Netherlands
The Journal of Medicine
PUBLISHED IN COLLABORATION WITH THE NETHERLANDS ASSOCIATION OF INTERNAL MEDICINE

SUPPLEMENT MARCH 2004

Genetic basis of host susceptibility to infections

Proceedings of the 14th GSK Kurhaus Workshop on Infectious Diseases
Scheveningen, the Netherlands, 8 March 2002

PREFACE

The wide variation in the incidence and severity of infectious diseases among individuals in the same population and between populations may be (largely) due to variation in socioeconomic circumstances, human behaviour and variation in microbial virulence. However, host susceptibility and severity of infectious diseases in humans can also be linked to polymorphisms in the human genome. This 14th Kurhaus Workshop on Infectious Diseases explores several important examples of genetic polymorphisms that have recently been linked to host susceptibility and infection. Although much remains to be discovered in this field the evidence presented at this Workshop clearly pointed toward the future impact of genomic information in the management of infectious diseases in individuals as well as in public health.

Scientific Committee
B.M. de Jongh
S. de Marie
J.W.M. van der Meer
C.M.J.E. Vandenbroucke-Grauls
H.A. Verbrugh

Editors
J.W.M. van der Meer
H.A. Verbrugh

VAN ZUIDEN communications
Alphen aan den Rijn, the Netherlands

MISSION STATEMENT

The mission of the journal is to serve the need of the internist to practice up-to-date medicine and to keep track with important issues in health care. With this purpose we publish editorials, original articles, reviews, controversies, consensus reports, papers on speciality training and medical education, book reviews and correspondence.

EDITORIAL INFORMATION

Editorial office 'The Netherlands Journal of Medicine'
Geeralien Derksen-Willemsen
University Medical Centre St Radboud
Department General Internal Medicine 541
PO Box 9101
6500 HB Nijmegen
The Netherlands
Tel.: +31 (0)24-361 04 59
Fax: +31 (0)24-354 17 34
E-mail: g.derksen@aig.umcn.nl

Contents

The role of mannose-binding lectin in health and disease 4
M.W. Turner

Discussion 9

Toll-like receptors contribute to host responses against mycobacterial infection 12
M.J. Fenton, K.A. Heldwein

Discussion 17

Human Fc receptor polymorphisms in relation to bacterial infection 20
T.W. Kuijpers

Discussion 25

Genetic susceptibility to *Neisseria meningitidis* infections 28
M. Emonts, R. de Groot, P.W.M. Hermans

Discussion 35

Genetics of type-1 cytokines in human intracellular infectious diseases: a spectrum of novel genetic deficiencies demonstrates the essential role of type-1 cytokines in immunity against nontuberculous mycobacteria, *M. bovis* BCG and salmonellae 38
T.H.M. Ottenhoff

Discussion 40

NRAMP1 (SLC11A1) and vitamin D receptor genes: disease associations 42
K.P.W.J. McAdam

Discussion 45

The role of interleukin-1 beta in *Helicobacter pylori*-associated disease 47
E.M. El-Omar

Discussion 53

The role of polymorphisms in chemokines and chemokine receptors in the clinical course of HIV-1 infection 55
H. Schuitemaker, R. van Rij

Discussion 63

List of participants 66

Subscriptions
General information
An annual subscription to The Netherlands Journal of Medicine (ISSN 0300-2977) consists of 11 issues. Issues within Europe are sent by standard mail and outside Europe by air delivery. Cancellations should be made, in writing, at least two months before the end of the year.
Subscription fee
The annual subscription fee within Europe is € 650,00, for the USA € 665,00 and for the rest of the world € 675,00. Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis.
Payment method
Please make your check payable to Van Zuiden Communications B.V., PO Box 2122, 2400 CC Alphen aan den Rijn, the Netherlands or you can transfer the fee to ING Bank, account number 67.89.10.872, Castellumstraat 1, Alphen aan den Rijn, the Netherlands, swift-code: ING BNL 2A. Do not forget to mention the complete delivery address of the Journal.

Claims
Claims for missing issues should be made within two months of the date of dispatch. Missing issues will be mailed without charge. Issues claimed beyond the two-month limit must be prepaid at back copy rates.

Orders, preprints, advertising, author or general enquiries
Please contact the publisher.

Van Zuiden Communications B.V.
PO Box 2122, 2400 CC Alphen aan den Rijn
The Netherlands
Tel.: +31 (0)172-47 61 91, fax: +31 (0)172-47 18 82
E-mail: zuiden@zuidencomm.nl

Acknowledgement
This Supplement has been made possible because of an educational grant by GlaxoSmithKline.

CITED IN:
BIOSIS DATABASE; EMBASE/EXCERPTA MEDICA; INDEX MEDICUS (MEDLINE)

The role of mannose-binding lectin in health and disease

M.W. Turner

Institute of Child Health, University College Londen, Londen, United Kingdom

ABSTRACT

Mannose-binding lectin (MBL) is an important element of the innate immune defence system. The protein binds to the sugars present on many microbial surfaces and subsequently activates the complement system through a family of specific proteases called the MASPs (MBL-associated serine proteases). Studies of MBL binding to selected Gram-negative organisms suggest that the lipopolysaccharide (LPS) structure is of primary importance. For a range of clinically relevant organisms MBL binding leads to activation and cleavage of C4 and C3, suggesting that this is a major pathway for opsonophagocytosis. MBL deficiency, resulting from three mutations in exon 1 and polymorphisms in the promoter region of the gene, is associated with both increased susceptibility to infections and autoimmune disease. Recent evidence indicates that the protein also modulates disease severity, possibly by influencing cytokine production.

INTRODUCTION

Mannose-binding lectin (MBL) is a pattern recognition molecule of the innate immune system.[1] It belongs to a family of proteins called the collectins,[2-4] in which lectin (carbohydrate recognition) domains are found in association with collagenous structures. In man these proteins include serum MBL, lung surfactant protein A (SP-A) and lung surfactant protein D (SP-D). Each of these proteins plays an important role in innate immune defence, but MBL is of particular interest because it is able to activate the complement system in an antibody- and C1-independent manner.

MBL STRUCTURE

MBL has a bouquet-like structure with many similarities to C1q. However, unlike C1q it can exist as dimers, trimers, tetramers or hexamers. All higher-order oligomers of MBL are based on subunits comprising three identical peptide chains of 32 kDa. Each chain is characterised by a lectin domain, an α-helical coiled-coil hydrophobic neck region, a collagenous region and a cysteine-rich N-terminal region.[5,6] Three such chains interact to give a collagenous triple helix,[7] but separate at the neck region to give three independent carbohydrate recognition domains.

MBL is a C-type (calcium-dependent) lectin that is able to interact with the 3- and 4-hydroxyl groups of various sugars, including N-acetyl-D-glucosamine, mannose, N-acetyl-mannosamine, fucose and glucose.[8] The repeating arrays or patterns of sugar groups expressed on microbial surfaces make ideal targets for MBL binding, since the three sugar-binding sites of one subunit provide a flat platform with a constant distance between the individual binding sites (45 Å for human MBL).[9] Because the Kd of each separate MBL-sugar interaction is relatively low (10^{-3} M)[10] such simultaneous multiple binding is critical in order to achieve a high avidity.

MBL FUNCTION

The activation of complement by MBL represents a pathway which is independent of both the classical and alternative pathways, but which has similarities to the classical pathway. In the circulation MBL is found in association with

four structurally related pro-enzymes. These are the MBL-associated serine proteases (MASPs) 1, 2 and 3[11-13] and a truncated version of MASP-2 called MAp 19.[14,15] In serum there is a 20-fold excess of MASP-1 over MBL[16] and some evidence that the enzyme cleaves C3 directly. MASP-2, which is present at much lower concentrations than MASP-1, appears to be the more important in complement activation.[12] The available data suggest that MBL – MASP-2 complexes become activated when bound to appropriate sugar arrays on microbial surfaces.[17] The enzyme specificity of MASP-2 is apparently identical to that of Cl esterase and results in the sequential cleavage of C4 and C2. The C4b fragments generated bind covalently either to the MBL itself or to the nearby microbial surface and act as a focus for C2 binding/activation. The resultant C4b2a complex has C3 convertase activity and cleaves C3 in a similar manner to the C3 convertases of both the classical and alternative pathways of complement activation (see *figure 1*).

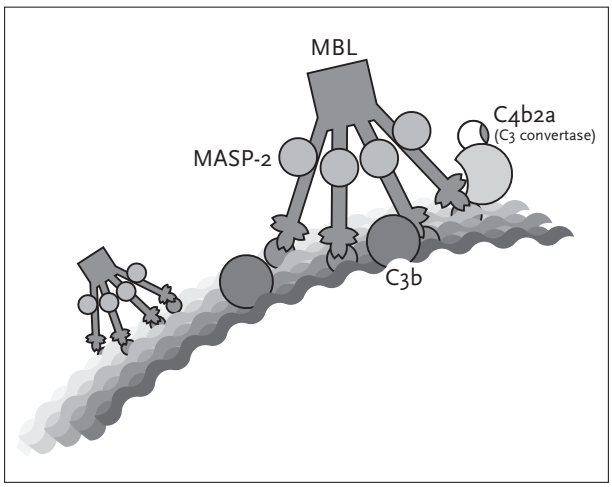

Figure 1
Mannose-binding lectin (MBL) complexed with the pro-enzyme MASP-2 is shown binding to a sugar array on the surface of a micro-organism

The MASP-2 becomes activated and is able to cleave sequentially both C4 and C2. A complex of the major fragments of these components, C4b2a, is a functionally-active C3-cleaving enzyme (or C3-convertase) and is able to generate large amounts of C3b, an important opsonin.

The C3b generated by the MBL – MASP pathway is fed into the positive feedback amplification loop of complement activation and results in the deposition of large amounts of opsonic C3b on the microbial surface.

There is some evidence to suggest that MBL is able to interact directly with cell surface receptors and promote opsonophagocytosis and other immune processes. Several putative MBL-binding proteins/receptors have been proposed, including cClqR/calreticulin,[18] $ClqR_p$[19] and CR1.[20,21]. However, it is unclear whether MBL acts as a direct opsonin for micro-organisms[22] or simply enhances other well-established pathways of complement and/or immunoglobulin receptor-mediated phagocytosis.[23]

MBL GENETICS AND POLYMORPHISMS

Within the human collecting gene cluster mapped to 10q 21-24[24] there is a single functional MBL gene comprising four exons. Exon 1 encodes the signal peptide, a cysteine-rich region and part of the glycine-rich collagen-like region. Exon 2 encodes the remainder of the collagenous region, whilst exon 3 encodes for the α-helical coiled-coil neck region. The fourth exon encodes the C-terminal carbohydrate recognition domain. Upstream of the MBL gene are a number of regulatory, promoter elements which are believed to enhance MBL transcription approximately threefold during acute-phase responses.[5,6]

MBL deficiency results predominantly from three single-point gene mutations in codons 52, 54 and 57 of exon 1 of the MBL gene.[25] These are commonly referred to as the D, B and C variants, with A indicating wild type. The B variant mutation occurs in approximately 26% of Caucasians, whereas the C variant mutation is characteristic of sub-Saharan African populations in whom it may reach frequencies of 50 to 60%. Both the B and C mutations result in the substitution of a dicarboxylic acid for an axial glycine, and this impairs correct oligomerisation.[26] In addition to the above structural gene mutations, several polymorphisms exist within the promoter region of the MBL gene. These polymorphisms are the H/L, X/Y and P/Q loci at positions -550, -221 and +4 of the MBL gene.[27] The alleles expressed at these loci are in linkage disequilibrium and four promoter haplotypes (LXP, LYP, LYQ and HYP) are commonly found. Of these the HYP haplotype is associated with high MBL levels, whereas the LXP haplotype is found in association with low levels of the protein.[28] The HXP haplotype has never been unequivocally identified.

The three structural gene mutations are also in linkage disequilibrium with the promoter polymorphisms and every individual expresses two of the following seven possible haplotypes – HYPA, LYQA, LYPA, LXPA, LYPB, LYQC and HYPD. The frequencies of these haplotypes differ markedly between different population groups.[27] Our observations on the distribution of the B and C alleles in African and non-African populations led us to suggest that the two mutations probably arose independently after the migration of hominids out of Africa some 100,000 to 150,000 years ago.[26] It is of interest that none

of the three structural gene mutations were introduced into Australia at the time of first settlement (c. 50,000 years ago)[29] whereas the B mutation was introduced into North America at the time of the last glaciation (c. 20,000 years ago). This suggests that the B mutation may have arisen between 20,000 and 50,000 years ago on the LYP promoter background.[29]

MBL BINDING TO MICRO-ORGANISMS

We have used flow cytometry to study MBL binding to a range of clinically relevant pathogens isolated from immunocompromised children and found large differences.[30] Some organisms such as *Candida albicans*, β-haemolytic Group A *Streptococci* and *Staphylococcus aureus* have consistently exhibited high binding, whereas others such as *Clostridium* sp., *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, β-haemolytic *Streptococcus* Group B and *Streptococcus pneumoniae* appear not to bind the protein. In between are other organisms with more variable patterns of binding, such as *Klebsiella* species and *Escherichia coli*. In our present state of knowledge such heterogeneity is not readily explained, and it has prompted us to explore in more detail the determinants of MBL binding to bacteria.

To this end we have studied the effect of LPS structure on MBL attachment to both *Salmonella enterica* serovar Typhimurium[31] and to the human pathogens *Neisseria gonorrhoeae*[31] and *N. meningitidis* (serogroups B and C).[32,33] In particular, we have examined the relative importance of LPS structure and capsule in determining MBL binding to the serogroup-B meningococcus. It was observed that the absence of sialic acid from the LOS of *Neisseria meningitidis* serogroup B,[32] serogroup C[33] and *Neisseria gonorrhoeae*[31] permitted MBL to bind to each of these organisms. In contrast, MBL appeared to bind very poorly or not at all to organisms with sialylated LOS. In the case of *Salmonella* species, organisms of the rough chemotype (not expressing the O-antigen) showed MBL binding whereas organisms with the smooth chemotype and expressing the O-antigen exhibited little or no MBL binding.[31] These results suggest that LPS structure exerts a major influence on MBL attachment to bacteria.

THE ROLE OF MBL IN DISEASE

The immunological significance of MBL deficiency was initially established in children,[34] but there are now numerous studies indicating a role for the lectin in later life and supporting the notion that it should be considered as an ante-antibody, a humoral factor playing a critical role in immune defence before the production of antibodies.[1]

There is increasing evidence that MBL – disease associations are very complex. At present the topic may be considered under the following separate headings: (a) MBL and disease susceptibility, (b) MBL and disease severity and (c) inappropriate activation of the MBL – MASP pathway.

MBL AND DISEASE SUSCEPTIBILITY

Several studies have shown that deficiency of MBL is associated with an increased overall susceptibility to infectious disease.[35,36] In terms of community health this may go some way towards explaining why some individuals suffer more infections than others.[37,38] However, in a hospital setting it also appears that MBL deficiency has an important influence on the occurrence and duration of febrile neutropenic episodes in children with malignancy.[39] In addition to such increased generalised susceptibility, other studies have identified an increased susceptibility to infection by specific pathogens in MBL-deficient individuals, including human immunodeficiency virus,[40,41] *Plasmodium falciparum*,[42] *Cryptosporidium parvum*[43] and *N. meningitides*.[44] However, in the case of intracellular parasites (e.g. *Leishmania*) it appears that MBL deficiency may actually protect against disease. It is suggested that since such parasites exploit C3b opsonisation to facilitate uptake by the C3 receptors of macrophages and thereby gain entry to those cells, any reduction in the complement-activating function of the host may help reduce the probability of infection. The most convincing evidence to date of such a mechanism is a study of patients with visceral leishmaniasis in Brazil.[45] The median MBL level of these patients was significantly higher than that of healthy individuals and MBL mutations were significantly more common in the healthy controls.

In addition to the above reports of associations with infectious disease, there have been several investigations of possible associations between MBL deficiency and susceptibility to autoimmune disease. There is strong evidence of such an association in the case of systemic lupus erythematosus (SLE). Cohorts of British,[46] Hong Kong Chinese,[47] American Black[48] and Spanish[49] SLE patients have all shown evidence of an increased frequency of mutant MBL alleles or deficiency of the serum protein. The interpretation of these findings has usually reiterated the hypothesis proposed in relation to components of the classical complement pathway, namely that an impairment of the mechanisms involved in the removal of immune complexes may predispose to the development of autoimmune disease.

MBL AND DISEASE SEVERITY

In addition to the mounting evidence that MBL deficiency influences susceptibility to disease there have been several reports suggesting that the protein can also modulate disease severity. In the field of autoimmunity there is evidence of such a modulatory role for MBL, and recent studies from two centres have indicated that MBL variant alleles are associated with both severity and early onset of disease in patients with rheumatoid arthritis.[50-53] The mechanism by which MBL exerts such effects is unclear but our recent studies on *Neisseria meningitidis*[54] suggest that one possible pathway may be through cytokine modulation. When *N. meningitidis* organisms were incubated with increasing concentrations of MBL and added to whole blood from an MBL-deficient donor, the release of the cytokines TNF-α, IL-1b and IL-6 from monocytes was enhanced at lower MBL concentrations (<4 μg/ml) but reduced at higher concentrations (>4 μg/ml). Further work is required to establish whether this complex modulation of proinflammatory cytokine release occurs with other infectious organisms.

INAPPROPRIATE ACTIVATION OF THE LECTIN PATHWAY

Pathology associated with unregulated or inappropriate activation of the classical and alternative pathways of complement has been well documented over many years and it is to be expected that similar reports involving the MBL – MASP pathway will appear. At the time of writing there have been a small number of such studies in the fields of renal disease and reperfusion injury.

In one of the first studies in this area Endo and colleagues reported that MBL – MASP activation contributed to the glomerular damage observed in a significant number of patients with IgA nephropathy.[55] However, in another study of renal biopsies from several patients with different forms of glomerulonephritis, Lhotta and co-workers claimed that the MBL deposition observed was of minor importance.[56] Subsequent studies have described MBL deposition in the glomeruli of a patient with poststreptococcal glomerulonephritis[57] and in ten patients with Henoch-Schönlein purpura nephritis.[58] Much further work is required to evaluate the role of the MBL – MASP system in these disorders.

Recently MBL depletion and anti-human MBL monoclonal antibodies have been used to establish a role for the MBL – MASP pathway in initiating the complement activation which occurs following hypoxia-reoxygenation of human endothelial cells.[59] In a separate study from the same group the MBL – MASP pathway was shown to be activated in rats following myocardial ischaemia reperfusion, suggesting that it is implicated in the subsequent tissue injury.[60] Blockade of the lectin pathway with inhibitory monoclonal antibodies protected the heart from ischaemia reperfusion, and suggests that this therapeutic approach should be explored in human patients.

