfusion refractoriness does not equal alloimmunization. In our experience, platelet transfusion refractoriness can be seen following the first platelet transfusion. These patients are usually very sick, with multiple complex problems, and the refractoriness could be related to infection in some patients, to immune thrombocytopenia in others, perhaps by the sort of bystander immune mechanisms we heard about earlier in the programme involving the red cells, etc. So my question is: Even if leukodepletion works, i.e., even if it prevents HLA-mediated alloimmunization, what do you think would be the real impact on platelet recoveries? Perhaps platelet recovery will continue to be fairly low, and perhaps ongoing non-alloimmune refractoriness will not justify the efforts to prevent just one of the causes of refractoriness. What are your comments?

E.J. Lee: I think dr. Brand alluded to the issue of the magnitude of the problem. The magnitude of the problem is what we are able to change and what we cannot. I think that there are many causes of platelet refractoriness, yet alloimmuni-

zation is often a non-reversible phenomenon, that limits the availability of treatment to patients in the future. For that reason, I think we should continue our efforts towards trying to influence those mechanisms of refractoriness than can be affected.

H.T. Meryman (Rockville, MD, USA): I have a comment on that. In a collaborative study of leukocyte-depleted platelet transfusions in bone marrow transplant patients at John Hopkins University, in both the control and in the experimental group, all of our patients who became refractory were patients who had developed HLA alloimmunization. !1

A comment also on dr. Houbiers' presentation, in which he implied that if anergy were to be the cause of the tumour-stimulating effect, one would have to assume that there was a specific antigen common both to the blood transfusion and the tumour. I do not feel that it is necessary to be that restrictive. We know that one can develop immunoresponses of very broad specifity from transfusions. There are reports of individuals exposed to a single donor who become positive to the majority of cells in a typing panel. If we assume that the anergy that one might develop would be the mirror image of the potential alloimmunization, one might therefore acquire a tolerance of equally broad specificity.

J.G.A. Houbiers (Leiden, NL): On your first comment: The difference in number of refractory patients between the study groups counts; it is never a black/white situation.

Well, to what dr. Smit Sibinga already said: The answer will take a new symposium. This was just one of several hypotheses for the observed clinical blood transfusion effect, which, besides, still has to be proven itself. Some of the underlying immunological mechanisms, effector arms, might however be similar. Specific as well as aspecific immunosuppressive effects of blood transfusion have been reported. In the case of alloimmunization quite a fraction of the recipient T cells are activated: The allo-precursor frequency is high. The precursor frequency for such rare antigens as tumour specific antigens is very low. Improper presentation by donor cells carrying such an antigen to these few recipient T cells will anergise, tolerise only a small fraction of the total T cell repertoire. So, I do not really see the model as a mirror image of alloimmune response suppression (like renal transplantation and blood transfusion).

C.Th. Smit Sibinga (Groningen, NL): I think, one of the elegant things in the set-up of dr. Houbiers' study is, that he started to clearly define the present information in what is called "correlation based on retrospective information", which just gives an idea of what might be a relationship and the unravelling of a real course of the relationship. In this respect indeed there is much still uncertain.

1. Briane HG, Meryman HT, Holland HK, et al. Alloimmunization to HLA antigens in bone marrow transplant patients receiving red cells and platelets with very low residual leukocyte counts. (Manuscript in preparation).

P. Kühnl (Hamburg, D): Dr. The, you presented the data of dr. Lagaaij which showed, that the transfusion of a unit of blood with at least one common HLA-DR type, gives an advantage over five years of some 24%. This is in controversy to what has been described in the Collaborative Transplant Study of Opelz[1] and co-workers, where they evaluated the donor specific transfusions of living related kidney donors. There was no benefit of a donor specific transfusion compared with a random or "third party" transfusion. Would you like to comment on the CTS data on one side and the Eurotransplant/Leiden data of dr. Lagaaij on the other?

T.H. The (Groningen, NL): In these clinical studies for me the problem is in the criteria patients had to meet to be included into the study. Dr. E.L. Lagaaij just eliminated a lot of patients, that had expectancies for instance of having other antibodies. I am not sure whether Opelz had done that, too.