REFERENCES

1. Ezekowitz RAB. Ante-antibody immunity. Curr Opin Immunol 1991;1(1):60-2.
2. Holmskov U, Malhotra R, Sim RB, Jensenius JC. Collectins: collagenous C-type lectins of the innate immune defence system. Immunol Today 1994;15(2):67-74.
3. Ezekowitz RAB, Sastry K, Reid KBM. Collectins and Innate Immunity. 1st ed. Texas, USA: R. G. Landes Co & Chapman & Hall, 1996.
4. Epstein J, Eichbaum Q, Sheriff S, Ezekowitz RAB. The collectins in innate immunity. Curr Opin Immunol 1996;8:29-35.
5. Sastry K, Herman GA, Day L, Deignan E, Bruns G, Morton CC, Ezekowitz RA. The human mannose-binding protein gene. Exon structure reveals its evolutionary relationship to a human pulmonary surfactant gene and localization to chromosome 10. J Exp Med 1989;170:1175-89.
6. Taylor ME, Brickell PM, Craig RK, Summerfield JA. Structure and evolutionary origin of the gene encoding a human serum mannose-binding protein. Biochem J 1989;262:763-71.
7. Weis WI, Drickamer K. Trimeric structure of a C-type mannose-binding protein. Structure 1994;2:1227-40.
8. Weis WI, Drickamer K, Hendrickson WA. Structure of a C-type mannose-binding protein complexed with an oligosaccharide. Nature 1992;360:127-34.
9. Sheriff S, Chang CY, Ezekowitz RAB. Human mannose-binding protein carbohydrate recognition domain trimerizes through a triple a-helical coiled-coil. Nature Struc Biol 1994;1(11):789-94.
10. Iobst ST, Wormald MR, Weis WI, Dwek RA, Drickamer K. Binding of sugar ligands to Ca2+-dependent animal lectins. I. Analysis of mannose binding by site-directed mutagenesis and NMR. J Biol Chem 1994;269(22):15505-11.
11. Matsushita M, Fujita T. Activation of the classical complement pathway by mannose-binding protein in association with a novel C1s-like serine protease. J Exp Med 1992;176:1497-502.
12. Thiel S, Vorup-Jensen T, Stover CM, et al. A second serine protease associated with mannan-binding lectin that activates complement. Nature 1997;386:506-10.
13. Dahl MR, Thiel S, Willis AC, et al. Mannan-binding lectin associated serine protease 3 (MASP-3) – A new component of the lectin pathway of complement activation. Immunopharm 2000;49:79.
14. Stover CM, Thiel S, Thelen M, et al. Two constituents of the initiation complex of the mannan-binding lectin activation pathway of complement are encoded by a single structural gene. J Immunol 1999;162:3481-90.
15. Takahashi M, Endo Y, Fujita T, Matsushita M. A truncated form of mannose-binding lectin-associated serine protease (MASP)-2 expressed by alternative polyadenylation is a component of the lectin complement pathway. Int Immunol 1999;11:859-63.
16. Terai I, Kobayashi K, Matsushita M, Fujita T. Human serum mannose-binding lectin (MBL)-associated serine protease-1 (MASP-1): determination of levels in body fluids and identification of two forms in serum. Clin Exp Immunol 1997;110:317-23.

17. Thiel S, Petersen SV, Vorup-Jensen T, et al. Interaction of C1q and mannan-binding lectin (MBL) with C1r, C1s, MBL-associated serine proteases 1 and 2, and the MBL-associated protein MAp19. J Immunol 2000;165(2):878-87.
18. Malhotra R, Thiel S, Reid KBM, Sim RB. Human leukocyte C1q receptor binds other soluble proteins with collagen domains. J Exp Med 1990;172:955-9.
19. Tenner AJ, Robinson SL, Ezekowitz RAB. Mannose-binding protein (MBP) enhances mononuclear phagocyte function via a receptor that contains the 126,000 m(r) component of the C1q receptor. Immunity 1995;3:485-93.
20. Klickstein LB, Barbashov SF, Liu T, Jack RM, Nicholson-Weller A. Complement receptor type 1 (CR1, CD35) is a receptor for C1q. Immunity 1997;7:345-55.
21. Ghiran I, Barbashov SF, Klickstein LB, Tas SW, Jensenius JC, Nicholson-Weller A. Complement receptor 1/CD35 is a receptor for mannan-binding lectin. J Exp Med 2000;192(12):1797-807.
22. Kuhlman M, Joiner K, Ezekowitz RA. The human mannose-binding protein functions as an opsonin. J Exp Med 1989;169(5):1733-45.
23. Kitz DJ, Stahl PD, Little JR. The effect of a mannose binding-protein on macrophage interactions with Candida albicans. Cell Mol Biol 1992;38:407-12.
24. Hansen S, Holmskov U. Structural aspects of collectins and receptors for collectins. Immunobiology 1998;199:165-89.
25. Turner MW. Mannose binding lectin: the pluripotent molecule of the innate immune system. Immunol Today 1996;17:532-40.
26. Lipscombe RJ, Sumiya M, Hill AV, et al. High frequencies in African and non-African populations of independent mutations in the mannose binding protein gene. Hum Mol Genet 1992;1(9):709-15. [published erratum appears in Hum Mol Genet 1993;2:342]
27. Madsen HO, Garred P, Thiel S, et al. Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. J Immunol 1995;155:3013-20.
28. Madsen HO, Satz ML, Hogh B, Svejgaard A, Garred P. Different molecular events result in low protein levels of mannan-binding lectin in populations from Southeast Africa and South America. J Immunol 1998;161:3169-75.
29. Turner MW, Dinan L, Heatley S, et al. Restricted polymorphism of the mannose-binding lectin gene of indigenous Australians. Hum Mol Genet 2000;9(10):1481-6.
30. Neth O, Jack DL, Dodds AW, Holzel H, Klein NJ, Turner MW. Mannose-binding lectin binds to a range of clinically relevant microorganisms and promotes complement deposition. Infect Immun 2000;68(2):688-93.
31. Devyatyarova-Johnson M, Rees IH, Robertson BD, Turner MW, Klein NJ, Jack DL. The lipopolysaccharide structures of Salmonella enterica serovar Typhimurium and Neisseria gonorrhoeae determine the attachment of human mannose-binding lectin to intact organisms. Infect Immun 2000;68(7):3894-9.
32. Jack DL, Dodds AW, Anwar N, et al. Activation of complement by mannose-binding lectin on isogenic mutants of Neisseria meningitidis serogroup B. J Immunol 1998;160(3):1346-53.
33. Jack DL, Jarvis GA, Booth CL, Turner MW, Klein NJ. Mannose-binding lectin accelerates complement activation and increases serum killing of Neisseria meningitidis Serogroup C. J Infect Dis 2001;184(7):836-45.
34. Super M, Thiel S, Lu J, Levinsky RJ, Turner MW. Association of low levels of mannan-binding protein with a common defect of opsonisation. Lancet 1989;ii:1236-9.
35. Summerfield JA, Sumiya M, Levin M, Turner MW. Association of mutations in mannose binding protein gene with childhood infection in consecutive hospital series. BMJ 1997;314:1229-32.
36. Garred P, Madsen HO, Hofmann B, Svejgaard A. Increased frequency of homozygosity of abnormal mannan-binding protein alleles in patients with suspected immunodeficiency. Lancet 1995;346:941-3.
37. Winkelstein JA, Childs B. Why do some individuals have more infections than others? JAMA 2001;285(10):1348-9.
38. Koch A, Melbye M, Sørensen P, et al. Acute respiratory tract infections and mannose-binding lectin insufficiency during early childhood. JAMA 2001;285(10):1316-21.
39. Neth O, Hann I, Turner MW, Klein NJ. Deficiency of mannose-binding lectin and burden of infection in children with malignancy: a prospective study. Lancet 2001;358:614-8.
40. Garred P, Madsen HO, Balslev U, et al. Susceptibility to HIV infection and progression of AIDS in relation to variant alleles of mannose-binding lectin. Lancet 1997;349:236-40.
41. Nielsen SL, Andersen PL, Koch C, Jensenius JC, Thiel S. The level of the serum opsonin, mannan-binding protein in HIV-1 antibody-positive patients. Clin Exp Immunol 1995;100:219-22.
42. Luty AJF, Kun JFJ, Kremsner PG. Mannose-binding lectin plasma levels and gene polymorphisms in Plasmodium falciparum malaria. J Infect Dis 1998;178:1221-4.
43. Kelly P, Jack DL, Naeem A, et al. Mannose-binding lectin is a component of innate mucosal defence against Cryptosporidium parvum in AIDS. Gastroenterology 2000;119(5):1236-42.
44. Hibberd ML, Sumiya M, Summerfield JA, Booy R, Levin M, the Meningococcal Research Group. Association of variants of the gene for mannose-binding lectin with susceptibility to meningococcal diseases. Lancet 1999;353:1049-53.
45. Santos IK, Costa CH, Krieger H, et al. Mannan-binding lectin enhances susceptibility to visceral leishmaniasis. Infect Immun 2001;69(8):5212-5.
46. Davies EJ, Snowden N, Hillarby MC, et al. Mannose-binding protein gene polymorphism in systemic lupus erythematosus. Arthritis Rheum 1995;38(1):110-4.
47. Lau YL, Lau CS, Chan SY, Karlberg J, Turner MW. Mannose-binding protein in Chinese patients with systemic lupus erythematosus. Arthritis Rheum 1996;39(4):706-8.
48. Sullivan KE, Wooten C, Goldman D, Petri M. Mannose-binding protein genetic polymorphisms in black patients with systemic lupus erythematosus. Arthritis Rheum 1996;39(12):2046-51.
49. Davies EJ, Teh L-S, Ordi-Ros J, et al. A dysfunctional allele of the mannose binding protein gene associates with systemic lupus erythematosus in a Spanish population. J Rheumatol 1997;24:485-8.
50. Garred P, Madsen HO, Marquart H, et al. Two edged role of manose binding lectin in rheumatoid arthritis: a cross sectional study. J Rheumatol 2000;27(1):26-34.
51. Graudal NA, Homann C, Madsen HO, et al. Mannan binding lectin in rheumatoid arthritis. A longitudinal study. J Rheumatol 1998;25(4):629-35.
52. Graudal NA, Madsen HO, Tarp U, et al. The association of variant mannose-binding lectin genotypes with radiographic outcome in rheumatoid arthritis. Arthritis Rheum 2000;43(3):515-21.
53. Ip WK, Lau Y-L, Chan SY, et al. Mannose-binding lectin and rheumatoid arthritis in southern Chinese. Arthritis Rheum 2000;43(8):1679-87.

54. Jack DL, Read RC, Tenner AJ, Frosch M, Turner MW, Klein NJ. Mannose-Binding Lectin Regulates the Inflammatory Response of Human Professional Phagocytes to Neisseria meningitidis Serogroup B. J Infect Dis 2001;184(9):1152-62.
55. Endo M, Ohi H, Ohsawa I, Fujita Ta, Matsushita M, Fujita Te. Glomerular deposition of mannose-binding lectin (MBL) indicates a novel mechanism of complement activation in IgA nephropathy. Nephrol Dial Transplant 1998;13:1984-90.
56. Lhotta K, Würzner R, König P. Glomerular deposition of mannose-binding lectin in human glomerulonephritis. Nephrol Dial Transplant 1999;14:881-6.
57. Ohsawa I, Ohi H, Endo M, Fujita Ta, Matsushita M, Fujita Te. Evidence of lectin complement pathway activation in poststreptococcal glomerulonephritis. Kidney International 1999;56:1158-60.
58. Endo M, Ohi H, Ohsawa I, Fujita Ta, Matsushita M, Fujita Te. Complement activation through the lectin pathway in patients with Henoch-Schönlein purpura nephritis. Am J Kidney Dis 2000;35(3):401-7.
59. Collard CD, Vakeva A, Morrisey MA, et al. Complement activation after oxidative stress: role of the lectin complement pathway. Am J Pathol 2000;156(5):1549-56.
60. Jordan JE, Montalto MC, Stahl G. Inhibition of mannose-binding lectin reduces postischemic myocardial reperfusion injury. Circulation 2001;104:1413-8.

Discussion following lecture by M.W. Turner

Verbrugh: This topic is now open for discussion. I would first like to discuss the binding of mannose-binding lectin (MBL) to the surfaces of micro-organisms, one of the first observations that you talked about. There was quite some heterogeneity in binding between species of micro-organisms and within species. You did not say much about that, except that there was some focal site of binding, but is there anything else that you or anybody else in this room may add on that phenomenon? Because that seems to be important for MBL's mode of action.

Turner: Well, we have looked at both *Neisseria meningitidis* B and C and a range of isogenic mutants of salmonella. The bulk of the work that has been published has been done in those organisms. There is a consistent story that the presence of sialic acid strongly inhibits the binding of MBL. But there are problems with some organisms regarding the expression of sugars. Sometimes there will be a terminal sugar accessible that ought to bind MBL and yet we do not see any binding. So I must say that there is still an awful lot of work to be done. We cannot simplistically take the sugar structure that has been published for a particular mutant and say with any certainty, without doing the experiments, that MBL will bind or not. The fact is that sugars are not fixed in space. They are waving around and there are all sorts of stereo configurations that need to be taken into account. It is more complicated than simple linear sequences would suggest. Not a very satisfactory answer, but I am afraid that is the way it is.

Verbrugh: Because microbes are also rather dynamic structures where cell walls are broken down and synthesised, and you do show some binding at certain sites of the cell surface, but not at other sites, that may have to do with the structuring of the cell wall at the time in that organism.

Turner: I should also say, by the way, that the flow cytometry is not that sensitive, because when we came to do the immuno gold staining, we found that the sialylated parent organism of *Neisseria meningitidis* serogroup B did show some foci of binding but in much lower numbers and with the serogroup C, the 8026 mutant, which we can exogenously sialylate. We have data showing that a little bit of binding does occur. So obviously, whatever one says about the flow-cytometry results, one has to bear in mind that there is a sensitivity issue there and that one can push that a little bit further by using better techniques.

Van Strijp: One thing that would be far more sensitive is looking at it functionally. Is there complement activation, yes or no? Is there a correlation between the binding assay and complement activation in all of these organisms that you showed?

Turner: That is a good question. First of all, two things we always do are to worry a bit about the possibility that the binding we see may be going in the opposite direction and that there is some lectin on the surface of the organism recognising a sugar on the MBL, because it is a sugar-bearing molecule itself. So we always do an EDTA chelation experiment to show that the binding is a calcium-dependent one. And we always do an inhibition with an inositol glucosamine or mannose and show that that competes. So we have two controls in all these systems to help us confirm that it really is a C-type lectin interaction. Whenever we looked for C4 binding, which we can do with purified C4, we always found an absolute correlation. If it binds MBL, we can go on. We have got data to show those strains which bind MBL, bind C4, and there is a strong correlation between the levels of binding, so the answer is, yes, there is a correlation. We have yet to see an exception to that.

Van Strijp: There are people stating that the old-fashioned alternative pathway may no longer exist and that most of it is mediated by MBL. Do you think that is true?

Turner: The thing about the alternative pathway, the real important point about it, is the amplification loop, which certainly exists. I think the only point at issue is whether there is circulating this small amount of precleaved C3 which will pick up factor B. Even Peter Lackman, who was very positive about that concept in the 1980s, was very happy to think that it might well be MBL causing some of that entrée into the system. I do not have a strong view on that, but I think the fact is that both MBL-associated serine proteases (MASP) and MBL have been shown in quite primitive organisms, and even in urochordates all the MASP proteins are present. So it is an ancient system, there is no doubt about it. Whether or not there is a separate mechanism whereby organisms can become coated by alternative pathway components without MBL playing a role, I do not know.

Degener: In your experiments you have probably worked with MBLs in a solution with a bacterial inoculum, but in the human body, or in the animal experiment, where does the binding take place then? Is that at the level of mucous membranes or is there anything more needed before the binding to MBLs takes place, for instance adherence of bacteria to a membrane?

Turner: We can say that the protein is essentially an intravascular protein most of the time, but in the presence of inflammation it certainly gets extravascular. It is present in the synovial fluids of rheumatoid arthritis patients for example, it is present in jejunal secretions of AIDS patients with cryptosporidial diarrhoea. We made Western blots of such aspirates and proved MBL to be there. So it gets to some mucosal surfaces when there is inflammation, but I do not think it would normally be present at such sites. Two other members of the same family, the collectin family, are lung surfactant protein A and protein D. Many like to think of them as innate immune equivalents of IgA on mucosal surfaces. The other area where there has been some work suggesting it might get into mucosal surfaces is with cystic fibrosis, because again you would not expect it to play any role at all in that disease. But there are data to suggest that it does get into alveolar fluids.

De Vries: You said that there might be some discussion about the suggested selective advantage for the mutations. And you had two possible explanations. One was the decreased complement-mediated immunopathology and the other was the decreased invasion of intracellular parasites. Those are quite testable hypotheses. Is there any data on the subject?

Turner: Well, on the question of the parasites there was a paper in *Infection and Immunity* last year from Alan Ezekowitz collaborating with a group in Brazil. MBL binding to *Leishmania* has been directly demonstrated to the premastigotes. But population studies in Brazilians with and without *Leishmania* support the concept that having the mutation protects against leishmaniasis. There is a small population study with that disease which does support it.

De Vries: That is the outcome. But is there any evidence in that study or in any other related study that the mutation decreases the invasion by *Leishmania* parasites for instance of macrophages?

Turner: Not that I am aware of.

De Vries: And about the complement-mediated immunopathology?

Turner: There is no direct data there, but there is with C6 deficiencies some very good work from South Africa. In the Cape C6 deficiency is very common among the coloured population. These people are more susceptible to Neisserial infections, but they are far less likely to die of those infections. Many years ago Ross and Denton suggested that this is a possible explanation of the fact that they are not activating the complement system through to the end of the lytic pathway, and therefore not releasing the mediators which are so damaging, and that this might be an advantage for them in their particular environment. So it is by analogy with that C6 deficiency story that this suggestion has been put forward. Obviously, MBL should be regarded as a complement component, there is no question about that. So it is not an outrageous extrapolation to think of it in the same sort of way, but there is no more direct supportive data.

Vandenbroucke: The mutation changes the structure of the protein, you said. I was wondering when you said MBL deficiency, because you also said that neutropenic patients have lower levels. What does this mean? Does it mean a less active MBL, or does it mean lower levels of protein? In other words, if you want to study MBL deficiency, should we look at the genes, should we look at active protein or should we look at protein levels?

Turner: Good question. We believe that it is advantageous to both genotype and measure the protein levels. There is detectable protein in individuals with the mutations. But what there is not, is higher-order oligomers, which is absolutely critical for MBL function. If you cannot build it up to those higher-order structures, then you have a functional deficiency. But if you analyse what is circulating

by SDS page, for example, from someone who is homozygous for a mutation compared with my own MBL, which shows normal levels, you can see that there is a spike in the monomeric region in the person with the homozygous mutation and then a series of higher-order oligomers in my serum. So there is detectable protein there, but it is functionally lousy protein.

Appelmelk: The protein is evidently rather promiscuously binding to a variety of sugars. We as humans are full of high-mannose chains. So why are we not killed by MBL? When there is flexibility, you only need mannose. Why do we stay alive?

Turner: I think there are two possible answers to that. First of all, we tend to cap sugars with sialic acid on most cell surfaces. As a generalisation, I think you would agree that most oligosaccharide chains on human cell surfaces, protein oligosaccharides and so on, are terminating with sialic acid. Secondly, the sorts of characteristic repeating sugars that one sees on *Candida* for example, with mannan particularly, are much less commonly seen on mammalian cell surfaces. So the fact of the matter is, you have to have a repeating array of sugar groups. It is about this position and separation between them that the MBL is beautifully organised to respond to.

Kullberg: I am still intrigued by the *Leishmania* issue, because when there is a 40% incidence of polymorphism, one would think that there would be a strong selection pressure to keep these people in the population. Leishmaniasis obviously is not a disease that occurs everywhere, which would lead to this selection. So would not the *Leishmania* story act as a paradigm for other intracellular organisms? And the first one you would think of is *M. tuberculosis*, and maybe *Salmonella* may have a large impact. Are there any studies on that?

Turner: The reason why I have not discussed other organisms is because it is at this stage a rather confusing story. There are groups working on both *Mycobacterium tuberculosis* and leprosy, and initially the data seemed to be supporting the hypothesis, but I have recently examined a thesis from the University of Hong Kong. There was a large cohort of Chinese patients with tuberculosis, and instead of those individuals having the expected high frequency of wild-type MBL genotypes, in fact there was an increased frequency of mutations. It was against the hypothesis. I am again not going to put my neck on the block and say, I am totally wedded to the concept, because there are enough worrying stories coming through to make me a bit reserved on that front. Obviously, other diseases have also been considered: malaria was a candidate very early on because of its high incidence in Africa. The frequency obviously varies enormously around the world, as you saw, but what is very striking is that it is in the tropical regions of the world that we get the highest frequencies. I personally have not worked on this at all. I am just acting as a mouthpiece for some of the work of others. It is an extremely interesting question, because clearly the mutations have arisen independently of each other, the 57 and the 54 mutation, in different parts of the world and then both have risen to high frequencies in the population. It is quite a striking thing that that should have happened. It does not seem very logical that it is simply genetic drift, and that is really about as far as I can go at this stage. The malaria story is equally confused. There is one study by Bellamy in The Gambia which came to the conclusion that there was no strong association.[1] Another study by a German group came to the contrary view. But that was in a much smaller cohort.

Kullberg: The other question is about the pathophysiology of this mechanism. Do we really know that the binding of a micro-organism like *Leishmania* and the subsequent invasion is the cause, or might it be that the macrophage activation is playing a role through differences in cytokine induction, which also would explain this fact?

Turner: I agree with you there.

REFERENCE

1. Bellamy R. Identifying genetic susceptibility for tuberculosis in Africans: a combined approach using a candidate gene and a genome-wide screen. Clin Sci (Lond) 2000;98:245-50.

Toll-like receptors contribute to host responses against mycobacterial infection

M.J. Fenton, K.A. Heldwein

Boston University School of Medicine, the Pulmonary Center, Boston, USA

ABSTRACT

Mammalian toll-like receptor (TLR) proteins are pattern recognition receptors that mediate cellular activation by a wide variety of bacterial products. TLR activation leads to the expression of numerous mediators of innate immunity. We used the nonpathogenic mycobacterium *M. bovis* BCG (BCG) to define the roles of TLR proteins in the normal development of an immune response against the bacilli. We found that TLR2$^{-/-}$ mice were unable to effectively control the growth of BCG *in vivo*, compared with normal and TLR4$^{-/-}$ mice. Furthermore, splenic T cells isolated from infected TLR4$^{-/-}$ mice could proliferate *in vitro* following antigen challenge, but were unable to generate a strong Th1-type response. In contrast, splenic T cells isolated from infected TLR2$^{-/-}$ mice could neither proliferate *in vitro* following antigen challenge nor generate a strong Th1-type response. Together, these studies indicate that TLR proteins participate in the development of both innate and adaptive immune responses.

INTRODUCTION

Infection of macrophages with mycobacteria is accompanied by activation of the transcription factor NF-κB, secretion of inflammatory mediators (e.g. TNF-α, IL-1b), release of the reactive nitrogen intermediate nitric oxide (NO), and secretion of several chemokines.[1] Until recently, the signalling pathways that elicit the production of these mediators have remained unknown. Members of the mammalian Toll-like receptor (TLR) family have been implicated in the activation of macrophages by a variety of chemically diverse bacterial products.[2,3] We previously demonstrated that viable *M. tuberculosis* (Mtb) bacilli contain distinct ligands that activate cells via TLR2 and TLR4, whereas heat-killed Mtb failed to activate cells via TLR4.[4] In contrast, *M. avium* appears to lack any TLR4 agonists.[5] Several purified mycobacterial products have now been identified as TLR2 agonists, including arabinose-capped lipoarabinomannan,[6] dimannosylated phosphatidylinosito,[7] and the 19 kDa lipoprotein antigen.[8] The identity of the Mtb TLR4 agonist remains unknown. Interestingly, TLR2 agonists activate macrophages to express only some of target genes activated by TLR4 agonists.[7,9] This difference is due, at least in part, to the capacity of TLR4 agonists, but not TLR2 agonists, to induce β-interferon (IFN-β) expression. IFN-β acts in an autocrine/paracrine manner to activate the transcription factor STAT1.[10] TLR4-dependent STAT1 activation, in combination with NF-κB, results in the expression of several genes that mediate host responses against bacterial pathogens. These include iNOS, IL-12p40, IL-6, and several chemokines.[10]

Given the spread of drug-resistant Mtb strains, there is a pressing need to develop treatments that augment host innate immunity rather than to rely on new antibiotics. One novel approach would be to develop therapeutics that antagonise TLR proteins. This has been accomplished for one TLR4 agonist, Gram-negative bacterial lipopolysaccharide (LPS) and its pharmacophore lipid A. Three lipid A analogues, lipid IVA, *Rhodobacter sphaeroides* lipid A (RSLA) and E5531 have all been reported to function as LPS antagonists when tested both *in vitro* and *in vivo*.[11-13] We subsequently demonstrated that RSLA could also block signalling by a TLR2 agonist, the mycobacterial glycolipid lipoarabinomannan.[14] Together, these data suggest that certain lipid A structural antagonists are capable of blocking TLR-dependent activation by molecules that are chemically dissimilar to LPS. We later showed that the synthetic lipid

A-like antagonist E5531 could block TLR4-dependent signalling induced by Mtb. E5531 could inhibit selected Mtb-induced macrophage responses, namely apoptosis and TNF-α secretion, but it did not block Mtb-induced NO production.[15] Subsequent studies revealed that induction of NO production by Mtb was mediated by a TLR-independent mechanism[15] (and unpublished observations). This underscores the concept that while purified bacterial TLR agonists can activate macrophages to express a variety of proinflammatory mediators *in vitro*, this is not predictive of TLR-dependent macrophage activation by intact bacteria. Here we have sought to determine the relative contributions of both TLR2 and TLR4 to innate and adaptive immune responses. In these studies we have used the nonpathogenic mycobacterium BCG to investigate the role of TLR proteins in host immune defence *in vivo*. We reasoned that the use of BCG would allow us to examine the contributions of TLR proteins to the normal development of host immunity in the absence of mycobacterial virulence factors that corrupt the normal development of these host responses.

METHODS AND MATERIALS

Reagents

M. bovis BCG (ATCC 35734) was purchased from the ATCC. Bacteria were grown in LPS-free Middlebrook 7H9 liquid medium, supplemented with AODC, Tween 80, and glycerol. Cultures were grown to a density of 0.5 to 0.6 (OD_{620}). Numbers of BCG per ml of culture were determined by colony counting.

Animals and cells

$TLR2^{-/-}$ and $TLR4^{-/-}$ mice (female, 5 to 7 weeks old) were provided by Dr Shuzio Akira (Osaka University), and have been previously described.[16] These mice had been previously backbred onto a C57Bl/6 background for four generations prior to their use in these studies. Inbred C57Bl/6 mice were used as control animals. Thioglycollate-elicited peritoneal exudate macrophages were isolated from uninfected mice, and cultured *in vitro*, as we have previously described.[6] Each mouse was infected with 10^6 CFU of *M. bovis* BCG by intraperitoneal injection, and sacrificed 14 days later. Spleens were removed from the infected mice, and total splenocytes were prepared for culture *in vitro*, as previously described. Contaminating LPS levels in all media components were <10 pg/ml final concentration as measured by *Limulus* amebocyte lysate kit (BioWhittaker).

Measurements of cytokine production by macrophages *in vitro*

Peritoneal macrophages from $TLR2^{-/-}$, $TLR4^{-/-}$ and normal mice were infected *in vitro* (5 BCG per macrophage) with live BCG for 18 hours. The culture supernatants were removed, filtered to remove any BCG, and then cytokine levels were measured using specific ELISA kits, as recommended by the manufacturer (R&D Systems and Pharmingen).

Measurement of bacterial loads in the lung

Lungs removed from infected mice were homogenised in a sterile blender, using a lysis buffer consisting of sterile water containing 0.025% SDS. Homogenates were diluted in the lysis buffer, and 1 ml aliquots were cultured on Middlebrook 7H11 agar plates supplemented with glycerol, L-arginine and cycloheximide (10 mg/ml). Colonies were counted 14 days later.

Splenic T cell restimulation assay

Spleens were recovered from uninfected and BCG-infected mice 14 days after inoculation. Total splenocytes were obtained by tissue disruption between sterile frosted glass slides, and erythrocytes were removed by lysis in Tris-buffered ammonium chloride (Sigma). Splenocytes were then cultured in 96 well plates (5×10^5 cells/well) in the presence or absence of heat-killed BCG (10^3 CFU/well). For proliferation measurements, splenocytes were cultured for two additional days and pulsed with ^3H thymidine (1 mCi/well) eight hours prior to harvesting. Cells were harvested using an automated cell harvester (Skatron Instruments) and incorporation of radiolabelled thymidine was measured by scintillation counting. For cytokine secretion, splenocytes were cultured for three additional days. Culture supernatants were then recovered and specific cytokine levels measured by ELISA.

RESULTS AND DISCUSSION

TLR proteins are necessary for specific macrophage responses to BCG *in vitro*

Peritoneal macrophages from $TLR2^{-/-}$, $TLR4^{-/-}$ and normal mice were infected *in vitro* with live BCG for 18 hours. Cytokine levels in the culture supernatants were measured using ELISA and the Greiss assay respectively. As shown in *figure 1*, BCG-induced TNF-α and IL-6 secretion was substantially lower in the $TLR2^{-/-}$ macrophages compared with both $TLR4^{-/-}$ and normal macrophages. In contrast, secretion of IL-12p70 was similar in BCG-stimulated macrophages from all three mouse strains examined. This suggests that TLR2, but not TLR4, is necessary for BCG-induced TNF-α and IL-6 production, whereas IL-12 production does not depend on these TLR proteins. In these experiments, TNF-α production was strongly dependent on the presence of TLR2. Numerous investigators have shown that TNF-α expression in macrophages can be induced using purified TLR2 agonists.[6,17-19] Intact bacteria,

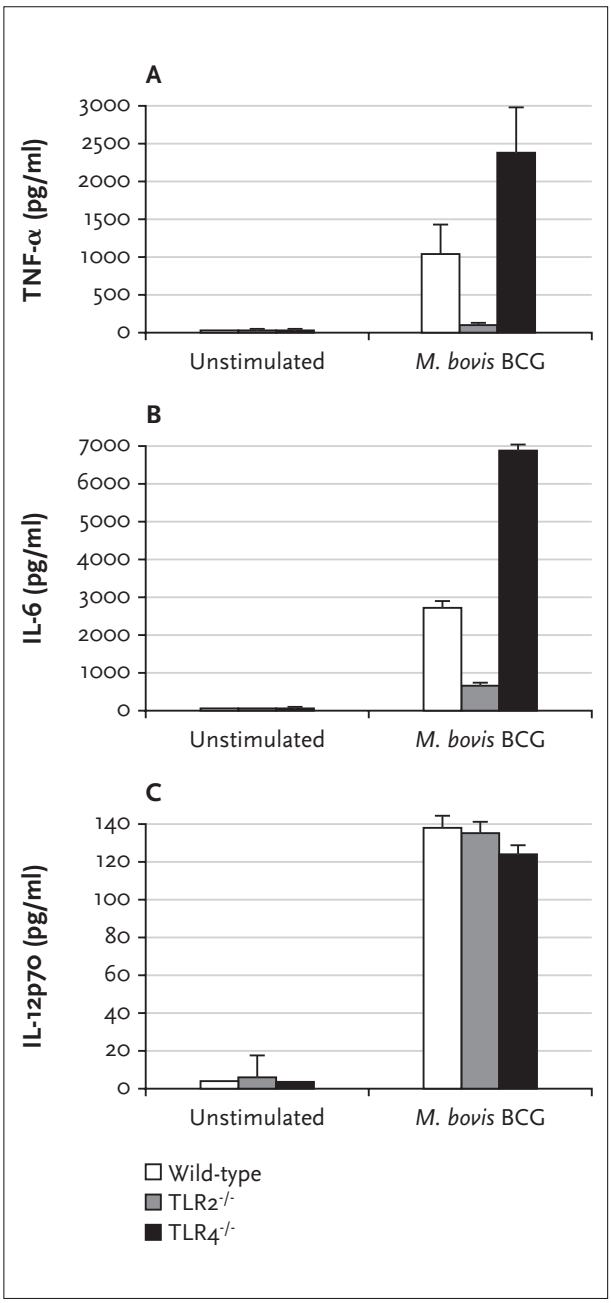

Figure 1
Cytokine production by normal and TLR-deficient macrophages stimulated with BCG

Thioglycollate-elicited peritoneal macrophages (5 x 10^5 cells/well) from wild-type, $TLR2^{-/-}$ and $TLR4^{-/-}$ mice were stimulated with live BCG (five bacilli/macrophage) for 24 hours, and supernatants assayed for TNF-α (A), IL-6 (B) and IL-12p70 (C) by quantitative ELISA. Data are presented as the mean values of triplicate wells ± SD. Results are representative of duplicate experiments.

such as Gram-negative organisms and Mtb, possess both TLR2 and TLR4 agonists, although it is likely that the relative expression of these agonists can vary greatly. Thus, the relative levels of TLR2 and TLR4 agonists expressed by different mycobacterial species are likely to dictate which TLR protein is most necessary for the induction of TNF-α expression. We had previously shown that Mtb possesses both TLR2 and TLR4 agonists, and that blocking TLR4 signalling with the lipid A-like LPS antagonist E5531 largely blocked TNF-α secretion induced by Mtb.[15] In contrast *M. avium* does not appear to be capable of activating cells via TLR4.[5] It has been reported that BCG possess both TLR2 and TLR4 agonists,[20] but the relative abundance of these agonists had not been determined. We hypothesise that BCG express lower levels (or fewer types) of TLR4 agonists than Mtb. Similarly, BCG may express higher levels (or more types) of TLR2 agonists, compared with Mtb. In either case, this could explain why TLR4 is not necessary for macrophage activation by BCG. In contrast to TNF-α and IL-6, IL-12 secretion by BCG-stimulated macrophages did not depend on the presence of TLR2 or TLR4. This finding was unexpected, but is reminiscent of Mtb-induced NO production, which is independent of TLR proteins.[15] It remains to be formally determined whether BCG-induced IL-12 production, like NO production, is also mediated by a TLR-independent mechanism.

TLR2 is necessary for resistance to mycobacterial infection *in vivo*

$TLR2^{-/-}$, $TLR4^{-/-}$ and normal mice were infected with live BCG for 14 days. The lungs were then removed and the number of bacteria in the tissues was counted. As shown in *figure 2*, the lungs from infected $TLR2^{-/-}$ mice contained more BCG bacilli than lungs from $TLR4^{-/-}$ and control mice.

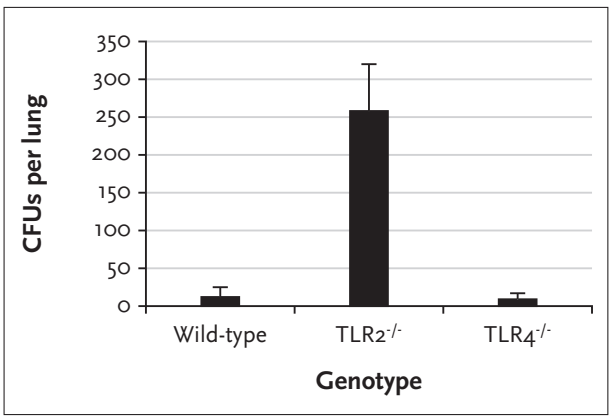

Figure 2
Bacterial loads in the lungs of normal and TLR-deficient mice infected with BCG

Normal and TLR-deficient mice were infected intraperitoneally with 10^6 CFU *M. bovis* BCG, and the lungs were harvested 14 days later. Lung homogenates were then prepared, diluted, and cultured on Middlebrook 7H11 agar plates for colony counting. Data are presented as mean of lung CFUs from three mice per genotype ± SEM. Results are representative of duplicate experiments.

Both normal and TLR4-deficient mice were fully capable of controlling the infection. Thus, the absence of TLR2 led to permissive growth of BCG in the lung. This finding is consistent with the inability of TLR2$^{-/-}$ macrophages to secrete substantial amounts of TNF-α following BCG challenge *in vitro* (*figure 1*). Studies performed using TNF-α$^{-/-}$ mice have demonstrated the importance of TNF-α in controlling mycobacterial growth *in vivo*.[21] It remains to be determined whether diminished production of TNF-α is responsible for the lack of mycobacterial growth control in the TLR2$^{-/-}$ mice. These data do indicate that TLR2 is necessary to generate an effective host response against mycobacterial infection. These findings do not completely exclude a role for TLR4 in the host response against mycobacterial infection. Our studies shown here used a background mouse strain that is genetically resistant to mycobacterial infection (i.e. C57Bl/6), as well as a relatively low inoculum of BCG. Preliminary studies performed using a mouse strain that is genetically sensitive to mycobacterial infection and lacks functional TLR4 (i.e. C3H/HeJ mice) have revealed that TLR4 does indeed contribute to the resistance to BCG infection *in vivo* (SN Vogel and MJF, unpublished observations). Furthermore, this protective role for TLR4 was only observed using a higher inoculum of BCG (>10^7 CFU/mouse).

Splenic T cells from both TLR2$^{-/-}$ and TLR4$^{-/-}$ mice fail to generate a potent Th1-type response *in vitro*

In order to characterise TLR-dependent control of BCG growth *in vivo*, we sought to determine whether TLR-deficient mice failed to elicit an effective innate and/or adaptive immune response following BCG infection. To test these possibilities, we isolated total splenocytes from uninfected and BCG-infected normal and TLR-deficient mice. Splenic T cells were then stimulated *in vitro* with heat-killed BCG as a source of antigen. Activation of the T cells was assessed 48 hours later by measuring both cytokine production and T-cell proliferation (^{3}H thymidine incorporation). As shown in *figure 3*, splenocytes isolated from uninfected mice did not respond to antigenic stimulus, as measured by either cytokine production or T-cell proliferation. In contrast, normal splenocytes from infected normal mice secreted IFN-γ following antigenic stimulation. This IFN-γ production was not observed using splenocytes from infected TLR2$^{-/-}$ or TLR4$^{-/-}$ mice. These findings suggest that TLR-deficient splenocytes fail to develop a potent Th1-type response *in vitro*. We do not believe that this failure to secrete IFN-γ represents a skewing of Th responses, as antigen-stimulated splenocytes from infected normal and TLR-deficient mice did not secrete measurable amounts of IL-4 or IL-5 *in vitro* (data not shown). In subsequent studies, we examined T-cell proliferation *in vitro* and observed that cells from normal and TLR4$^{-/-}$ mice proliferated in response to antigenic stimulation, whereas cells from TLR2$^{-/-}$ mice did not (*figure 3B*). Taken together, these data suggest that TLR2 and TLR4 contribute in a fundamentally distinct manner to the development of an effective Th1 response. Antigen-specific T cells are generated in BCG-infected TLR4$^{-/-}$ mice, as indicated by the proliferation of these cells *in vitro* following antigenic challenge. Nevertheless, these T cells fail to develop into effective Th1 responder cells, as indicated by the absence

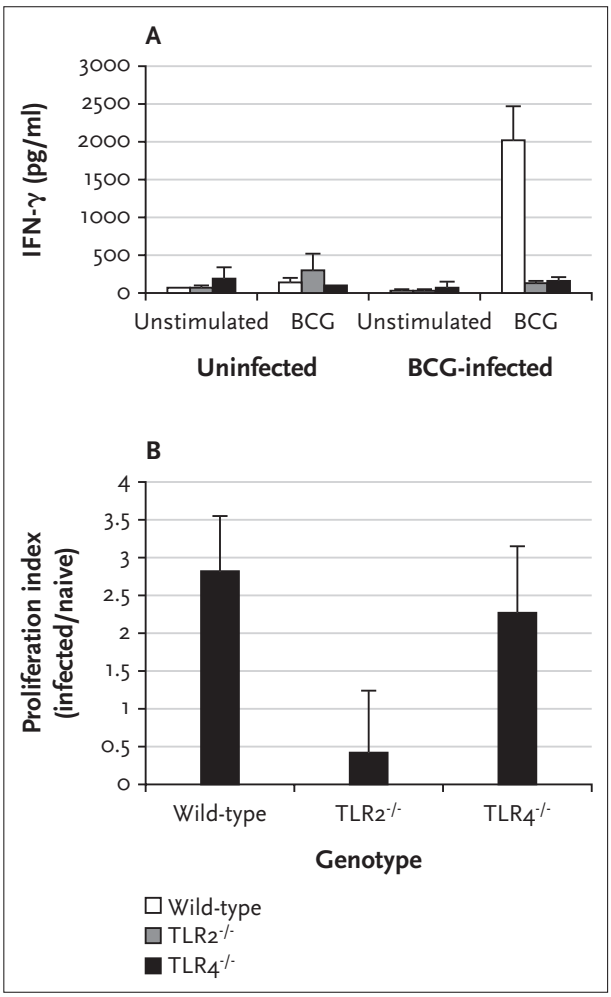

Figure 3

Antigen-dependent activation of splenocytes from BCG-infected normal and TLR-deficient mice

Splenocytes isolated from uninfected and *M. bovis* BCG-infected mice were cultured (5 x 10^5 splenocytes/well) in the presence or absence of heat-killed BCG (10^3 CFU/well). (A) After three days, culture supernatants were harvested and IFN-γ levels were measured by specific ELISA. (B) After two days in culture, splenocytes were pulsed with ^{3}H-thymidine for eight hours. Background values of isotope incorporation in unstimulated cells were subtracted from values of stimulated cells, and data are expressed as a ratio of specific isotope incorporation in stimulated cells from infected mice over uninfected mice. Triplicate wells of cells from each mouse were assayed. Data are presented as the mean of values from three uninfected or three infected mice per genotype ± SEM. Results are representative of duplicate experiments.

of IFN-γ secretion. This is not a consequence of skewing towards a Th2 phenotype, as there is no increase in IL-4 and IL-5 production by the antigen-stimulated $TLR4^{-/-}$ splenocytes. Thus, the immune defect in these cells appears to come from the inability of Th0 cells to commit to a Th1 phenotype. It should be noted that the absence of a strong Th1 response did not detract from the ability of $TLR4^{-/-}$ mice to control BCG infection *in vivo* (*figure 2*). This may be due, in part, to effective innate immunity (as evidenced by normal proinflammatory cytokine production, *figure 1*) additional to Th1-independent adaptive immunity. Furthermore, Th1 responses may not be absent in these mice, only diminished relative to control animals.