J.G.A. Houbiers: This is important, but moreover, the chance of having an HLA-DR matched blood transfusion is 50%. Receiving one blood transfusion gives a 50% change for the effect dr. Lagaaij demonstrated. If Opelz' patients received two transfusions or three they must be at least as immunosuppressed as dr. Lagaaij's patients.

C.Th. Smit Sibinga: Dr. Cottler, from the list of limitations it is quite evident that the adverse effects of interleukin-2 are basically the most limiting factor at this point in time, but also the cost and the availability of IL-2 play a role. I recently became aware of the change in the regime in specific the kidney tumours, in which IL-2 is not given systematically but subcutaneously reducing quite some of the systemic side-effects. Could you comment on that? Maybe you also could give us your view on the retargeting phenomenon as discussed by dr. de Leij, which is a very promising, active and new tool in the targeting towards tumour cells.

M. Cottler-Fox (Bethesda, MD, USA): On IL-2, I think you state the question and the answer quite clearly, that there is data to suggest that low dose continuous infusion of IL-2 is as effective if not more effective than high-dose bolus and that it is considered less toxic. I think the subcutaneous injection simply follows along that line: A slower more continuous release, a lower dose and much less toxicity. Those studies are ongoing.
I do not know what to say, unfortunately, about the retargeting.

T.H. The: You made a plea for HLA tied CTL clones to treat CMV infections. That is very interesting. I have an intellectual hesitation since clones represent a

1. Opelz G. HLA antigen sensitization: A problem in graft survival. Transplant Proc 1989;(suppl 2):39-41.

small specificity on epitope level. What is the specificity to choose to be effective. I do not know anything about that.

M. Cottler-Fox: The people in Seattle, who are doing the work, claim that they are specifically trying to select multiple clones, which would give them a broad specificity.

T.H. The: Then, of course, the problem is the indication and when to start. I think that is a major investment and one should apply that for tailor-made situations with regard to the CMV host circuit and the antigenemia. I think they have adopted that technology also in Seattle.

M. Cottler-Fox: The Seattle current study is specifically a prophylactic trial to look both at toxicity and therapy as well as prevention of CMV disease since even with acyclovir and IVIg therapy, you still have something like a 50% mortality rate with CMV pneumonia. It seemed a reasonable patient population in which to try a very new and speculative therapy.

P.L. Yap (Edinburgh, UK): Dr. Cottler-Fox, I thought your figure for ADA deficiency of 1:100,000 was actually about ten times higher than my impression of the incidence. Maybe there are geographical differences. The children with ADA deficiency, that you are treating by gene therapy, do you envisage the fact, that you will have in fact to carry on with the therapy for the rest of their lives, unless you manage to put the gene into a truly long-lived cell? Also, how do you envisage such a large group like the group of dr. Rosenberg be involved in a routine blood bank in the development and implementation of gene therapy?

M. Cottler-Fox: The percentage of ADA deficient children, that I showed is the one used by the Blease and Anderson group at NIH, I believe they mean it to refer to the most severely effected children. There are children with reasonable levels of ADA although not normal levels and they are not normally as ill as the people that the protocol was meant to treat although I have to admit the first two children were not terribly ill when they entered the protocol. I think that it will be unavoidable that medicine in the very near future will wish to involve many new trials of gene therapy. Although the expansion of cells with new genes in them is a very large undertaking, there are a sufficient number of researchers interested in making the technique easier. Than eventually a smaller group would be possible. But more importantly I think it is a mistake to let the production and the issuing of these new products for transfusion into immunocompromised hosts fall into the hands of people, who do not have the training and quality control to really control and care in issuing the appropriate product to the correct patient that blood bankers have as a reflex. I think, if this kind of new transfusion therapy, if you will, is undertaken by other groups of people, there will be far more mistakes made early on than would be the case if transfusion medicine people became involved early. So that I think even a small blood bank which

cannot undertake to collect, grow and expand these cells themselves should nonetheless be involved in the procedures used and certainly in the quality control and issuing of them. Every institute will have to work it out among themselves, but I think there must be a transfusion medicine person involved as early as possible.