The finding that $TLR2^{-/-}$ splenocytes failed to both proliferate and secrete IFN-γ following antigenic challenge *in vitro* contrasts with the phenotype of the $TLR4^{-/-}$ cells. The BCG-infected $TLR2^{-/-}$ mice appear to possess a defect in the development of antigen-specific T cells. The inability of TLR2-deficient cells to secrete IFN-γ would then simply be a consequence of the lack of antigen-responsive T cells, rather than (or in addition to) a defect in Th1 commitment. This possibility is consistent with our finding that antigen-independent activation of $TLR2^{-/-}$ splenocytes, using cross-linked anti-TCR antibodies, induced both T-cell proliferation and IFN-γ secretion (data not shown). We do believe that $TLR2^{-/-}$ mice are capable of developing antigen-specific T cells, and published data report that these mice contain normal numbers and types of immune cells.[16] Taken together, our data suggest that the absence of TLR2 renders the mice incapable of responding to the adjuvant activity manifested by the mycobacteria themselves. This adjuvant activity is critical for the development of T cells that recognise mycobacterial antigens, and for the ability of the host to mount an effective adaptive immune response. Because $TLR2^{-/-}$ macrophages fail to secrete critical proinflammatory cytokines in response to mycobacterial infection *in vitro*, it is likely that these cells also fail to express critical cytokines and co-stimulatory molecules *in vivo* during the course of infection. Dendritic cell maturation and function may also be defective in $TLR2^{-/-}$ mice due to the inability of these cells to respond to mycobacterial TLR2 agonists. Several laboratories have previously shown that TLR agonists induce dendritic cell maturation and activation.[22-24] Furthermore, those dendritic cells that are incapable of responding to TLR agonists fail to mature and become functional *in vitro*.[25] Studies are currently underway in the laboratory to characterise the immune defects observed in $TLR2^{-/-}$ and $TLR4^{-/-}$ mice.

REFERENCES

1. Fenton MJ. Macrophages and tuberculosis. Curr Opin Hematol 1998;5:72-8.
2. Heldwein KA, DT Golenbock, MJ Fenton. Recent advances in the biology of Toll-like receptors. Mod Asp Immunobiol 2001;1:249-52.
3. Akira S, K Takeda, T Kaisho. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2001;2:675-80.
4. Means TK, Wang S, Lien E, Yoshimura A, Golenbock DT, Fenton MJ. Human Toll-like receptors mediate cellular activation by M. tuberculosis. J Immunol 1999;163:3920-7.
5. Lien E, Sellati TJ, Yoshimura A, et al. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. J Biol Chem 1999;274:33419-25.
6. Means TK, Lien E, Yoshimura A, Wang S, Golenbock DT, Fenton MJ. The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. J Immunol 1999;163:6748-55.
7. Jones B, Means TK, Heldwein KA, et al. Different Toll-like receptor agonists induce distinct macrophage responses. J Leuk Biol 2001;69:1036-44.
8. Brightbill HD, Libraty DH, Krutzik SR, et al. Host defense mechanisms triggered by microbial lipoproteins through Toll-like receptors. Science 1999;285:732-6.
9. Hirschfeld M, Weis JJ, Toshchakov V, et al. Signaling by TLR2 versus TLR4 agonists results in differential gene expression in murine macrophages. Infect Immun 2001;69:1477-82.
10. Toshchakov V, Jones BW, Zhang S, et al. Differential Stat1a/b Phosphorylation Secondary to IFN-b Production Mediates the Distinct Pattern of Macrophage Gene Expression Observed in Response to TLR4 and TLR2 Agonists. Nature Immunol 2002;3:392-8
11. Kovach NL, Yee E, Munford RS, Raetz CR, Harlan JM. Lipid IVA inhibits synthesis and release of tumor necrosis factor induced by lipopolysaccharide in human whole blood ex vivo. J Exp Med 1990;172:77-84.
12. Golenbock DT, Hampton RY, Qureshi N, Takayama K, Raetz CR. Lipid A-like molecules that antagonize the effects of endotoxins on human monocytes. J Biol Chem 1991;266:19490-8.
13. Christ WJ, Asano O, Robidoux LC, et al. E5531, a pure endotoxin antagonist of high potency. Science 1995;268:80-3.
14. Savedra R Jr, Delude RL, Ingalls RR, Fenton MJ, Golenbock DT, et al. Mycobacterial lipoarabinomannan recognition requires a receptor that shares components of the endotoxin signaling system. J Immunol 1996;157:2549-54.
15. Means TK, Jones BW, Schromm AB, et al. Differential effects of a Toll-Like receptor antagonist on macrophage responses to M. tuberculosis. J Immunol 2001;166:4074-82.
16. Takeuchi O, Hoshino K, Kawai T, et al. Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. Immunity 1999;11:443-51.
17. Underhill DM, Ozhinsky A, Smith KD, Aderem A. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. Proc Natl Acad Sci USA 1999;96:14459-63.
18. Werts C, Tapping RI, Mathison JC, et al. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. Nature Immunol 2001;2:346-52.
19. Hirschfeld M, Kirschning CJ, Schwandner R, et al. Inflammatory signaling by Borrelia burgdorferi lipoproteins is mediated by Toll-like receptor 2. J Immunol 1999;163:2382-6.
20. Tsuji S, Matsumoto M, Takeuchi O, et al. Maturation of human dendritic cells by cell wall skeleton of M. bovis bacillus Calmette-Guerin: involvement of Toll-like receptors. Infect Immun 2000;68:6883-90.
21. Bekker LG, Moreira AL, Bergtold A, Freeman S, Ryffel B, Kaplan G. Immunopathologic effects of tumor necrosis factor-a in murine mycobacterial infection are dose dependent. Infect Immun 2000;68:6954-61.

22. Michelsen KS, Aicher A, Mohaupt M, et al. The role of Toll-like receptors (TLRs) in bacteria-induced maturation of murine dendritic cells (DC). Peptidoglycan and lipoteichoic acid are inducers of DC maturation and require TLR2. J Biol Chem 2001;276:25680-6.
23. Re F, Strominger JL. Toll-like receptor 2 (tlr2) and tlr4 differentially activate human dendritic cells. J Biol Chem 2001;276:37692-9.
24. Thoma-Uszynski S, Kiertscher SM, Ochoa MT, et al. Activation of Toll-like receptor 2 on human dendritic cells triggers induction of IL-12, but not IL-10. J Immunol 2000;165:3804-10.
25. Schnare M, Barton G, Holt A, Takeda K, Akira S, Medzhitov R. Toll-like receptors control activation of adaptive immune responses. Nature Immunol 2001;2:947-50.

Discussion following lecture by M.J. Fenton

Van der Meer: I enjoyed that very much. Just to go back to the concept of the Toll-like receptor (TLR) as first line of response, can you tell us a bit more about the molecular interaction between bacterial components and TLR? Do they actually bind, and if so, how?

Fenton: Some of you may have noticed that I tried to stick to the word 'agonist' rather than to the word 'ligand'. That is not a mere coincidence. With the exception of Gram-negative bacterial lipopolysaccharide (LPS) there has really been no clear biochemical demonstration of any of these molecules serving as classic ligands and we have little sense of what the affinity of ligands for these receptors is. In the matter of LPS, Richard Ulevitch's lab and a few others have sought to demonstrate a physical interaction in using cross-linking approaches. They have been able to show a physical association between Gram-negative LPS and TLR4 in the presence of the coreceptors CD14 and MD2. If you transfect the cell with an expression plasmid encoding TLR4 in the absence of the coreceptors CD14 and MD2 you basically have to throw in milligram amounts of LPS to get an activation. So from the point of view of ligand-receptor interaction all these Toll-receptor agonists are very poor ligands by themselves. They probably operate in the context of a larger receptor complex that includes coreceptors such as CD14. I think it is reasonable to assume that there are a variety of co-receptors that may help TLR2 agonists or TLR9 agonists to also recognise the receptors and engage a signal. The other part of this paradigm relates to how strongly the receptor is engaged and what the kinetics of this interaction are. Several studies that have been published using FACS analysis to look at receptor complex interaction have shown that, if you use FITC-labelled LPS as your classic ligand, you can demonstrate a rapid and transient interaction between LPS, TLR4 and CD14. But interestingly, a few seconds or minutes after this initial interaction and activation the receptor components seem to move to different places on the cell and in some cases some of the components are internalised. Many of you here are familiar with Sam Wright's work and the story of LPS internalisation. I think that if you take this together for LPS it suggests that there is a multireceptor component that is not assembled and only engaged on the cell surface for a very short period of time to allow a signal to be generated. The receptor components then disassemble and go elsewhere following the activation. There does not seem to be a strong high-affinity interaction between TLR proteins and any of their agonists in the absence of coreceptors.

Hermans: I have a question about the polymorphisms that are known to occur both in TLR4 and TLR2. In TLR4 there are amino acid substitutions known to lead to hyporesponsiveness. But as far as I am aware they do not show much of a link with the severity of or susceptibility to infectious diseases.

Fenton: As far as I am aware there is not a strong correlation in this case between TLR4 mutations and susceptibility to disease. In experimental models with normal human volunteers looking at the susceptibility to LPS by inhalation, David Schwartz has been able to show a correlation between certain polymorphisms in TLR4 and hyporesponsiveness of humans to LPS.[1] But at least so far there does not seem to be an increased incidence of disease. The very few studies that have been published so far looked at the extracellular domains of the Toll-like receptor proteins. Most of the work today is focused on the intracellular domains looking at intracellular signalling. Some new studies in the Journal of Immunology have shown that the extracellular domain of TLR4 possesses a hypervariable domain and most of the polymorphisms seen in different human populations or amongst different species seem to cluster in this hypervariable region. It does seem to have an impact on the ability of TLR4 to recognise LPS or different forms of LPS as well. So we are beginning to get a sense of where within the molecule recognition really occurs, but so far the correlation with disease has been minimal.

Ottenhoff: I have a question about the IL-12. You said that TLR2 knock-out mice produce normal IL-12 upon BCG

stimulation. *In vitro* there is little interferon-α production. So my question actually is, what is the mechanism to explain that?

Fenton: That is a good point. Dr van der Meer said last night at dinner that we were supposed to expose all of our Achilles heels to the panel today. So it turns out to be a very complicated story. You really need to approach it from three different angles. One is to look at the question of how purified TLR agonists induce specific responses in macrophages *in vitro*. The next would be then to look at the whole bacterium as well, because those responses are going to be very different. A great example of this is if you look at nitric oxide (NO) production induced by mycobacteria. I am a bit slow maybe in getting to the answer to your question, but you can add purified LPS for example as a classic TLR4 agonist and show that you can activate nitric oxide production and that occurs through the TLR4 pathway. If you then use live mycobacteria to induce NO it turns out that in that setting it is a TLR-independent process that drives the NO production. We published those results a few years ago. So you have got to try to keep the two pathways apart. With the data I showed for IL-12, we have not yet determined whether the IL-12 production is dependent on TLRs or not in that setting. It may be in the setting of whole live mycobacteria that the macrophages do not need TLRs to make IL-12. Experiments are going on right now in the laboratory to determine that. Now *in vivo* you would have expected that the presence of IL-12 would have correlated with a fine ability to produce γ-interferon. So I think that is going to be the biggest Achilles heel of the data I showed you today, which are all unpublished and very preliminary, which is why do we see good IL-12 production and not good γ-interferon production? I think the reasons for that can differ for the two kinds of mice. In the case of the TLR2 knock-out mice, I think we are failing to get γ-interferon production because we failed to develop a large panel of antigen-specific T cells. The T cells are just not there. You can give them all the IL-12 you want and they will not do anything with it. The TLR4 knock-out animal is a bigger mystery because we – presumably – have antigen-specific T cells there but they are not making γ-interferon. I think part of that answer comes from the process of a Th0 cell maturing into a Th1 cell. So one possibility is that in the absence of TLR4 we are unable to fully activate the development of a commitment to the Th1 phenotype. I should point out that we do not have Th skewing here. We are not skewing the animals towards Th2. There is no production of IL-4 and IL-5 in these TLR4 knock-out animals. We therefore think that there is a defect in Th1 commitment and we are testing that possibility now. The other issue may be a threshold issue. We have not looked at IL-18 or at IL-23 as other components of these three cytokines leading to α-interferon production. It may be that again we have plenty of IL-12 but not sufficient IL-18 and IL-23 to lead to a threshold of γ-interferon production. Lastly, in the C3H/HeJ model, where we now have a higher infective dose and we do see disseminated bacterial growth when you look at IL-12 levels in the serum of these mice, you do find in this case that the IL-12 levels in the animal are lower. I think that under those conditions we are seeing a correlation and γ-interferon levels are way down in these animals as well. So under the right conditions we can see it. All I can say to answer your question directly is that the splenocyte assay gives us an answer with regard to T-cell responses in the dish, but I do not think that it will really surprise anyone here that that does not necessarily correlate with what happens *in vivo*.

Netea: I have two questions. One is going back to the coreceptors for TLRs. When you mentioned all the TLRs and the agonists that are known, you did not include TLR6 and TLR1. They are described as possibly serving as coreceptors. Do you think that there are two classes of TLRs? TRLs which are really signalling and TLRs which are coreceptors? That is my first question, and a second one relates to the Toll reacting to Spätzle, an endogenous substance in *Drosophila*. In humans most of the data are done in the context of pathogens and we get more or less the same signals as in the context of IL-1 stimulation. My question is, why do you think that we also need an IL-1 system? We do have more or less the same intracellular signalling going through TLRs. Do you think that we in fact developed a kind of intention to amplify our TLR system by using IL-1?

Fenton: The TLR2 and coreceptor question is of course an important one. Those of you who are familiar enough with the data will probably agree that TLR2 probably does not work alone. TLR proteins probably function as dimers. In the case of TLR2 it probably functions only as a heterodimer in association with either TLR1 or TLR6. We do not have any data so far to answer the question whether TLR2-dependent responses also utilise TLR6 *vs* TLR1, so I cannot answer that question. In terms of whether they are coreceptors or actually contribute differently to the signalling in the intracellular responses, it is quite likely that they will. I think that if you look at responses mediated through TLR2.1 *vs* 2.6 you are likely to find some differences. Most people who have looked at TLR-dependent responses have focused on a similar set of cellular responses such as IL-6 or TNF production, IL-1 production. Those seem to be shared in common with all of the TLR receptors. It is only when you begin looking at different responses that you start to see differences: for example our findings that TLR4 engagement leads to type-1 interferon production, but engagement of TLR2

does not. I think you are likely to see differences between TLR2.6 and TLR2.1, but the data have not been published in that regard. It is also – just before I leave the topic of coreceptors – quite likely that non-TLR coreceptors are going to be involved as well. I think there is indirect evidence to support complement receptors as being TLR coreceptors, perhaps the macrophage mannose receptor or the scavenger receptor, maybe even certain Fc receptors as well.
To answer your second question, I think it ties in a bit to what I have just said, which is that there are certain common responses that seem to occur when you activate all of these receptors, not only all of the TLR receptors but also the IL-1 and IL-18 receptors. But clearly the functional responses of the cells are different. You can induce for example apoptosis in some cells triggered through TLR4, TLR2, but certainly not in cells stimulated through IL-1 or IL-18. So as usual the devil is in the details. There are certain responses that seem to be Toll dependent and certain responses that are specific for Tolls and not for IL-1 or IL-18 receptors. So what does that mean in the big picture of the biology? I think that like the IL-1 and IL-18 receptors, Toll receptors play an important role in the inflammatory response. The inflammatory response as a result of a pathogen invasion, but perhaps an inflammatory response mediated by endogenous factors as well. There certainly is an amplification loop going on here. IL-1 and IL-18 production is certainly going to be important in maintaining the inflammatory response and the duration of the response, and there is also the likely possibility that endogenous factors are being made that feed back into the Toll receptors themselves.
You pointed out the ligand for the *Drosophila* Toll, the Spätzle protein. Spätzle is produced and cleaved and feeds back into the Toll itself, but just as in the mammalian Toll agonist, no-one has actually demonstrated Spätzle binds as a ligand to Toll. It is all indirect evidence that supports that hypothesis. There are some studies suggesting that mammalian proteins can feed back into Toll receptors, heat-shock proteins being an important example.
Cells that are damaged in the course of bacterial invasion may release these heat-shock proteins, they may be an endogenous danger signal as well. There is no evidence that these proteins can recognise IL-1 or IL-18 receptors. So I think we can draw a series of circles some of which will overlap. I think the IL-1 and IL-18 receptors and the Toll receptors play similar roles in the development and maintenance of inflammation. But I think they also play highly specific roles depending on the type of pathogen and the type of response and whether it is an organism possessing TLR4 antigens or not. I think it is almost as if we can say that the innate immune response is adaptive in that it can recognise a variety of different motives through this large variety of different TLR proteins that are available.

Verbrugh: I have three more discussants who want to ask a question.

Appelmelk: What is your explanation that a knock-out in TLR2 completely blocks all T-cell development? I can understand it for lipoarabinomannan within the CD1 environment but not for Toll-independent proteins in the CD4 environment.

Fenton: Hopefully it will be the simple answer that in the absence of TLR2 you fail to get the expression of a basic set of cytokines and costimulatory molecules that are necessary to clonally activate T-cell populations. Let's hope it is just as simple as that. We have tested the antigen presentation function in the TLR2-deficient cells and antigen presentation appears normal. So we think it is a defect in either costimulus or cytokines.

Kuijpers: My question is related to the fact that a moderate dose of BCG could be coped with in the TLR4 knock-out mice. Is it due to the fact that there is an increased level of other cytokines available (thinking about TNF), or has that been dealt with experimentally?

Fenton: We have only looked at a few cytokines in that model. The circulating TNF levels appeared to be normal in those animals. So far, we have just seen the deficiency in α-interferon and IL-12.

McAdam: I have a question similar to the last one. Did you see any granulomatosis inflammation in the TLR2-deficient mice?

Fenton: We have not looked into the model long enough to test that out. The other problem is, we cannot do that with the BCG model, because the mice clear the infection, even the TLR2-deficient mice at that initial inoculum of bacteria that we would use. So where I would normally want to look at granuloma formation 40 days after infection even the TLR2 knock-out mice have cleared the infection by then. In the case of using higher inocula, we see a disseminated infection, as you saw on the one slide. We did not see anything that looked like a true granuloma. We did not see any organisation in either the TLR4 or the TLR2 knock-out mice. So I did not want to jump to any quick conclusions, but from looking at the initial data we have, there seems to be very poor granuloma formation.

REFERENCE

1. Arbour NC, Lorenz E, Schutte BC, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet 2000;25:187-91.

Human Fc receptor polymorphisms in relation to bacterial infection

T.W. Kuijpers

Emma Children's Hospital, Academic Medical Centre, Amsterdam, the Netherlands

ABSTRACT

Human IgG receptors are very heterogeneous. Currently there are three Fc gamma receptor classes specifying at least 12 receptor isoforms to be distinguished. On top of this complexity, Fc gamma receptors (FcγRs) differ between different individuals. Polymorphisms have been identified for two FcγR classes, representing allelic variation of the FcγRIIa (CD32), FcγRIIIa and FcγRIIIb (CD16). The FcγRIIa polymorphisms are now considered to be a heritable risk factor for infectious diseases and some manifestations of autoimmune disorders. A relevant role of the IIIb polymorphism in infectious disease has been suggested, though less convincingly. Detailed analysis of the exact contribution of each of these polymorphisms in relation to previously implicated risk factors for infectious or immunological disease should unravel the pathophysiological contribution of FcγR polymorphisms in the wide variety of factors now being investigated. The information obtained about the multiple genes that impact inflammatory responses of the host – and most likely in reaction to exogenous triggers and pathogens – is becoming overwhelming. The rapid development of molecular techniques makes it possible to determine the incidence of all these individual genetic polymorphisms. Although its relevance will be more difficult to ascertain, the information on all the different genes and their allelic variations involved in the host immune response will be very important for our understanding of infectious disease in modern times.

INTRODUCTION

Human genomics and the rapidly growing insight into the host inflammatory response explain the increasing interest in infectious disease genetics over the last five to ten years. Host genetic factors are major determinants of susceptibility to infectious diseases in humans. Candidate gene studies by association and human genome-wide analysis have been used to identify susceptibility and resistance genes in infectious diseases. However, a single gene defect has rarely been directly related to a devastating diseases, such as interferon-gamma receptor (IFNγR) or interleukin-12 receptor (IL-12R) mutations leading to severe or fatal infections with mycobacterial strains.
In clinical terms, gene polymorphisms of the host immune defence factors appear to have a much broader role and contribution to health and disease. These genetic variants, which modify the regulation or function of the mediators, have been associated with susceptibility to and/or outcome of severe sepsis and septic shock. All steps of the host response to bacteria may be affected by genetic factors. For example, Fcγ receptors (FcγRs), Toll-like receptors (TLRs), CD14 and mannose-binding lectin (MBL) mutations have all been shown to modify the host detection of pathogens leading to pneumococcal infections, Gram-negative bacteria septic shock, and meningococcal disease. Polymorphisms of cytokine genes (TNF-α, IL-1 receptor antagonist (IL-1RA), IL-10, etc.) have been reported to influence the level of secreted mediators and to unbalance the inflammatory cascade. Coagulation response to sepsis may also be affected by gene variants such as the plasminogen activator inhibitor 1 (PAI-1) common functional polymorphism that increases the risk of death from meningococcal infection.
In this paper we will largely focus on the contribution of FcγRs in infectious disease.

PHAGOCYTES

The expression of FcγRs is limited to cells of the haematopoietic lineage (*table 1*). The consequence of IgG binding

Table 1
Opsonin receptors on leucocytes

RECEPTOR	CD	GENE	EXPRESSION
Fc receptors			
FcγR-I	CD64	*FCGRIA*	Monocytes, macrophages, IFN-γ- or G-CSF-stimulated neutrophils
		FCGRIB	-
		FCGRIC	-
FcγR-II	CD32	*FCGRIIA*	Neutrophils, eosinophils, basophils, Monocytes, macrophages Platelets
		FCGRIIB	B lymphocytes Macrophages (neutrophils?)
		FCGIIC	NK cells
FcγR-III	CD16	*FCGRIIIA*	Macrophages, NK cells
		FCGRIIIB	Neutrophils
FcRn		*FCRN*	Syncytiotrophoblasts, intestinal epithelial cells
FcαR	CD89	*FCAR*	Neutrophils, monocytes, mesangium cells
Complement receptors			
CR1	CD35	*CR1*	Erythrocytes, granulocytes, monocytes
CR2	CD21	*CR2*	B lymphocytes
CR3	CD11b/CD18	*CD11B and INTG2*	Neutrophils, eosinophils, basophils, monocytes, macrophages, NK cells
CR4	CD11c/CD18	*CD11C and INTG2*	Monocytes, macrophages, neutrophils, eosinophils, basophils
C1qR?	CD91?		Monocytes, neutrophils, eosinophils, endothelial cells?

to these receptors varies among the cells in relation to their primary function. In the case of infectious disease, it is clear that the phagocytes have the greatest impact on clearance and elimination of bacterial and fungal pathogens, both from tissues and the circulation. The professional phagocytes to be distinguished are mainly formed of neutrophilic granulocytes, monocytes and monocyte-derived tissue macrophages. Neutrophils constitute the major type of leucocytes in peripheral blood, with counts ranging from 40 to 70% under normal conditions. Neutrophils contain numerous proteases and other proteins in their granules and can produce – when activated – a large quantity of toxic oxygen metabolites through the NADPH oxidase activity to kill tissue-invading pathogens.[1,2] Neutrophils protect our bodies against so-called extracellular bacterial and fungal micro-organisms, whereas the macrophages are particularly involved in and equipped for the killing of intracellular pathogens. Neutrophils have a high turnover rate. Once these cells have egressed from the bone marrow, their half-life in the peripheral circulation is about six to eight hours before they leave rather randomly into the extravascular tissue where the neutrophils roam for another 24 hours. In the case of inflammation, circulating neutrophils sense the site of infection, rapidly extravasate and crawl in large quantities towards the invading micro-organisms to ingest and kill them.[2-4] During an infection monocytes follow only later to perform the role of scavenging cell, to engulf the remaining apoptotic neutrophils, and to prepare the local environment for recovery from the tissue damage inflicted during the first wave of neutrophil-mediated inflammation. Macrophages are tissue-dwelling cells derived from monocytes with an estimated half-life of three to four months. Macrophages can be found in any organ or tissue, though in different forms and endowed with different functions. These cells are well situated and actively involved in liver and spleen clearing the blood compartment from aged cells, oxidatively damaged material, as well as filtering out any bacterial pathogen that escaped the defence system of skin or mucosal lining, including the destructive power of tissue-infiltrated neutrophils. Thus, for proper functioning of this first line of defence by phagocytes, a number of prerequisites have to be fulfilled.

PHAGOCYTOSIS AND MICROBICIDAL ACTIVITY

Phagocytes operate in concert with antibodies and complement factors, so-called opsonins. Micro-organisms covered with these opsonins are bound through specific receptors for these opsonins on the plasma membrane (*table 1*). Antibodies bind with their Fab regions to microbial antigens. In this way, the Fc regions of these antibodies are closely packed together. This spatial arrangement enhances complement activation, thus leading to binding of C3b and C3bi to the micro-organisms and subsequent binding of the microbes to the complement receptor type 1 (CR1)

and complement receptor type 3 (CR3) respectively. On the other hand, the proximity of the antibody Fc regions also promotes direct binding of the opsonised micro-organisms to the Fc receptors on the phagocytes.[5]

Two types of Fcγ receptors are present on neutrophils: FcγRIIa and FcγRIIIb. Only after long-term activation of neutrophils by interferons or growth factors is a third Fcγ receptor expressed, i.e. the FcγRI, which binds monomeric IgG with high affinity (*table 2*). In contrast, the constitutively expressed FcγRIIa and FcγRIIIb bind monomeric IgG only with low avidity, but can efficiently bind immune complexes containing multiple IgG molecules. A polymorphism in FcRγRIIa defines the intrinsic ability to recognise all four IgG subclasses or not. IgG2 antibodies, often formed as the most predominant IgG subclass of antibodies against the microbial carbohydrate structures, only react with the so-called L131 type of FcγRIIa (with a leucine at amino acid position 131). As will be discussed later, it is generally assumed that individuals with this isotype are better protected against infections with certain micro-organisms than individuals with the R131 type of FcγRIIa (arginine at position 131).
FcγRIII has two variants, a transmembrane form (FcγRIIIa) expressed on macrophages and natural killer cells, and a neutrophil-specific form (FcγRIIIb) linked to the plasma membrane by a lipid anchor, which allows very rapid redistribution and early localisation of opsonised material. Although there is a polymorphic site in the FcγRIIIb on neutrophils (NA1/NA2 depending on differences in glycosylation), its effect on clinical outcome is as yet not as clear as in the case of the FcγRIIa. In contrast to all other transmembrane FcγR members, the FcγRIIIb on neutrophils can be rapidly shed from the membrane during functional activation or during the process of programmed cell death (apoptosis) in the tissues. The presence of soluble FcγRIIIb plasma in combination with its relatively long half-life renders this molecule a prime candidate for the estimation of total neutrophil mass.[6]
Monocytes express the FcγRI and FcγRIIa whereas macrophages show FcγRI, reduced FcγRIIa and a high level of FcγRIIIa. FcγRIIIa has two polymorphic sites of which the FcγRIIIa-158V/F allotypes show some functional influence on the avidity of IgG subclass binding, of IgG4 in particular.[7] The FcγRIIIa-V/F158 allelic variation possibly influences autoimmune disease in its presentation, course and outcome. Even in one type of disease, the published data of groups of patients with various racial backgrounds are contradictory or at best inconclusive.[8,9]
Studies on the inhibitory action of FcγRIIb were largely limited to B-cell functions. With the current insight from experimental FcγRIIb-knockout models,[10,11] its expression on macrophages (and other phagocytes) and – as a consequence – its impact on the outcome in inflammatory responses as well as on the efficacy of intravenous immunoglobulin infusions in an experimental model for immune thrombocytopenia[12] has been demonstrated. The precise working mechanism of the disease-mitigating role of FcγRIIb should be elucidated in the near future, also in human disease.

MICROBICIDAL ACTIVITY

Binding of opsonised material to surface receptors leads to concentration of such receptors around the area of contact. Subsequently, the cell extends pseudopods that engulf the particle. By consecutive receptor binding, these pseudopods fit tightly around the particle and finally fuse with each other to form a closed membrane vesicle (phagosome) around the particle, within the phagocyte. Neutrophils may overeat themselves in infected areas and die of congestion. Macroscopically, this is manifested as pus formation. Apart from phagocytosis, receptor binding may also start two other processes, i.e. the generation of reactive oxygen compounds and the release of granule

Table 2
Function of Fcγ and complement receptors on phagocytes

			EFFECT		
NOMENCLATURE	EXPRESSION	LIGAND	PHAGOCYTOSIS	MEDIATOR RELEASE	CYTOTOXICITY
FcγRI (CD64)	Constitutive*/long-term activation**	IgG	+	+	+
FcγRII (CD32)	Constitutive, stable expression	IgG	+	+	+
FcγRIII (CD16)	Shed upon activation	IgG	-	+	+
FcγR (CD89)	Constitutive, stable expression	IgA	+	?	?
CR1 (CD35)	Upregulated upon activation	C4b/C3b	+	+	+
CR3 (CD11b/18)	Upregulated upon activation	C3bi	+	+	+
CR4 (CD11c/18)	Upregulated upon activation	C3bi/C3dg	+	+	?

** Constitutive expression on monocytes and macrophages, ** activation-dependent expression on neutrophils.*

contents. Both reactions are localised events in that they are restricted to the release of microbicidal products into the phagosome. However, the secretion of these products begins before the phagosome is closed, and some of the oxygen compounds and granule enzymes may thus escape into the extracellular environment of the neutrophils. Moreover, neutrophils adhering to opsonised material that is too large to be ingested (e.g. immune complexes deposited along basement membranes) may secrete these products in large quantities into the extracellular space. Under such conditions, macrophages can fuse with each other forming large tissue multinuclear histiocytes as an important cell type, as is often present in granulomata.

Degranulation does not occur in resting neutrophils, monocytes, or macrophages. During phagocytosis or adherence of neutrophils to large substrates, intracellular signalling events induce the fusion of granules with the plasma membrane. Neutrophils contain at least two different types of granule; most likely the same holds true for monocytes. In macrophages, the granular content is less apparent. The azurophil granules resemble the lysosomes in other cell types in that they contain acid hydrolases, with a low pH optimum. Moreover, these granules also contain myeloperoxidase (MPO) and a number of serine proteinases (elastase, cathepsin G, proteinase 3). Further, the azurophil granules also contain large numbers of defensins, small peptides with a broad range of bactericidal activity, and bactericidal permeability-increasing protein (BPI), a very potent antibiotic against Gram-negative bacteria. Lysozyme, an enzyme that hydrolyses certain peptidoglycans of Gram-positive bacteria, is present in the azurophil as well as in the specific granules of the phagocytes. Proteins specifically found in the specific granules comprise lactoferrin, an iron-binding and therefore bacteriostatic protein, vitamin B_{12}-binding protein, and the metallo-proteinases collagenase and gelatinase. Neutrophils possess far more granules than monocytes or macrophages. Finally, neutrophils also contain so-called secretory vesicles, which actively exchange their membrane-bound receptors and enzymes with the plasma membrane. In contrast, macrophages have a vesicular system of endosomal trafficking – although not as elaborate as the dendritic cells – for exchange and presentation of antigen as an antigen-presenting cell, which is absent in neutrophils.

Simultaneous with degranulation, a membrane-bound oxidase enzyme complex located in the membrane of secretory vesicles and specific granules can be activated to generate reactive oxygen compounds needed in the killing process. This NADPH oxidase complex is composed of several subunits in the plasma membrane (cytochrome b_{558} α and β subunit: p22-*phox* and gp91-*phox*) and a number of activity-regulating proteins in the cytoplasm (e.g. p47-*phox*, p67-*phox*). Phagocytes at rest do not generate superoxide. Only after opsonin/ligand binding to cell surface receptors is the active NADPH oxidase assembled to generate superoxide in phagocytes. Superoxide spontaneously dismutates into hydrogen peroxide that may then react with chloride ions to form hypochlorous acid (HOCl). This reaction is catalysed by MPO, when released into the phagosome or the extracellular space. HOCl is very toxic for a broad range of micro-organisms but is rather short-lived. However, it can react with primary and secondary amines, and thus give rise to *N*-chloramines, some of which are very stable microbicidal agents. Under normal phagocytosing conditions, neutrophils convert more than 75% of their superoxide into hypochlorous acid and *N*-chloramines, and thus create a highly toxic environment within the phagosomes and in the cell surroundings.

MEANING OF FCγR POLYMORPHISMS IN INFLECTION

Since the FcγRIIa (CD32) is the sole IgG FcR capable of interacting with human IgG2, the main IgG subclass of bacterial capsular polysaccharides, most studies on infections with encapsulated bacteria have focused on the FcγR allotypes: i.e. FcγRIIa-R131 and IIa-H131.
The retrospective study by Bredius *et al*. first showed in a small cohort of 25 children with prior fulminant meningococcal septic shock that almost half of the children were homozygous for FcγRIIA-R/R131, the poor IgG2-binding allotype. This allele frequency was significantly different from its frequency in a healthy white population (44% *vs* 23%, p=0.03). The relevance of this finding was further supported by the fact that neutrophils with the FcγRIIa-R/R131 allotype phagocytised *N. meningitidis* opsonised with polyclonal IgG2 antibodies less effectively than did IIa-H/H131 neutrophils.[13] Another Dutch group had determined the FcγRIIa and FcγRIIIb phenotypes of 48 children with recurrent bacterial respiratory tract infections. FcγRIIa-H/H131 was less than half that observed in 123 healthy adults (p=0.01). IgG2 responses were low in 25 out of 48 patients after immunisation with pneumococcal vaccine. The authors' conclusion was that FcγRIIa polymorphisms contribute to increased susceptibility to infections with encapsulated bacteria in a childhood population with low IgG2 anti-carbohydrate antibodies.[14]

Similar variation in allele frequency was studied in small groups of patients with additional opsonisation defects in the complement system. The distribution of FcγRIIa and IIIb allotypes in 15 individuals with a deficiency in one of the late complement components (LCCD) and in 15 properdin-deficient patients with/without previous meningococcal disease was analysed. The combination of FcγRIIa-R/R131

with FcγRIIIb-NA2/NA2 allotypes was associated with previous meningococcal disease (OR=13.9, p=0.036). No such relation was observed in the properdin-deficient patients.[15] Another study in LCCD patients had previously shown that the distribution of FcγRIIa genotypes and disease demonstrated an apparently clear age effect. The R/R131, R/H131, and H/H131 genotype distribution was 0.14:0.29:0.57 for patients with their first disease episode <10 years of age, as compared with the distribution of 0.21:0.64:0.14 for those with their first episode >10 years (OR=8.0, p<0.05). Meningococcal disease had a more severe course in four out of 31 episodes in patients with the R/R131 or R/H131 allotypes, in contrast to one out of 18 in patients with H/H131 (OR=14.2, p<0.01). Thus, CD32-mediated phagocytosis may restrict the severity of meningococcal disease in LCCD patients with the H/H131 genotype.[16]

In a rather heterogeneous cohort of paediatric and adult HIV-positive individuals, skewing in FcγRIIa R/H131 allele frequency was not observed in relation to pneumococcal invasive disease.[17] In 60 black children with sickle-cell anaemia (SCA) and well-documented bacterial infections, the R/H131 genotype distribution in the 51 individuals with a history of *Streptococcus pneumoniae* infection was also not statistically significantly different from that of the control population. In contrast, however, the H/H131 genotype was unexpectedly overrepresented in the 11 individuals with a well-documented history *of Haemophilus influenzae* type-b infection (64% H/H131, 27% H/R131, 9% R/R131, p=0.002) when compared with ethnically matched controls (14% H/H131, 60% H/R131, 26% R/R131).[18]
A case-control study on the risk and outcome of meningococcal disease in 130 patients with microbiologically proven meningococcal disease and 260 asymptomatic sex-matched controls indicated a lack of skewing in allele distributions of FcγRIIA allotypes.[19] In comparison with meningococcal meningitis, however, both the fulminant meningococcal disease (OR=3.9, 95% CI 1.0 to 16, p=0.04) and sepsis without meningitis (OR=3.0, 95% CI 1.4 to 7.8, p=0.004) were associated more commonly with the FcγRIIA-R/R131 allotype than to those with meningococcal meningitis. Of the 42 patients with the R/R131 allotype, 31 (74%) had an adverse prognostic score, compared with 7% (4 of 59) of those with the R/H131 allotype and 3% (1 of 29) of those with the H/H131 allotype (p<0.0001).

Thus, the FcγRIIA-R/R131 allotype may not act as a strict susceptibility gene but – instead – a severity marker, associated with the more severe forms of meningococcal disease. This remains to be confirmed for other encapsulated pathogens.

CONCLUDING REMARKS

The meaning of polymorphic variations in the genes for the FcγRIIa, FcγRIIIa and FcγRIIIb is far from clear at the moment. Most studies describe small and heterogeneous groups, and may at best tell us something about differences within a certain cohort. Then, the polymorphisms indeed show their most characteristic features, such as an age effect, a difference in outcome and symptomatology, and – presumably – a different treatment effect. The role of these polymorphisms seen as a separate issue in the host defence response indicates a subtle impact, more interesting from the perspective of pathophysiology than for the therapeutic options to date. This may change rapidly as soon as monoclonal antibodies enter the clinics for the reasons outlined above.

In the first phase of inflammatory responses or other forms of stress, chemotaxins such as C5a, lipid mediators, and so-called chemokines are produced. The secretion of chemokines can be firmly induced by the proinflammatory cytokines derived from tissue macrophage and T cells such as TNF-α, IL-1, and IFN-γ, or by bacterial products such as lipopolysaccharide (LPS) from Gram-negative bacteria, peptidoglycans from Gram-positive bacteria, or lipoarabinomannans from mycobacteria.
The process of recruitment is complex and still incompletely understood. Once recruited, the phagocytes – and neutrophils foremost – have a wide range of toxic mechanisms to fight any invading micro-organism, as described. These mechanisms are strongly regulated and delicately controlled because an over-excessive or premature induction of the toxic activities may result in the inactivation of protease inhibitors and activation of several cascades of activating substances (e.g. coagulation and fibrinolysis, the complement system). As a consequence, bacteraemia may progress to septic shock and disseminated intravascular coagulation, a community-acquired pneumonia may develop into acute or adult respiratory distress syndrome, and hypoxia/reperfusion injury can lead to fatal circulatory collapse.

In many of these early steps of inflammation, molecules are involved in which allelic variation has been characterised, often in the coding or promotor regions, as well as in the nontranscribed intronic sequences or 3'-regions. Their meaning in biological and – as a consequence – in clinical terms remains to be clarified. The FcγR allotypic variation is only a minute factor within the complex sequence of reactions in the host immune reaction to an invading pathogen.