C.Th. Smit Sibinga: I think, you are quite right. It is another plea for a serious reconsideration on a minimal size and a minimal infrastructure in the blood banking community to really bridge both the shores from donor to the patient, including these developments, which are no longer on the horizon, they have become reality. That means that we have to change our own scenery and load up our batteries for this future which has started already today.

P.L. Yap: What about those children with ADA deficiency on gene therapy? Will they need to continue for the rest of their lives.

M. Cottler-Fox: Well, first of all it was a question of how long the T-cells would survive. They now appear to survive far longer in good numbers than anyone initially anticipated they would. After six months off therapy completely, the first trial still had very good levels of ADA transduced T-lymphocytes in the peripheral blood. However, we are all aware that we would like to be able to stop giving them repeated infusions. There is a group in Italy which has already undertaken to put the ADA gene into bone marrow derived stem cells. I do not know with what success, although the protocol was ongoing. My own institute within the next two months will attempt to put the gene into peripheral blood derived stem cells in the hope, that we may eventually be able to do it once and for all.

C.Th. Smit Sibinga: Dr. van Dijk, could it be that this bystander immune phenomenon could play a role not only in cytolysis, but also in the suppression as a result of regular transfusion?

B.A. van Dijk (Nijmegen, NL): Yes I think so, the report of Kaminsky[1] I showed is one example of it. It was not only a transfusion, but it was also retrospectively seen in many transfusions in aplastic anemia patients. They all had more or less autoreactivity against cytotoxic T-cells. That is not because they were recently transfused, but also in the past.

C.Th. Smit Sibinga: There might be a clue in this phenomenon to the questions that were left open by dr. Houbiers: Eventually through the homologous transfusion an autologous phenomenon of tolerance might be induced.

1. Kaminsky ER, Hows JM, Goldman JM, Batchelor JR. Lymphocytes from multi-transfused patients exhibit cytotoxicity against cells. Brit J Haematol 1992;81:23-6.

B.A. van Dijk: I think it is very intriguing to see the autoantibody production against red cells after transfusion reactions. It is a phenomenon, that you see more and more in a hospital transfusion setting. If you find an alloantibody in the patient and you follow it up, you will find sometimes positive direct antiglobulin test long persistent plus the induction of autoantibodies. I think it is very regular, if you look for it.

C.Th. Smit Sibinga: There seems to be a parallel to dr. Watson's presentation in what happens in these hemophiliacs when we repeatedly burden them with foreign proteins. What are your thoughts about the immune bystander phenomenon, so the autologous lymphocytes being stimulated in their autoimmune function of suppression by the load of proteins over the time.

H. Watson (Edinburgh, UK): As a comment I think it is very speculative.

C.Th. Smit Sibinga: I was intrigued by the information, although very preliminary as you said, on the difference in behaviour between those patients being treated with ion exchange material versus those treated with immune adsorption material. Is that in the purity eventually or do you think there is anything changed to the molecules or what would you think would be the mechanism.

H. Watson: I showed you from the laboratory work, basically the problem confronting hemophilia centre directors. The inhibitory effects of high purity concentrates on lymphocyte proliferation appear in general to be less than those of intermediate purity concentrates, however, you know as I said, that the improved profile of the monoclonal purified products may not be seen in similar high purity products produced by ion exchange method.[1] It may not be simply a matter of purity, it may relate to another mechanism. The differences between such products may clearly be important.

C.Th. Smit Sibinga: It might be interesting to watch that for the near future, because specifically in Europe the market is more or less 50% divided into ion exchange and the immune adsorption chromatography type of materials.