REFERENCES

1. Delves PJ, Lydyard PM. Leukocyte development. In: Cellular Immunology. Delves PJ (ed). Oxford: Blackwell Scientific Publication, 1994:33-43.
2. Gallin JI, Goldstein IM, Snyderman R (eds). Inflammation: Basic Principles and Clinical Correlates. 2nd edition. New York: Raven Press, 1992.
3. Bokoch GM. Chemoattractant signalling and leukocyte activation. Blood 1996;86:1649-60.
4. Murphy PM. The molecular biology of leukocyte chemoattractant receptors. Annu Rev Immunol. 1994;12:593-633.
5. Borregaard N, Cowland JB. Granules of the human neutrophilic polymorphonuclear leukocyte. Blood 1997;89:3503-21.
6. Ravetch JV, Bolland S. IgG Fc Receptors. Annu Rev Immunol 2001;19:275-90.
7. Koene HR, Kleijer M, Algra J, Roos D, Borne AE von dem, Haas M de. Fc gammaRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48L/R/H phenotype. Blood 1997;90:1109-14.
8. Koene HR, Kleijer M, Swaak AJ, et al The Fc gammaRIIIA-158F allele is a risk factor for systemic lupus erythematosus. Arthritis Rheum 1998;4:1813-8.
9. Dijstelbloem HM, Winkel JGJ van de, Kallenberg CGM. Inflammation in autoimmunity: receptors for IgG revisited. Trends in Immunol 2001;22:510-5.
10. Clynes R, Maizes JS, Guinamard R, Ono M, Takai T, Ravetch JV. Modulation of immune complex-induced inflammation *in vivo* by the coordinate expression of activation and inhibitory Fc receptors. J Exp Med 1999;189:179-85.
11. Yuasa T, Kubo S, Yoshino T, et al. Deletion of fcgamma receptor IIB renders H-2(b) mice susceptible to collagen-induced arthritis. J Exp Med 1999;189:187-94.
12. Samuelson A, Towers TL, Ravetch JV. Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor. Science 2001;291:484-6.
13. Bredius RG, Derkx BH, Fijen CA, et al. Fc gamma receptor IIa (CD32) polymorphism in fulminant meningococcal septic shock in children. J Infect Dis 1994;170:848-53.
14. Sanders LA, Winkel JG van de, Rijkers GT, et al. Fc gamma receptor IIa (CD32) heterogeneity in patients with recurrent bacterial respiratory tract infections. J Infect Dis 1994;170:854-61.
15. Platonov AE, Kuijper EJ, Vershinina IV, et al. Meningococcal disease and polymorphism of FcgammaRIIa (CD32) in late complement component-deficient individuals. Clin Exp Immunol 1998;111:97-101.
16. Fijen CA, Bredius RG, Kuijper EJ, et al. The role of Fcgamma receptor polymorphisms and C3 in the immune defence against Neisseria meningitidis in complement-deficient individuals. Clin Exp Immunol 2000;120:338-45.
17. Abadi J, Zhong Z, Dobroszycki J, Pirofski LA. Fc gammaRIIa polymorphism in human immunodeficiency virus-infected children with invasive pneumococcal disease. Pediatr Res 1997;42:259-62.
18. Norris CF, Surrey S, Bunin GR, Schwartz E, Buchanan GR, McKenzie SE. Relationship between Fc receptor IIA polymorphism and infection in children with sickle cell disease. J Pediatr 1996;128:813-9.
19. Domingo P, Muniz-Diaz E, Baraldes MA, et al. Associations between Fc gamma receptor IIA polymorphisms and the risk and prognosis of meningococcal disease. Am J Med 2002;112:19-25.

Discussion following lecture by T.W. Kuijpers

De Groot: I appreciate your remark about susceptibility and severity at the end of your presentation, which brings me to my question. It also relates to the first speaker, Dr Turner, who said, as I understand, that in the study on *Mycoplasma pneumoniae* infection there was a question of susceptibility. I fully agree with your statement that these molecules of innate immunity are more likely related to the severity of infectious diseases than to susceptibility. But if that is the case, could you comment on the type of studies that we have done so far, which are skewed, because most of them deal with retrospectively acquired material in highly selected populations from university settings and they are not prospectively done, i.e. they are not population-based. Can you give us your perspective on this? Maybe Dr Turner has a response to this question?

Kuijpers: First of all, we are now trying to do all these studies in a larger cohort of about 200 patients with recurrent respiratory tract infections more or less similar to the study already mentioned by Summerfield in which Dr Turner cooperated for his study of mannose-binding lectin (MBL). Once again, by checking all the known genotypic or allelic variation in these children, there is no clear-cut skewing in any of the polymorphisms presently known, not even for the MBL mutations. You can count the number of infections that these 200 children have, but indeed once again this is an anamnestic response by parents, and thus prone to recall bias. The only way to find out the truth is in a proper prospective study, preferably not in a tertiary centre, in order to have the broadest population possible. But what surprised us was that even the MBL mutations were definitely not skewed to a particular mutation as compared to controls.

Turner: I would not disagree with that. I think most of the studies on MBL today should have been done in centres where there have been tertiary referral hospitals collecting the samples. That is one of the reasons why we are doing a large study in the West of England – it is called ALSPAC, the Avon Longitudinal Study of Pregnancy And

Childhood – which Professor Jean Golding set up in the early 1990s to follow 14,000 pregnancies, and we are just completing a study of 8000 children to see what correlates we can find for disease associations in those children. That is a true prospective study. These sorts of studies are desperately needed for these innate immune markers.

Van der Meer: Just to get back to the point of the *Mycoplasma* infection as a complication in hypogammaglobulinaemic patients, the question there as well is whether it is a severity or a susceptibility marker.

Turner: You are right, it was mainly infection due to *M. hominis*, and the patient cohort was a mixture of common variable immunodeficiency and also some X-linked agammaglobulinaemia patients who had started off as a study of common variable immunodeficiency – the *Mycoplasma* association was discovered as an incidental part of that. So we then enlarged the cohort for *Mycoplasma*.

Kuijpers: I want to point out the study already mentioned by Peter Garred in cystic fibrosis. Considering a drop of eight years in lifespan, it is crucial when you are also MBL-deficient. Then you may think about prospective treatment options being available nowadays for those CF patients who are MBL-deficient. But that can only be done in a proper setting with a multicentre study. Without such collaboration we probably remain stuck with the question, is it susceptibility or severity? A severity marker is already important, but only in certain cohorts.

Appelmelk: Dr Kuijpers, first can you explain to me, when you have an allele in a low-affinity receptor causing inactivity, why doesn't the high-affinity receptor take over? It sounds as though there is some redundancy in the system. So how does that work?

Kuijpers: I think you are completely right in mentioning this. Of course, all comes down to the initial thought and idea that these polymorphic sites in case of IgG2 opsonising mechanisms were of importance because there was a strict difference in whether you had the one or the other genotype, FcγRIIa. Of course, the other studies were a kind of follow-up to that, first of all trying to confirm their ideas, and then suddenly these genotypes for the IIIa were showing different expression patterns and especially so in the autoimmune diseases. If a macrophage expresses so many Fcγ-receptors, having the opportunity to bind IgG-laden material, it is indeed questionable why it would not be taken over by other Fcγ receptors. I think a lot of the work in autoimmune diseases in fact confirms the genotype skewing. There are more molecules involved in immune reactivity as such. Think about the IL-6 or proteins related to apoptosis. Nobody knows in fact. This might simply be a way to look at something different in that particular genetic locus, not meaning that it is that particular receptor as such.

McAdam: Are these same receptors expressed on a placenta to facilitate the passage of immunoglobulin across the placenta? Would it be useful to look at babies with neonatal infections?

Kuijpers: The only receptor believed to be important in the transport of IgG from the mother to the child is thought to be the FcRN, so the neonatal receptor, which shows homology to the HLA class-I molecules. This is not even related to the FcγRI, II or III. So it is a very different mechanism. There is, as far as I know, no information about allelic variation in that receptor. Of course, I just mentioned the polymorphic sites in the FcγRIIb: they might be another thing to study, and especially that it is not in the extracellular part, where it may have an effect on the function of the other receptors. That is of course something that may or may not affect the outcome of an inflammatory reaction. But once again, I wonder whether that would tell us very much about susceptibility as such.

Van Agtmael: I would like to ask you a question from a clinical point of view. In March 2001 there was a study in Cochrane Review on the use of intravenous immunoglobulins (IVIG) for sepsis and septic shock and it showed a moderate effect.[1] It would be interesting to look at this group of patients to find factors in those patients who would benefit from the intravenous immunoglobulins. Do you think that it would be useful to look at the Fc polymorphisms in this broad group of patients with sepsis and to identify those who might benefit from the IVIG?

Kuijpers: Well, it would not be the first molecule I would look at, although potentially IVIG may be beneficial. I think it will be very hard to see in which patients they are effective. Of course, I am talking very much as a paediatrician seeing a lot of meningococcal infections – that is a very homogeneous population. But when you are practising internal medicine, there is a huge variation. There are so many parameters that you have to exclude or include to come up with a firm idea about which group is definitely helped by IVIG. The follow-up of those patients studied is difficult. Did they show a normal humoral response etc? All that crucial information is completely lacking in these cohorts. So I don't know.

De Groot: A small remark on the question concerning the neonatal infection, which brought me back to the presentation by Dr Turner in the beginning, where he said that surfactants may play a role as part of innate immunity. There is data to show that mutations in the genes for

surfactants A and D are associated with an increased risk of chronic recurrent lower respiratory tract infection. It is all very preliminary, and that actually brings me to my question. I will turn the gun onto myself immediately after I have said this because so far all these studies – if you have read Kimman's nice book – have been done on associations between single-gene polymorphisms and diseases. You have already said so, but knowing the literature for instance on leucocytes or chronic granulomatous disease (CGD), even in a clear-cut serious mutation disease as CGD there is a tremendous variability in the expression of the disease. This varies from patients who do not become ill at all to those who are very seriously ill with many in between. The way we study these diseases as clinicians is at this point in time very simplistic. We try to take one gene mutation and apply it to a population and look for an association, whereas it might very well be possible that there are a hundred genes involved and that the effects of twenty genes are upregulating severity and there are thirty down-regulating it.

Verbrugh: I have to intervene as Chairman, because we have a time problem here. Could you answer that briefly, because we would like to have two more comments from speakers before we go on to the next topic?

Kuijpers: This is one of the main points set out by the Dutch Society for Science, that you have to enter this field by doing gene arrays and snip arrays, and that may not even bring a clear answer to your question, because if you have twenty different genes or snips being characterised in a certain cohort of patients you come up with the same question. You have then characterised only a limited number of these snips. You may be more susceptible, but then also of course it is a matter of meeting the agent that can potentially affect you.

Kimman: Are Fcγ-receptors involved in the response to vaccinations, and has anybody looked for associations between response to vaccination and Fcγ-receptor polymorphisms?

Kuijpers: I would say yes, most likely, but whether somebody has studied that, I am not aware of that.

Van Furth: Just in emulation of the discussion with Van Agtmael and you, Dr Kuijpers, is there a difference between persons regarding the turnover of IgG, given intravenously or just autologous? Have these studies been done?

Kuijpers: I don't think anybody has done that in a detailed fashion, and the only thing I could say is that of course the turnover of IgG very much relates to the FcRN effect. So I think that a different receptor is involved in rescuing IgG from being cleared and broken down.

REFERENCE

1. Alejandria MM, Lansang MA, Dans LF, Mautaring JB. Intravenous immunoglobulin for treating sepsis and septic shock. Cochrane Database Syst Rev 2001;2:CD001090.

Genetic susceptibility to *Neisseria meningitidis* infections

M. Emonts, R. de Groot, P.W.M. Hermans

Erasmus Medical Centre, Department of Paediatrics, Rotterdam, the Netherlands

ABSTRACT

The clinical presentation of infections by *Neisseria meningitidis* is highly diverse. Some patients with invasive meningococcal infections develop meningitis, while others present with sepsis or even septic shock. Three major host defence systems are activated after transepithelial passage and invasion of the bacteria into the bloodstream. These include the complement pathway, the inflammatory response by cytokines and chemokines, and the coagulation and fibrinolysis pathway. These three systems are mutually dependent. Genetic polymorphisms among components of these pathways (co)regulate the susceptibility, severity and outcome of meningococcal disease. In this paper the current knowledge of polymorphisms which are known to be associated with susceptibility to and severity of meningococcal infection is reviewed.

INTRODUCTION

Despite ongoing improvements in the treatment of infections by *Neisseria meningitidis* the mortality rates in patients with invasive disease are still very high, ranging from 4 to 40%. This is a reflection of the different clinical presentations of infection. The disease spectrum varies from meningitis to sepsis and septic shock. The first entity has a mortality rate of 4 to 6%, while in septic shock mortality rates up to 40% have been reported. The observation that septic shock can develop within several hours of onset of meningococcal invasive disease implies a major role for the innate immunity in host defence. Meningococcal lipopolysaccharide (LPS) is one of the major factors to induce the host response during bacterial invasion. This response is complex and involves the activation of three major host response systems (*figure 1*). The first is the complement pathway that apart from contribution to phagocytosis of the bacteria also functions as an inducer for the inflammatory reaction through C3a and C5a. The second system is the inflammatory reaction mediated by different chemokines and cytokines among which TNF-α and IL-1β play a central role. The third cascade is activation of the coagulation and fibrinolysis, which result in a prothrombotic stage. Activation of the host response in patients with sepsis results in a sudden onset of fever and a petechial or purpuric rash followed by hypotension. In patients with septic shock, disseminated coagulation and multiple organ failure develop. For an extensive review of the pathophysiology we refer to previous reports.[1,2]

The broad range of clinical presentations of *N. meningitidis* infections raises the question why some patients show hardly any clinical symptoms while others die within several hours of onset of symptoms. Variations in host genetic factors are known to contribute to these differences. Genetic polymorphisms are relatively stable in the human population. Single-nucleotide polymorphisms (SNPs) are distributed throughout the human genome at an estimated overall frequency of one in every 2 kb.[3] It has been shown that these polymorphisms may affect susceptibility to and severity and outcome of infectious disease. This review focuses on the current knowledge of genetic variability in the susceptibility to and severity of meningococcal infections (*table 1*).

INNATE IMMUNITY

TLR

The innate immunity plays an important role in the initial recognition of invading pathogens. This recognition was, until recently, believed to be nonspecific. However, elucidation of the function of Toll-like receptors (TLR) has shown

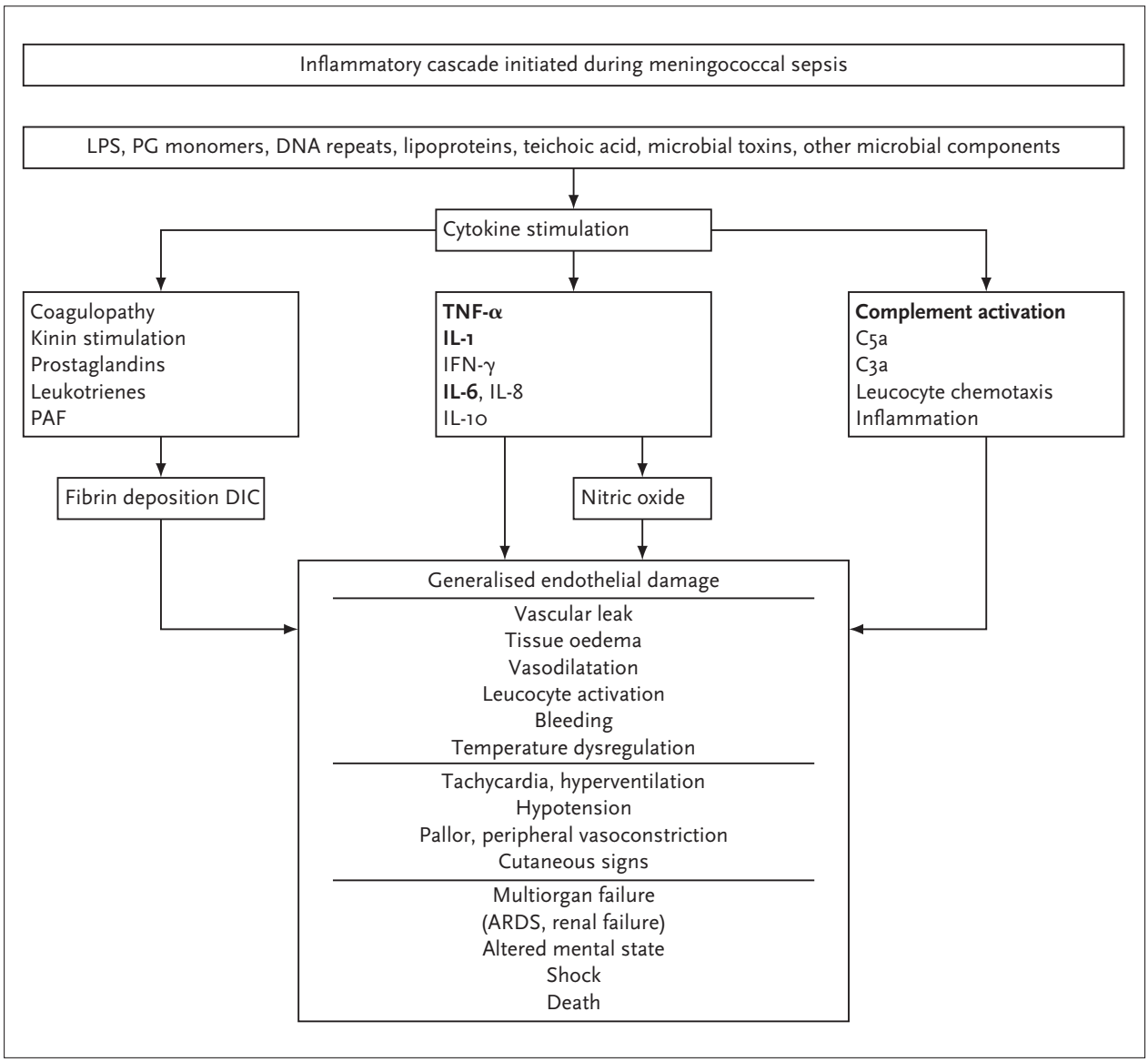

Figure 1
The three main cascades involved in the pathophysiology of meningococcal infection

The factors depicted in bold are discussed in the text. Adapted from Harcourt Publishers Ltd.

otherwise. TLRs sense different microbial molecules, covering a range of pathogens that cause infections in the host. TLR4, for example, recognises bacterial LPS. C3H/HeJ mice, having a point mutation in *tlr4* that abolishes LPS responses, are hyporesponsive to Gram-negative infections.[4,5] The common Asp299Gly polymorphism in TLR4 alters the extracellular domain of the receptor. Airway epithelial cells of patients carrying this polymorphism are hyporesponsive to LPS. However, no association has been found between the Asp299Gly polymorphism and the susceptibility to or severity of meningococcal infection.[6] Smirnova *et al.* have found an excess of rare amino acid polymorphisms in TLR4 of which the clinical relevance has not yet been studied.[7] TLR2 recognises other bacterial components, such as lipopeptides and peptidoglycan.[4] Until now the relation between polymorphisms in TLR2 and meningococcal infections has not been studied. TLRs recognise ligands in the presence of CD14 and MD-2 (TLR4) and activation of the diverse TLRs results in the activation of different signalling pathways, leading to NF-αB activation. Polymorphisms in the factors which are involved in these signalling pathways may also result in different phenotypes.

LBP and BPI

In blood LPS binds to lipopolysaccharide-binding protein (LBP) to form a complex that binds TLR4. In a recent study the Cys98Gly and Pro436Leu polymorphisms of LBP were not correlated with the susceptibility to or severity

Table 1
Genetic polymorphisms possibly associated with meningococcal infection (for details see text)

PATHWAY	GENE	POLYMORPHISM
Innate immunity	TLR4	Asp299Gly
	LBP	Cys98Gly
		Pro436Leu
	BPI	A645G
		G545C
		PstI in intron 5
	MBL	Codon 52
		Codon 54
		Codon 57
Acquired immunity	FcγRIIa	His131Arg
	FcγRIIIa	Val158Phe
	FcγRIIIb	NA1/NA2
Coagulation/ fibrinolysis	t-PA	Alu repeat insertion/deletion
	PAI-1	4G/5G insertion/deletion
	Factor V	FV^{L} G1691A
Cytokines	TNF-α	G-308A
	IL1RN	86 bp repeat in intron 2
		T2018C
	IL1B	C-511T
	IL-6	G-174C

of bacterial sepsis in all patients but a relationship was found with the outcome of bacterial sepsis in males. It has been proposed that these polymorphisms may partially explain the worse outcome of bacterial infections observed in males. In the same study the clinical effects of polymorphisms in bactericidal/permeability-increasing (BPI) protein were also evaluated. BPI is another protein binding to LPS which inhibits LPS-induced host cell responses. Neither the A645G, the G545C silent polymorphism, nor the *Pst*I in intron 5 were associated with outcome and severity of bacterial sepsis. However, in this study the limited number of patients prevented a separate analysis of patients with Gram-negative *versus* Gram-positive infections, thus introducing a bias with respect to LPS-related susceptibility. This may have limited the sensitivity in detecting an association.[8]

COMPLEMENT

MBL

Activation of the complement system forms a significant part of the innate immunity. Besides beneficial, also deleterious effects are associated with complement activation. The severity of hypotension is in part regulated by complement activation. At present, three activation pathways are considered (*figure 2*).[9] Firstly, the classical pathway which is activated by antibody-antigen interactions, and secondly, the two pathways of the innate immunity, which do not require antibody.

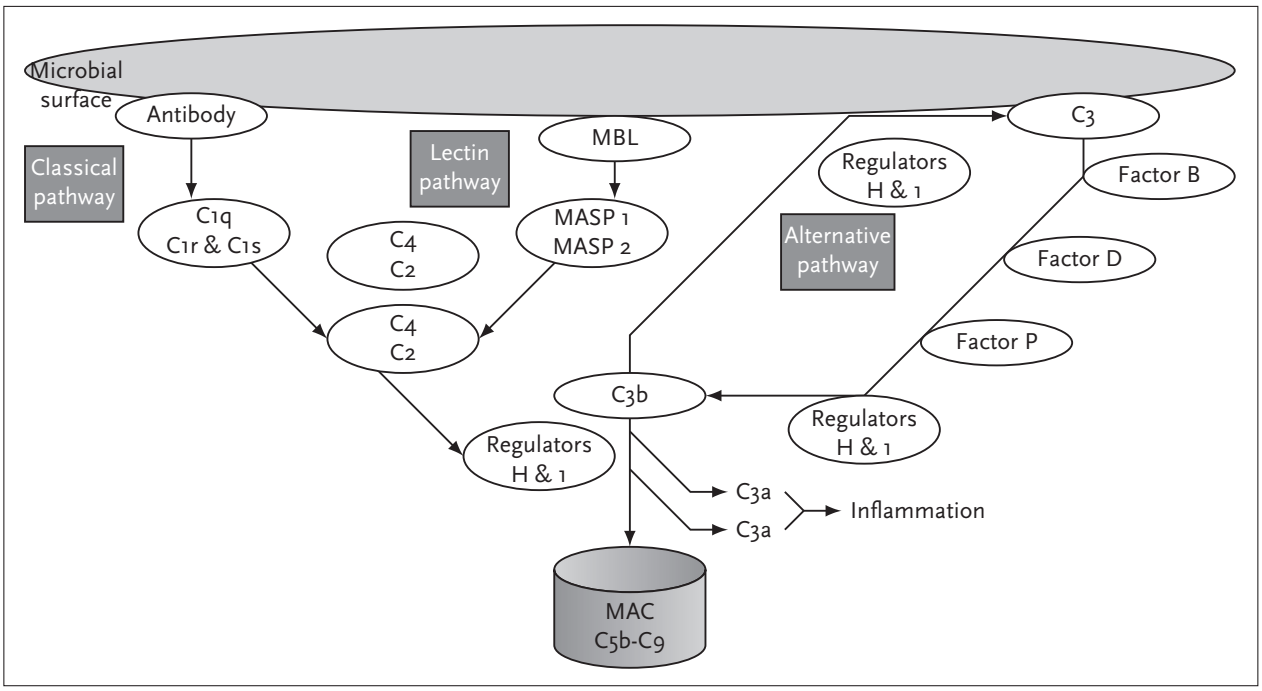

Figure 2
The three pathways of complement activation, connecting into a common late pathway generating the membrane attack complex (MAC) and inflammatory mediators C3a and C5a

Adapted from Hibberd et al.[9]

The alternative pathway of innate immunity is activated through interaction of C3 with factor b, factor d and properdin to generate C3b. The additional innate activation pathway is activated through mannose-binding lectin (MBL). All three pathways converge in a common final pathway. MBL first forms trimers, which in turn further assemble to form multimers. The MBL multimers bind to sugars and LPS on the bacterial surface. This complex then activates the mannose-associated serine proteases MASP1 and MASP2. MASPs activate C4 of the classical pathway.
Three allelic variants of MBL have been described in codons 52, 54 and 57 of exon 1. A mutation in codons 54 and 57 causes the disruption of the axial glycine repeats (Gly-Xaa-Yaa) resulting in aberrant trimers. These trimers cannot form correct multimers and are unstable. Through an unknown mechanism, the codon 52 mutation also results in unstable trimers. Heterozygotes for the variant alleles show a decrease in MBL serum levels to 10% of normal, while in people homozygous for any of the three mutations levels drop to 1% of normal.[9,10] In children the variant alleles were associated with an increased susceptibility to meningococcal infections. Patients homozygous for the variant alleles showed a trend towards slightly less severe disease than heterozygous and wild-type individuals. This observation, however, did not reach significance.[11]

LCCD and factor D

Patients with late complement component deficiency (C5-C9) (LCCD) are known to suffer from recurrent Gram-negative bacterial infections. Failure to form a membrane attack complex (MAC) might underlie this increased susceptibility. Interestingly, these infections result in milder disease severity than observed for the total patient population, suggesting that there are also adverse effects of complement activation during meningococcal infections. Complement D deficiency was found in members of a family suffering from severe *N. meningitidis* infections. The alternative pathway for complement activation was impaired. This pathway, in contrast to the classical pathway, appears to be prominent in meningococcal infection.[12,13]

ACQUIRED IMMUNITY

Fcγ receptors

Fcγ receptors (FcγRs) belong to a heterogeneous family of receptors and are grouped in three classes (FcγRI, II and III). Three subtypes of receptors responsible for IgG-mediated signalling, FcγRIIa, FcγRIIIa and FcγRIIIb, are thought to be important in host defence against meningococci. FcγRIIa is located on leucocytes and mononuclear macrophages and is sensitive to IgG2 and IgG3. Two alleles are known to differ at amino acid position 131 because of single-nucleotide polymorphism (SNP) in exon 4. The 131Arg allotype confers lower interaction efficiency on IgG2 and IgG3 than the 131His haplotype. Two allotypes known for FcγRIIIa, 158Phe and 158Val show different binding of IgG1, IgG3 and IgG4, as interaction with FcγRIIIa-158Val is stronger. FcγRIIIb is expressed on neutrophils and has the neutrophil antigen (NA) polymorphism representing four amino acid substitutions in the membrane-distal loop of the receptor. FcγRIIIb-NA2 binds IgG1 and IgG3 less efficiently than FcγRIIIb-NA.[14] One can imagine that differences in efficiency in binding IgGs result in different host response effectiveness and thereby variable susceptibility to and/or severity of disease.
A study in survivors of meningococcal disease and first-degree relatives of survivors and nonsurvivors revealed no differences in FcγR distribution. The FcγRIIa-131Arg allele was more often found in meningitis patients compared with sepsis patients. The FcγRIIIa-158Val allelic frequency was markedly increased in relatives of meningitis patients compared with relatives of patients presenting with haemodynamic instability. The Arg/Arg-Phe/Phe-NA2/2 frequency that represents the least efficient FcγR combination, and is responsible for diminished phagocytosis, was tripled in first-degree relatives compared with healthy nonrelated controls. These data suggest an association between FcγR haplotype and susceptibility to and severity of meningococcal disease.[14]

COAGULATION

Activation of coagulation and fibrinolysis is the result of the acute inflammatory response in patients with invasive meningococcal disease. The prothrombotic endothelium surface results from activation by cytokines.
Subsequently, tissue factor production results in activation of the extrinsic pathway of coagulation and the production of platelet-activating factors. The fibrinolytic system is initially activated but is subsequently inhibited. This results in a marked imbalance in coagulation and fibrinolysis resulting in a net procoagulant state and ultimately disseminated intravascular coagulation (DIC). This leads to deposition of fibrin, the formation of microthrombi and bleeding. Multiorgan failure and death are the most severe clinical findings regarding this imbalance.

t-PA

Tissue-type plasminogen activator (t-PA) is a serine protease that converts plasminogen into its active form, plasmin, which in turn leads to fibrinolysis. Impaired t-PA function leads to insufficient lysis of thrombi. Differences in t-PA production or impaired function can therefore affect the severity of meningococcal disease. An insertion/deletion polymorphism of an Alu element in t-PA considered to affect the basal levels of t-PA has been investigated in

relation to meningococcal disease. No association could be observed between the different alleles and the severity or outcome of meningococcal disease.[15] The authors have regarded the use of an intensive care unit (ICU) as a criterion for severe meningococcal disease. In our opinion this only holds partially true, since not all patients admitted to the ICU for observation because they are at risk of septic shock actually develop septic shock. This might have prevented them from finding significant differences between the study populations. Further research regarding polymorphisms in t-PA with respect to meningococcal disease is therefore indicated.

PAI-1

Plasminogen activator inhibitor type 1 (PAI-1) is responsible for the inhibition of fibrinolysis both directly and indirectly through inhibition of t-PA (*figure 3*). In turn PAI-1 is inhibited by activated protein C. In septic shock, laboratory findings show decreased levels of all coagulation factors including protein C.[16] The gene encoding PAI-1 has several polymorphic loci, including a 3'-HindIII site, a CA(n) repeat in intron 3 and a 4G/5G insertion/deletion polymorphism at -675 in the promoter. PAI-1 activity has been shown to be significantly higher in control subjects homozygous for the 4G allele than in subjects homozygous for the 5G allele.[17] The production of PAI-1 mRNA after IL-1 stimulation appeared to be higher in HepG2 cells bearing the 4G allele.[18] In patients with meningococcal sepsis the levels of PAI-1 are positively related to severity of disease, outcome, cytokine levels, acute-phase proteins and coagulation parameters.[16] In nonsurvivors the PAI-1 levels were shown to be 1.9 times higher for the same TNF-α levels than in survivors. In children suffering from meningococcal disease a relationship has been observed between genotype, PAI-1 levels and outcome of disease. The subjects with the 4G homozygous genotype had higher PAI-1 levels and had an increased risk of death. No association was observed with severity of disease.[19]

A similar study performed by Westendorp *et al.* showed a higher incidence of the 5G/5G genotype among relatives of patients with meningitis. Patients whose relatives were carriers of the 4G/4G genotype had a sixfold higher risk of developing septic shock than meningitis.[20]

FV^L

Factor V Leiden (FV^L) is associated with thrombotic events, and is therefore an interesting candidate for involvement in the development of meningococcal purpura fulminans. A study comparing children with meningococcal disease, healthy controls and parents of children with meningococcal disease did not reveal an association between the FV^L mutation and susceptibility. Patients heterozygous for the mutation showed increased complications, as assessed by requirement for skin grafting, referral to plastic surgeon and/or amputation. A significant effect on mortality was not observed.[21]

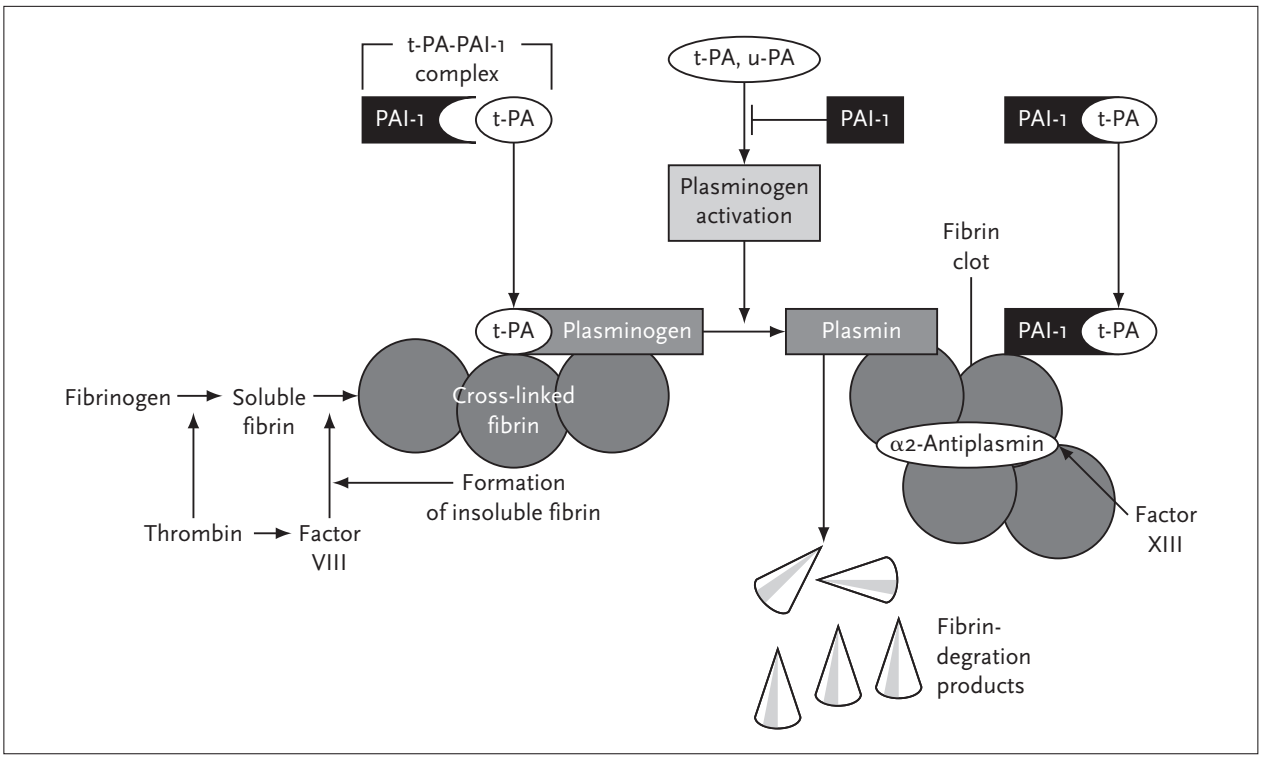

Figure 3
Involvement of PAI-1 and t-PA in coagulation and fibrinolysis

CYTOKINES

TNF-α

Tumour necrosis factor α (TNF-α) plays a central role in the activation of the inflammatory response, and serum levels are raised in all patients with meningococcal disease. TNF-α activates adhesion-promoting receptors and ligands enabling leucocytes to cross the endothelium and reach the site of inflammation. Other inflammatory mediators such as IFN-γ, IL-6 and IL-8 are activated by TNF-α. A procoagulant state is promoted by the induction of tissue factor release. TNF-α blood levels are positively associated with disease severity, coagulopathies and outcome.[22] Westendorp *et al*. found a higher TNF-α response in *ex vivo* blood samples after stimulation with endotoxin in patients who had experienced a moderately severe disease course compared with patients with a mild course. The TNF-α response was low again in survivors of fulminant disease. On admission for meningococcal disease, patients who did not survive had initial TNF-α levels three times higher than those in survivors with a clinical disease presentation of similar severity.[23,24]

The G-308A polymorphism in the TNF-α promoter region has been shown to be associated with outcome of meningococcal disease. The TNF2 allele (-308A) is associated with an increased risk of death. Controversial opinions exist about the role of this polymorphism and the severity of disease. The diverse results of the various studies may reflect the differences in patient and control populations. Some researchers study meningococcal sepsis only, while others look into the relevance of the G-308A polymorphism in patients suffering from sepsis on an ICU after surgery.[25-27] No association has been observed for the G-308A polymorphism and susceptibility to meningococcal disease.[25,26] It is clear that TNF-α production is influenced by genetic factors in meningococcal sepsis, but the relative importance of the known polymorphisms is still under discussion.

IL-1 family

The interleukin-1 (IL-1) family consists of both proinflammatory and counterinflammatory members. The genes IL1A and IL1B encode the proinflammatory proteins IL-1α and IL-1β, respectively. The IL-1 receptor antagonist (IL-1RA) represents the anti-inflammatory component. IL-1RA can bind the IL-1 receptor without inducing signal transduction. Several polymorphisms are known in the IL-1 family. Five alleles of IL1RN, the gene encoding IL-1RA, are known to differ by a variable number repeat of 86 bp in intron 2.[28] This repeat contains transcription factor binding sites. The IL1RNA2 allele has two repeats and is in linkage disequilibrium with SNP T8006, also known as T2018C.[29,30] The A2 allele has been shown to be associated with susceptibility to severe sepsis in patients in a surgical intensive care unit.[31] Read *et al*. have investigated whether variants of the IL-1 and TNF gene families are associated with severe manifestations of meningococcal infection. All patients included had microbiologically proven infection by *N. meningitidis*. A significant association has been found between the outcome and the IL1B C-511T polymorphism. Patients homozygous for either the common or the rare allele had increased odds ratios for death, compared with heterozygous individuals. The combination of heterozygosity of IL1B (-511) together with homozygosity of the common allele of IL1RN at +2018 was significantly associated with survival. In this study no association between the TNF -308 genotype and fatal outcome was demonstrated.[30] These data suggest that IL-1 genotype influences the outcome of meningococcal disease.

IL-6

Interleukin 6 (IL-6) is a major pyrogen and is responsible for the induction of hepatic acute-phase proteins and antibody production by B cells. IL-6 blood levels are increased in meningococcal infection.[32] The G-174C polymorphism in the promoter region of IL-6 is associated with outcome in meningococcal infection, since the -174G/G genotype is associated with an increased mortality risk.[33] However, this polymorphism is part of a complex haplotype associated with differences in IL-6 production. The -579G, -572G, -373A9/T11, -174G haplotype shows higher transcription of IL-6 in an ECV304 cell line after IL-1 induction than the other polymorphism combinations. This study clearly shows that different polymorphisms can have an influence on transcription, but they are not functioning independently.[34] When assessing the contribution of the G-174C polymorphism in meningococcal disease, the complete haplotype should be considered.

DISCUSSION

Meningococcal disease comprises a complex pathophysiology resulting in a spectrum of disease presentation in affected individuals. Assessing the contribution of host factors in infection therefore requires a strict definition of the patient population. Patients suffering from meningococcal meningitis might have different 'susceptibility genotypes' to septic shock patients, as different pathophysiological pathways are activated. Combining these study populations is therefore not advisable. The same problems occur when comparing patients with sepsis of unknown microbiological origin. Host response to Gram-positive microbes might differ from the response towards Gram-negative bacteria.[4] It must be noted, however, that in approximately 10% meningococcal infection cannot be proven with microbiological techniques, while the clinical presentation is typical.
In addition, it might be difficult to obtain patient groups of sufficient numbers to show an association between disease

susceptibility, severity and outcome when investigating genotypes with extremely low allele frequencies. Ethnic differences in the study population might also interfere with the supposed associations. Studies including relatives of patients as controls might overcome this problem. These studies compare the observed and expected inheritance of the different alleles. One must, however, consider the possibility that disease is not linked to the studied gene, but to another gene in linkage with the first.
Another interesting focus of investigation is the interaction of the polymorphisms and the cumulative effect on the course of meningococcal disease. These interactions might be either synergistic or counteracting. In the IL-1 family such interactions have been found for IL-1B(-511) and IL1RN (2018). The development of high-throughput molecular genetic techniques is of great importance in our efforts to unravel the immense complexity of genetic interactions.