P. Kühnl: Dr. Houbiers, concerning the CRAB study results, in Germany there are about 50,000 new cases of colorectal cancer every year and there is a strong legal pressure on doctors to provide autologous blood transfusion to patients, if this is a less risky procedure. I am very curious about any early recommendations derived from the intermediate results the CRAB study,[2] should we offer

1. Hay CTM, McEvoy P. Purity of factor VIII concentrates. Lancet 1992;i:1613.
2. Busch ORC, Hop WCJ, Hoynck van Papendrecht MAW, Marquet RL, Jeekel J. Autologous versus allogeneic blood transfusion in colorectal cancer surgery; first results from a randomized multicenter trial. Informationstagung über Eigenbluttransfusion. April 24-25, 1992.

this alternative to a fraction of (elderly) patients, who suffer from colorectal cancer? In Germany, there is one small study from Munich,[1] which shows prospectively in some 50 patients, that homologous blood may be detrimental concerning the infectivity rate and the metastasis rate in these patients. Could you possibly give a comment on the CRAB study recommendations; are there any?

J.G.A. Houbiers: There are no final recommendations. A Rotterdam based multicentre clinical trial with over 500 patients, compared patients receiving standard homologous blood with patients receiving predeposit autologous blood. They could not demonstrate a difference in recurrence or survival rate. In this respect autologous blood seems not a saver solution. Analyzing their data retrospectively the disadvantage of transfused patients in general again was observed. It was shown that donation of blood was followed by a 2 weeks period of depressed natural killer cell activities. Avoidance of blood transfusion still must be the main concern. The results of our clinical trial (the CRAB project) in which we hypothesize that depletion of leukocytes prevents the deleterious blood transfusion effect will be available next year.

1. Heiss MM, Mempel W, Jauch KW, Dieterich W, Peter K, Schildberg FW. Multizentrische Studie zur Auswirkung der Eigenbluttransfusion auf das Ergebnis der Tumoroperation. Informationstagung über Eigenbluttransfusion. April 24-25, 1992.

INDEX

α-1-antitrypsin deficiency, 227
A23187, 3–4, 6
accessory signal, 14–15
acute graft-versus-host disease, 93–94
acyclovir, 292
ADA deficiency, 242, 292–293
ADA deficient, 292
adoptive immunotherapy, 239, 241–242
αFP, 269
AIDS, 173, 280
AIHA, 199
albumin, 137
allogeneic blood transfusions, 266
allogeneic bone marrow, 45
allogeneic bone marrow transplantation (BMT), 46, 51–52, 87, 93–94, 246, 257
allogeneic peptides, 81
allograft, 85, 87, 91
allograft rejection, 14–15
alloimmunization, 11–15, 21, 31, 37, 39–40, 42–43, 57, 60–61, 65, 67, 69, 71, 74, 77, 101–102, 104, 121, 124, 161–163, 208, 245–252, 289–290
allosensitization, 46
anaphylatoxins, 141
ankylosing spondylitis, 200
anti-C3d, 256
anti-CD10, 87, 91
anti-CD19, 87, 91
anti-CD2, 87
anti-CD20, 87
anti-CD3, 92
anti-CD34, 93
anti-CD5, 87
anti-HCV, 183
anti-HIV, 173
anti-HLA antibodies, 57–61
anti-idiotypic antibodies, 57–61, 68, 70
anti-IgG, 256
antibody dependent cell-mediated cytotoxicity, 141
antibody dependent cellular cytotoxicity, 240
antigen presenting cells (APC), 3, 41, 49, 65, 69, 72–73, 82, 161, 240, 246–247, 270
antigen zygosity, 205
antigen-binding capacity, 146
antigenic mimicry, 60
apheresis, 205, 239–240, 242, 245–246, 248, 250
aplastic anemia, 46
auto-cytotoxicity, 256
auto-immune hemolytic anemia, 199
autograft, 85, 87
autologous blood transfusion, 266, 294
autologous bone marrow transplantation (BMT), 49, 68–69, 94
autoreactive T-cells, 50
azathioprine, 11

B-cell, 276
β-thalassemia, 208
β2-microglobulin, 191, 276–277
BFU-E, 91

Biomedical Excellence for Safer Transfusion (BEST), 104, 235
BIS-1, 133–135
bispecific monoclonal antibody, 129, 131–132, 135
bone marrow purging, 85, 88–89, 91–92, 94
bone marrow transplantation (BMT), 45–46, 49–51, 85, 101, 201, 236, 245–248, 257
bystander hemolysis, 256

cancer recurrence, 261, 263
cap formation, 22–23, 25–29, 31, 33
capillary leak syndrome, 130
CD10, 88
CD14, 21
CD19, 269
CD20, 166
CD24, 164–165
CD3, 132, 276
CD3 receptor, 3, 6
CD33, 92
CD34, 210, 236
CD38, 210
CD4, 6, 14–15, 65, 133, 187, 189, 196, 276–277, 280
CD4+, 49–50, 77, 79, 81, 240
CD4/CD8 ratio, 268, 275–276
CD71, 278
CD8, 6, 72, 133, 187, 191
CD8+, 49–51, 240–241
CD9, 164
CEA, 134–135, 269
cell culture techniques, 239
cell mediated immunity, 277
cell mediated lympholysis, 80
cellulose-acetate column filter, 109–111, 113, 115–117
cervical carcinoma, 269
CFU-GM, 91–92
Chagas' disease, 203
chemoattractants, 141
chimerism, 82
Christmas disease, 277
chromogenic substrate, 154
class I HLA antigens, 49
class I MHC, 13
class II HLA antigens, 49
class II major histocompatibility complex, 14, 21
classical pathway, 143
CML, 80
CMV, 99, 102, 104, 121, 124, 232, 241, 291–292
CMV IE-2, 286
CNBr-activated resins, 156, 158
CNBr-activated Sepharose 4B, 152
cold ethanol fractionation, 137, 145–148
colorectal cancer, 263, 265–266, 294–295
complement induced cell lysis, 140
complement-mediated cytolysis, 85, 92–93
complement-mediated immune hemolysis, 257
concavalin-A, 3–4, 6, 276
cotton-wool column filter, 109
counterflow centrifugation, 50
CR3 heterodimer (complement receptor 3), 112
cryoprecipitate, 280
CTL, 269–270, 291
CTLp, 80
cutaneous anergy, 277
cycloheximide, 3
cyclosporin, 11, 13, 50–52, 257
cyclosporin A, 129
cystic fibrosis, 227
cytokine expression, 5–7
cytokine receptor, 3, 5
cytokines, 3, 5–7, 52, 61, 69, 77, 129–130, 236, 239–241, 245
cytomegalovirus, 99, 225, 241
cytomegalovirus immediate early antigen-2, 286
cytotoxic effector T-lymphocytes, 129
cytotoxic T cell assays, 242
cytotoxic T cell precursor, 80
cytotoxic T lymphocyte precursor, 286
cytotoxic T lymphocytes, 77, 81, 132, 268–269

cytotoxic T-cell response, 276
cytotoxic T-lymphocyte precursor
frequency analysis, 256

DAT, 255–256
DEAE, 146
DEAE-Sephacel, 152
delayed serologic transfusion reactions, 255
DHR 123 method, 23, 25, 29
DHTR, 255–257
dimethylsulfoxide (DMSO), 22, 25, 29
dinitrochlorobenzene, 277
direct antiglobulin test, 255
disseminated intravascular coagulation, 255
DNA fingerprints, 219, 226–228
DNA fluorophore propidium iodid, 102
double-antigen sandwich EIA, 178–179, 183
DR-sharing *see* HLA-DR sharing
DSTR, 255–256

E-2, 286
EBV, 269
effector cell retargeting, 132
EIA, 180–181
ELISA, 175
endotoxin, 5–6, 86
enzyme-linked immunosorbent assay, 175
Epstein-Barr virus, 225
erythropoietin, 69
eukaryotic cells, 91

F-reticulocytes, 208
factor II, 156, 158
factor VII, 156, 158
factor VIII, 275–276, 278, 280
factor VIII concentrates, 234, 275–278, 280
factor VIII inhibitors, 275
factor IX concentrate, 277, 278
factor IX, 151–152, 154, 156–158, 275, 280
factor X, 156, 158
Fc receptor, 14
Fc receptor-mediated phagocytosis, 140
Fc-mediated effector function, 146
Fc-related effector functions, 147
febrile non-hemolytic transfusion reations, 255
Felty's syndrome, 200
fibrinogen, 275
fibronectin, 113, 275
Ficoll Hypaque gradient, 86
FIX preparation, 158
FK506, 51, 129
flow cytometry, 23, 58, 92, 121, 195–198, 200–201, 203, 205, 208–209, 211, 236
fluorescence in situ hybridization: FISH, 209
FMP-activated resins, 156
FNHTR, 255
free radical reactions, 21–22, 29, 33
fresh frozen plasma, 265

G-CSF, 69
gelonin, 91
gene insertion, 205, 210
gene therapy, 205, 241–242, 292–293
gene transfer, 241
glycosyl-transferase, 258
GM-CSF, 69
GMP-140, 113, 117
GMP-140 (CD62), 208
graft survival, 13
graft-versus-host disease (GvHD), 31, 45–47, 49–52, 66, 69, 71, 164, 191, 232, 236

Hanks' balanced-salt solution (HBSS), 22–25
HCV-RNA, 183
HCV-RNA detection, 183
hematopoietic stem cells, 51
hemolytic transfusion reactions (HTR), 255
hemolytic-uremic syndrome, 51

hemophilia A, 275, 277–278, 280
hemophilia B, 277–278, 280
hepatitis B virus (HBV), 223, 277
hepatitis C virus (HCV), 183, 223, 233–235, 277–278
herpes simplex virus, 225
Hgb F, 208

HIV, 173, 175, 180–183, 188–189, 201, 223, 231, 233–235, 241, 275–281
HIV-1, 173, 175–177, 179–182, 233–234
HIV-1 proviral genome, 173
HIV-p24, 173, 181–182, 233
HIV-RNA analysis, 180
HIV-RNA detection, 233
HL60 myeloid cell line, 92
HLA, 164, 246, 291
HLA antigens, 49, 77
HLA class I antigens, 37, 41–42, 79, 81, 246
HLA class II, 77, 79, 226, 286
HLA class II antigens, 50, 81, 278, 285–286
HLA matched platelet transfusion, 40, 247
HLA typing, 226–228
HLA-alloimmunization, 77
HLA-B, 268
HLA-B27, 200
HLA-DP, 226
HLA-DQ, 188, 226
HLA-DR, 41–42, 50, 80–81, 163–164, 188, 226, 268, 291
HLA-DR sharing, 41, 268, 270
HLA-DR6, 42
HLA-matched platelet transfusions, 249
HPA-1a, 259
HPA-1a antigen, 259
HPV, 269
HSR, 22, 24–25, 27
HTLV, 269
HTLV-I, 124
human albumin, 86
human immunodeficiency virus, 173, 201, 223, 275
hydrazide immunosorbents, 158
hydrazide-activated resins, 156, 158
hydrogen peroxide, 21, 23, 25, 29
hypergammaglobulinemia, 276
hypotonic shock response, 22
hypotonic shock treatment, 22

IAC *see* immune adsorption chromatography
ICAM-1, 21, 68, 189
ICAM-2, 189
idiopathic thrombocytopenic purpura (ITP), 147, 200
idiotype-anti-idiotype interactions, 143
idiotypic interactions, 57
IFNτ, 188, 190
IgA, 142–144
IgD, 142, 144
IgE, 142–144
IgG, 92, 137–148
IgG1, 140–141, 143
IgG2, 87, 140–143, 147
IgG3, 140–141, 143–144, 147
IgG4, 87, 140–141, 143, 147
IgM, 87, 92, 142–144, 148
IIb-IIIa, 200
IL-1, 21, 49, 52, 187–188, 191
IL-1 mRNA, 187
IL-1 receptor, 52, 69
IL-1b, 188
IL-2, 49, 51–52, 65, 73, 80, 92, 167, 191, 205, 239–240, 242, 270, 278–279, 291
IL-2 receptor, 52, 65
IL-3, 69
IL-5, 187
IL-6, 187, 190
IL-8, 190
immune adsorption chromatography (IAC), 151, 155–159
immune tolerance, 11
immunoaffinity chromatography, 158
immunoaffinity column chromatography, 159
immunoblot, 175, 180–181, 183
immunoglobulin, 187

Immunoglobulin A, 142
Immunoglobulin D, 142
immunoglobulin E, 142
immunoglobulin G, 137, 140
immunoglobulin M, 142
immunoglobulins, 187
immunomagnetic beads, 92
immunomagnetic purging, 92
immunopurification, 168
immunotherapy, 129–130, 269, 271
immunotoxins, 85, 91
innocent bystander cytolysis, 259
innocent bystander hemolysis, 257–259
interferon, 276
interferon gamma, 240
interferon receptors, 276
interferon-τ, 52
interleukin, 236
interleukin receptors, 276
interleukin-1 *see* IL-1
interleukin-2 *see* IL-2
International Bone Marrow Transplant Registry (IBMTR), 93–94
intravenous immunoglobulin concentrates, 144–148
ion exchange chromatography, 146, 152, 277–278
ITP *see* idiopathic thrombocytopenic purpura
IVIg, 58, 60, 68, 70, 292

Ki-67 antigen, 201
kidney transplant, 13

LAK, 130, 240–241
LAK cells *see* lymphokine activated killer cells
LAK cell precursors, 239
LCR, 221
LCT, 39–40
leukoagglutinins, 77
leukocyte depletion, 41, 43, 50, 71, 111, 113–118, 125, 161, 247–251, 289
leukocyte depletion filter, 109–112
leukocyte-depleted platelets, 12
LFA-1, 189, 191
ligase chain reaction, 221
lymphocytotoxic antibody, 245, 247, 250
lymphocytotoxicity, 58
lymphocytotoxicity test, 39
lymphokine activated killer (LAK) cells, 130, 166, 205, 239–240
lymphosurge, 133

mAb-coated magnetic bead, 85
MAIPA, 39–40
malaria, 203
mdr genes, 205
melanoma, 130, 135, 269
meta-analysis, 265–266
methotrexate, 52
methylene blue, 23, 26
MHC, 82, 232, 239
MHC class I antigen, 50
MHC class I molecules, 187, 191
MHC class II antigen, 5–6
MHC class II molecules, 187, 190
MHC-restricted T-lymphocytes, 130
microaggregate filter, 101
minimal residual disease, 261, 267
mitogenesis, 276
mixed leukocyte reaction (MLR), 22, 24–29, 31, 33, 80
MOC31, 132
monoclonal antibody (mAb), 85, 87–89, 91–92, 94, 151–152, 154, 156–158
monoclonal antibody immobilization of platelet antigens, 39
mouse IgG, 156
mRNA, 3–7
mytomycin C, 22

natural killer cell (NK cell), 49, 82, 130, 268–269, 276, 295
neo-epitopes, 183
neoantigen, 277–278
non-hemolytic febrile transfusion reactions (NHFTR), 99, 101, 103

oncogene mRNA, 228
oncogenes, 228
open window phase, 181–182
organ transplantation, 77, 80, 285

P-glycoprotein, 205
p24, 231, 234
P24 antigen, 280
p24-only reactors, 179
p40, 4
p53, 269
PAP, 91
parentage testing, 219–221, 227–228
paroxysmal nocturnal hemoglobinuria (PNH), 256
passenger lymphocyte syndrome, 257
PC, 151–152, 154, 156, 158
PCNA or proliferating cell nuclear antigen, 201
PCR, 94, 103, 124, 175, 179–183, 203, 210, 219, 221, 223–226, 233–236
PEG, 146
pentoxiphylin, 66
per-operatively spilled tumourcells, 271
peri-operative blood transfusion, 261, 263, 265, 267
peripheral blood stem cells, 69, 236
peripheral blood-derived hemopoietic stem cells, 242
phenylketonuria, 227
phytohemagglutinin, 276
PIFT, 39–40
PKC, 188, 190–191
platelet immunofluorescence test, 39
platelet refractoriness *see* refractoriness
platelet-specific antibodies, 57
PMA, 3–4, 6
pokeweed antiviral protein, 91
polyester flat-bed filters, 110–113
polyethylene glycol, 146
polyethylene glycol precipitation, 145
polymerase chain reaction *see* PCR
posttransfusion purpura, 259
primary hypogammaimmunoglobulinemia, 137, 145, 147–148
prostaglandin, 74
prostaglandin release, 14
protein A chromatography, 133
protein C, 151, 168
protein kinase C, 3–4, 49, 188, 190
protein tyrosine kinases, 190
PTK, 190–191
pulmonary tuberculosis, 279

Qβ replicase, 223
quality assurance, 195, 208

radioimmunoprecipitation assay *see* RIPA
RAS, 269
RBC, 109
RCC, 130
recombinant immunoblot (4-RIBA), 183
refractoriness, 21, 37, 39, 43, 57, 66, 68, 71, 99, 101, 245–247, 250–251, 289–290
REH6 cell line, 88
renal cell carcinoma, 130, 135, 269
repair chain reaction (RCR), 221
restriction fragment length polymorphism (RFLP), 219, 226
ricin A-chain, 91
rIL-2, 129–130, 133, 135
RIPA, 175, 178–179, 183

3SR, 221
SAM, 92
saporin, 91
sCD8, 134–135
SDS-PAGE analysis, 156
SE, 188–191
secretory IgA, 142, 144
self sustained sequence replication, 221
Sepharose CL-2B, 152
serum sickness, 137, 145
sheep anti-mouse IgG antibody, 92
sickle cell anemia, 227, 257
sickle cell disease, 208
signal transduction, 187, 191

single donor platelets, 250–251
single donor transfusions, 41
SOD *see* superoxide dismutase
solid-phase red cell adherence, 58
Southern blots, 221, 226
soybean agglutination, 50
Staphylococcal exotoxins (SE), 188
Staphylococcal toxic shock syndrome toxin 1, 188
staurosporin, 4
superoxide dismutase, 22, 25, 29, 73
suppressor cell network, 14
syngeneic BMT, 49
syngeneic bone marrow, 45
systemic lupus erythematosus, 200

T cell activation, 77
T cell-depletion, 94
T-cell subset, 50, 275
T-cell-receptor/CD3 (TCR/CD3) complex, 132
T4/T8 ratio *see* CD4/CD8 ratio
TA GvHD, 102
TBARS, 25, 27, 29, 32
TCR/CD3, 132
thalassemia, 101, 227
thiobarbituric acid reacting substances, 25
TIL, 240–242, 269–270
TNF, 5, 7, 49, 66, 130, 134–135, 188, 191, 236, 242
TNFα, 49, 52, 188–189
TNFα mRNA, 188
transfusion medicine, 195, 198, 201, 204–205, 208, 210–211, 242–243, 285–287, 292–293
transfusion-associated graft-versus-host disease *see* TA GvHD
transfusion-induced GvHD, 49–51
Trial to Reduce Alloimmunization to Platelets (TRAP), 102
Trypan Blue exclusion test, 88
trypanosomiasis, 203
TSA, 269–270
TSST-1, 188, 190
tumour associated antigen (TAA), 131–132, 269
tumour infiltrating leukocytes, 269
tumour necrosis factor *see* TNF
tumour specific antigen *see* TSA
tumour-infiltrating lymphocytes *see* TIL
tumour-reactive T-lymphocytes, 130
tumour-specific peptides, 131
tyrosine kinase, 49

ultraviolet B (UV-B), , 241, 247
UV irradiation, 67, 99
UV-B irradiation, 21–2 27–31, 33, 251, 289
UV-irradiation, 79

VNTR, 226–227
von Willebrand factor, 113

WBC depletion, 100–101
Western blot analysis, 156