REFERENCES

1. Kornelisse RF, Groot R de, Neijens HJ. Bacterial meningitis: mechanisms of disease and therapy. Eur J Pediatr 1995;154(2):85-96.
2. Kleijn ED de, Hazelzet JA, Kornelisse RF, Groot R de. Pathophysiology of meningococcal sepsis in children. Eur J Pediatr 1998;157(11):869-80.
3. Taylor JG, Choi EH, Foster CB, Chanock SJ. Using genetic variation to study human disease. Trends Mol Med 2001;7(11):507-12.
4. Underhill DM, Ozinsky A. Toll-like receptors: key mediators of microbe detection. Curr Opin Immunol 2002;14(1):103-10.
5. Akira S. Toll-like Receptors and Innate immunity. Adv Immunol 2001;78:1-55.
6. Read RC, Pullin J, Gregory S, et al. A functional polymorphism of toll-like receptor 4 is not associated with likelihood or severity of meningococcal disease. J Infect Dis 2001;184(5):640-2.
7. Smirnova I, Hamblin MT, McBride C, Beutler B, Di Rienzo A. Excess of rare amino acid polymorphisms in the Toll-like receptor 4 in humans. Genetics 2001;158(4):1657-64.
8. Hubacek JA, Stuber F, Frohlich D, et al. Gene variants of the bactericidal/permeability increasing protein and lipopolysaccharide binding protein in sepsis patients: gender-specific genetic predisposition to sepsis. Crit Care Med 2001;29(3):557-61.
9. Hibberd ML, Summerfield JA, Levin M. Variation in the Mannose Binding Lectin (MBL) Gene and Susceptibility to Sepsis. Sepsis 2001;4(3):201-7.
10. Madsen HO, Garred P, Kurtzhals JA, et al. A new frequent allele is the missing link in the structural polymorphism of the human mannan-binding protein. Immunogenetics 1994;40(1):37-44.
11. Hibberd ML, Sumiya M, Summerfield JA, Booy R, Levin M. Association of variants of the gene for mannose-binding lectin with susceptibility to meningococcal disease. Meningococcal Research Group. Lancet 1999;353(9158):1049-53.
12. Biesma DH, Hannema AJ, Velzen-Blad H van, et al. A family with complement factor D deficiency. J Clin Invest 2001;108(2):233-40.
13. Bjerre A, Brusletto B, Mollnes TE, et al. Complement Activation Induced by Purified Neisseria meningitidis Lipopolysaccharide (LPS), Outer Membrane Vesicles, Whole Bacteria, and an LPS-Free Mutant. J Infect Dis 2002;185(2):220-8.
14. Pol WL van der, Huizinga TW, Vidarsson G, et al. Relevance of Fcgamma receptor and interleukin-10 polymorphisms for meningococcal disease. J Infect Dis 2001;184(12):1548-55.
15. Kondaveeti S, Hibberd ML, Levin M. The insertion/deletion polymorphism in the t-PA gene does not significantly affect outcome of meningococcal disease. Thromb Haemost 1999;82(1):161-2.
16. Hazelzet JA, Risseeuw-Appel IM, Kornelisse RF, et al. Age-related differences in outcome and severity of DIC in children with septic shock and purpura. Thromb Haemost 1996;76(6):932-8.
17. Eriksson P, Kallin B, Hooft FM van 't, Bavenholm P, Hamsten A. Allele-specific increase in basal transcription of the plasminogen-activator inhibitor 1 gene is associated with myocardial infarction. Proc Natl Acad Sci USA 1995;92(6):1851-5.
18. Dawson SJ, Wiman B, Hamsten A, Green F, Humphries S, Henney AM. The two allele sequences of a common polymorphism in the promoter of the plasminogen activator inhibitor-1 (PAI-1) gene respond differently to interleukin-1 in HepG2 cells. J Biol Chem 1993;268(15):10739-45.
19. Hermans PW, Hibberd ML, Booy R, et al. 4G/5G promoter polymorphism in the plasminogen-activator-inhibitor-1 gene and outcome of meningococcal disease. Meningococcal Research Group. Lancet 1999;354(9178):556-60.
20. Westendorp RG, Hottenga JJ, Slagboom PE. Variation in plasminogen-activator-inhibitor-1 gene and risk of meningococcal septic shock. Lancet 1999;354(9178):561-3.
21. Kondaveeti S, Hibberd ML, Booy R, Nadel S, Levin M. Effect of the Factor V Leiden mutation on the severity of meningococcal disease. Pediatr Infect Dis J 1999;18(10):893-6.
22. Hackett SJ, Thomson AP, Hart CA. Cytokines, chemokines and other effector molecules involved in meningococcal disease. J Med Microbiol 2001;50(10):847-59.
23. Westendorp RG, Langermans JA, Bel CE de, et al. Release of tumor necrosis factor: an innate host characteristic that may contribute to the outcome of meningococcal disease. J Infect Dis 1995;171(4):1057-60.
24. Westendorp RG, Langermans JA, Huizinga TW, et al. Genetic influence on cytokine production and fatal meningococcal disease. Lancet 1997;349(9046):170-3.
25. Stuber F, Petersen M, Bokelmann F, Schade U. A genomic polymorphism within the tumor necrosis factor locus influences plasma tumor necrosis factor-alpha concentrations and outcome of patients with severe sepsis. Crit Care Med 1996;24(3):381-4.
26. Nadel S, Newport MJ, Booy R, Levin M. Variation in the tumor necrosis factor-alpha gene promoter region may be associated with death from meningococcal disease. J Infect Dis 1996;174(4):878-80.
27. Booy R, Nadel S, Hibberd M, Levin M, Newport MJ. Genetic influence on cytokine production in meningococcal disease. Lancet 1997;349(9059):1176.
28. Tarlow JK, Blakemore AI, Lennard A, et al. Polymorphism in human IL-1 receptor antagonist gene intron 2 is caused by variable numbers of an 86-bp tandem repeat. Hum Genet 1993;91(4):403-4.
29. Clay FE, Tarlow JK, Cork MJ, Cox A, Nicklin MJ, Duff GW. Novel interleukin-1 receptor antagonist exon polymorphisms and their use in allele-specific mRNA assessment. Hum Genet 1996;97(6):723-6.
30. Read RC, Camp NJ, Giovine FS di, et al. An interleukin-1 genotype is associated with fatal outcome of meningococcal disease. J Infect Dis 2000;182(5):1557-60.

31. Fang XM, Schroder S, Hoeft A, Stuber F. Comparison of two polymorphisms of the interleukin-1 gene family: interleukin-1 receptor antagonist polymorphism contributes to susceptibility to severe sepsis. Crit Care Med 1999;27(7):1330-4.
32. Waage A, Brandtzaeg P, Halstensen A, Kierulf P, Espevik T. The complex pattern of cytokines in serum from patients with meningococcal septic shock. Association between interleukin 6, interleukin 1, and fatal outcome. J Exp Med 1989;169(1):333-8.
33. Balding J, Livingstone WJ, Healy M, et al. G to C Transition in the Promotor Region of the IL6 Gene is Associated with Disease Outcome in Meningococcal Sepsis (abstract). In: XVIII International Society on Thrombosis and Haemostasis. Paris, France: 2001:P1065.
34. Terry CF, Loukaci V, Green FR. Cooperative influence of genetic polymorphisms on interleukin 6 transcriptional regulation. J Biol Chem 2000;275(24):18138-44.

Discussion following lecture by P.W.M. Hermans

Netea: I would like to ask you two questions; one is regarding the tumour necrosis factor (TNF) levels in meningococcal sepsis. You told us that they are high and that this increases the severity and the risk of death. Is this really an increased production capacity leading to death and leading to increased circulating levels, or is there actually a reduced production capacity leading to multiplication of bacteria, which leads to a lot of stimulus, which then leads to high amounts of cytokines? I am thinking of first-degree relatives of patients with meningococcal sepsis. If I remember rightly, they have a reduced production capacity of TNF. My second question is a more conceptual one, which is more or less for everybody. As we are now looking at Toll-like receptor (TLR) polymorphisms in various diseases, what are our chances of finding something important with a genetic approach? Because at the moment we find a very significant polymorphism or a very significant mutated gene, the host is already dead. So the closer we are to the truth in looking for a determinant gene, the smaller the chance we will ever find it. Only less significant polymorphisms are found which are compatible with survival, whereas the really significant ones are not detected since the hosts are already dead. I am thinking of TLRs, because if it is really a polymorphism which leads to a TLR-2 defect, I do not think this is compatible with survival. It may be the same with some important polymorphisms for TNF. For example, I do not know anyone who is really deficient in TNF.

Hermans: Quite complex comments, but starting with the last part, I think it is absolutely true that quite a few interesting polymorphisms have been studied now, and as soon as new polymorphisms pop up, I immediately get the feeling, OK, we have to increase the number of patients again to see any relevance in interaction. You are in fact driving towards a complete human-specific genetic passport for that particular human being. If I have got your point right, I think that these extremely complex and difficult studies might not give us the appropriate answers we were hoping for. Coming back to TNF, TNF-α expression actually, for many cytokines, when patients come in, as far as I understand from the clinicians, the cytokine responses and the cytokine levels are extremely high. Immediately after admission they tend to decrease, do you agree, Dr van Deuren?

Van Deuren: Yes.

Hermans: The turnover is extremely high, which means that the half-life is quite short.

Netea: I am just thinking that the circulating levels that you are measuring are in fact a result of the interaction between our capacity to make it and how many bugs are multiplying in our system. It is very difficult to discern and to say, a sick person has a lot of TNF, so that is a bad thing. It might very well be possible he was unable to make enough in the beginning to suppress the multiplication of the bacteria.

Hermans: I agree with that.

Van Deuren: I would like to ask a few questions and make a few remarks on meningococcal disease. Of course, it is a blood-borne infection with an impressive clinical picture and blood is the most easily obtained tissue. So a lot of measurements have been done in blood. But it is really the question whether that is the most important site of the body to look at. The gate for the meningococcal bacterium is the oronasal mucosa. At that site there are several interactions between the bacterium and our immune system: the meningococcal pylus is attached to CD46 for instance, the meningococcal pore A outer membrane protein is attached to the carcinoembryogenic antigen CEA, CD66a, b and c. And there are a lot of polymorphisms in these types of receptors or ligands too, for instance relating to the intramucosal survival of the meningococcus, because it is able to survive passing through the cells. There are

some studies showing that they are influencing bactericidal proteins, enabling them to survive and to pass through the bloodstream. Are you aware of any studies looking at the polymorphisms in this site of the body? Because that will really determine our susceptibility, because all the rest in the blood really is a matter of severity.

Hermans: As far as I know, such studies have not been undertaken, but the initial point you made that has not been studied either, in detail at least, is the genetic differences among these bugs, and it is definitely not only man C *versus* man B, but there are many more molecules showing a huge variety within this particular species.

Van Deuren: That is the second important property of the bug, of course, its switching off and switching on molecules so rapidly enabling it to enter the bloodstream. A second remark about the levels of TNF: the higher the concentration of endotoxin in your blood, the more TNF you produce. And really the best predictor of mortality is not the TNF level but the endotoxin level, as already mentioned by Brandtzaeg in his series of 150 patients. There is a good correlation between the level of TNF and endotoxin. So for me it is not the genetic trait or the capacity of the person to produce TNF, but it is the amount of endotoxin that he tolerates, and that will determine the amount of TNF produced and, thus, the amount of procoagulant and anticoagulant activities.

Appelmelk: You showed that you get an impressive increase in relative risk when you add the various polymorphisms. Where will this end? From a scientific point of view you can go on, but do we need to know all this? Which knowledge regarding polymorphisms will lead to clinical actions? You read in the papers about DNA passports and you see companies eager for dollars, but how important is it for the actual patient management?

Hermans: Your question actually was the main reason for me to show this study on tissue-type plasminogen activator (t-PA) therapy, which is in part guided by the existing polymorphisms and genetic variability. Indeed, when you have these details, there is a possibility of performing patient-specific therapy in the long run. At the moment that is not feasible. But I think such data are of definite value for the patient. It is not so that you are just born with a 'genetic passport' and that nobody can change anything about that. I think when you have these data in advance and you know where to interact, you can intervene in these processes.

Appelmelk: Do you think that it is realistic when we have a patient and are at the bedside to take his blood, for us to put it on a chip and then decide what to do?

Hermans: By that time it is too late!

Kusters: Can I give one brief comment or ask a question? You are now looking at only one type of disease and one type of bug trying to find the ideal 'genetic passport', but obviously evolution has gone a long way and the so-called wrong passport may in fact be an ideal passport for survival. Maybe an increased risk of a certain type of disease is the flip side of a better survival of other types of disease.

Hermans: When a patient comes in with a meningococcal sepsis, having an excellent overall survival passport, still at that time his survival clock has started ticking towards zero. When you know the disadvantages of that particular passport, if you can alter it, that would be a major advantage. Of course, such an intervention might be disadvantageous with another infection.

Kuijpers: Whether lipopolysaccharide (LPS) and the levels of TNF strictly correlate, depends on which test system you use, which ELISA, or especially which firm you have addressed to purchase this ELISA system from. Just comparing TNF ELISA test systems as such would already give you two or three papers probably. It is very difficult. With genes you cannot lie – that is an advantage, but on the other hand it is of course a disadvantage that if you then start to shift from genes to concepts, as for instance TNF-induced responses, and one observes the staggering lack of effect of blocking TNF for instance in meningococcal disease or sepsis, I very much doubt whether this approach will have any impact in the short run on how to treat patients.

Kimman: It is nice to comment on this issue of course, whether we should do this work in order to improve the treatment of individual patients. I do not see a future in which patients will be treated only after their genotype has been assessed, but on the other hand, you nicely showed that this kind of work can lead the way to pathways which can be influenced whatever the genotype of the patient is. For instance, you identify a PAI/t-PA pathway and then you have a lead for treatment irrespective of the genotype of the patient. Unfortunately it did not work, but you identified something which you can tackle.

De Groot: I would like to support Dr Hermans in relation to the question from Dr Appelmelk and the point that is raised here. I will give you a practical example why I think this genetic approach will have additional value and how it could already have in the current situation. Let's consider a polytrauma patient and suppose you knew the genetic profile in advance when he came in, thus being able to predict whether he was in a high-risk group or in a low-risk one. That would be helpful in the choice of therapy. Now Dr Hermans mentioned one therapy, t-PA, but another one

is known to all clinicians from a publication in the New England Journal of Medicine last year, namely activated protein C.[1] Both therapies have serious potential side effects in terms of cerebral haemorrhage. The selection of patients who would benefit from these very invasive therapies would therefore be balanced depending on their risk. So this is the way of thinking I believe we should pursue as clinicians in terms of making a choice actually for these drugs where we know that in these desperately ill patients there certainly will be a very narrow kind of safety margin.

Van Deuren: Just to address a limitation of this approach. In meningococcal disease I have looked at the moment – I call it the kinetics of dying – the patient dies from that disease. From literature and from my own experience I have collected approximately 300 deaths, not cases, but deaths, and from that it can be seen that one-third of the patients who will die have already died within six hours of admission, half have died within 12 hours, 66% within 24 hours and 80% within 48 hours. So already one-third of your patients have been lost within six hours. I do not know how long it takes to run a chip array.

Verbrugh: It is quite interesting that we are here discussing the value of our core business as a science, the creation of new knowledge. We would rather like to pose the question beforehand or we would like to know which knowledge we can use and what knowledge we cannot. This is a poor question to ask. Sometimes we do have to ask, because there are financial constraints, but primarily the business is to get new insights and new knowledge.

REFERENCE

1. Bernard GR, Vincent JL, Laterre PF, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. N Engl J Med 2001;344:699-709.

Genetics of type-1 cytokines in human intracellular infectious diseases: a spectrum of novel genetic deficiencies demonstrates the essential role of type-1 cytokines in immunity against nontuberculous mycobacteria, *M. bovis* BCG and salmonellae

T.H.M. Ottenhoff

Leiden University Medical Centre, Department of Immunohaematology and Blood Transfusion, Leiden, the Netherlands

ABSTRACT

Human genetic factors play an important role in determining the outcome of infections caused by intracellular pathogens, including mycobacteria and salmonellae.[1] The genetic elements involved and the mechanisms by which these control disease susceptibility *vs* resistance, however, remain incompletely characterised. Recent studies on patients with idiopathic severe infections due to otherwise poorly pathogenic mycobacteria (nontuberculous mycobacteria or *Mycobacterium bovis* BCG) or *Salmonella* species have revealed that many of these patients are unable to produce or respond to IFN-γ. This inability results from causative, deleterious genetic mutations in any one of five different genes in the type-1 cytokine cascade, encoding IL-12p40, IL-12Rβ1, IFN-γR1, IFN-γR2 or STAT1. The mutational events can lead to complete or partial deficiency, and are mostly autosomal recessive but can be dominant negative as well. The immunological, clinical and histopathological phenotypes resulting from the 11 groups of genetic type-1 cytokine (receptor) deficiencies that have been identified thus far differ significantly. These findings are summarised, discussed and placed in a broader context in relation to genetic disease predisposition and molecular and cellular mechanisms of protective immunity to these intracellular pathogens.

OUTLINE

Protective immunity to intracellular pathogens such as mycobacteria and salmonellae depends on effective cell-mediated immunity (CMI) in which T-cell-macrophage interactions play a crucial role.[2] A major effector mechanism of CMI is the activation of infected macrophages by type-1 cytokines, particularly interferon-gamma (IFN-γ).[2] IFN-γ is produced by antigen-specific T-helper 1 (Th1) cells and natural killer (NK) cells, and binds to IFN-γR1/R2 receptor complexes at the cell surface of macrophages.

The production of IFN-γ by T cells and NK cells itself is tightly regulated by another cytokine, interleukin-12 (IL-12). IL-12 is a heterodimer, composed of a p40 and a p35 subunit, and is produced by antigen-presenting cells such as macrophages, monocytes and dendritic cells[2] following the activation of Toll-like receptors by bacterial ligands.[3] Thus, IL-12 plays a major role in promoting and linking both innate and adaptive immunity. IL-12 binds to IL-12 receptor β1/β2 complexes at the surface of Th1 and NK cells. Whereas IL-12p40 predominantly interacts with the IL-12Rβ1 chain, IL-12p35 primarily binds to IL-12Rβ2. Two additional cytokines with IL-12-like activities have recently been identified, namely IL-18 and IL-23, both of which are produced by antigen-presenting cells. IL-18 acts mainly in synergy with IL-12 and plays an accessory role in promoting optimal IFN-γ production.[4] Interestingly, IL-23 shares one subunit

with IL-12, notably p40, which is coupled to a unique second chain, p19.[5] Similarly, the receptor for IL-23 consists of an IL-12Rβ1 subunit which is complexed to an as yet unidentified p19R chain. As expected, IL-12 and IL-23 display similar functions, including the stimulation of IFN-γ production, although their functional profiles are not completely identical.[5] Other cytokines can act as additional factors in promoting Th1 development and IFN-γ production, such as the recently identified TCCR ligand,[6] interferon-α and chemokines.[7]

IFN-γ, in synergy with TNF-α, is able to activate microbicidal mechanisms with antimycobacterial activity in murine macrophages.[8] Although IFN-γ is clearly able to affect the growth of *M. bovis* BCG also in human macrophages, reports on a similar role for IFN-γ in *M. tuberculosis*-infected human macrophages have been conflicting.[8] Recent evidence now suggests that IFN-γ may require the presence of other factors, such as 1,25-dihydroxy-vitamin D3 and TNF-α, or human lymphocytes, in order to exhibit anti-*M. tuberculosis* activity in human macrophages, but this issue clearly needs further study.[9] Furthermore, IFN-γ is well known to enhance MHC class I and II expression and to modulate the expression of other molecules involved in antigen presentation, such as proteasomes and transporters-associated-with-antigen-processing (TAP), thus promoting optimal CD4 and CD8 T-cell activation.[10] An additional component of CMI is the activation of cytolytic effector T cells that are able to kill infected macrophages, thereby inhibiting bacterial proliferation through a variety of mechanisms.[11] Effective CMI typically leads to the local containment of the pathogen inside well-organised granulomatous lesions, with epitheloid and giant cell formation and relatively few detectable micro-organisms.

Recently, several patients have been reported with unusually severe and sometimes fatal infections due to usually poorly pathogenic mycobacteria, in the absence of any recognised primary or secondary immunodeficiencies. These patients mostly suffered from infections due to *M. avium*, other nontuberculous mycobacterial species or *M. bovis* BCG, but also other pathogens have been reported including salmonellae and *M. tuberculosis*. The patients described thus far often fail to form well-organised granulomata at the sites of their lesions. A common feature of these individuals was their inability to produce or respond to IFN-γ *in vitro*. This appeared to be due to deleterious mutations in any one of five different genes in the type-1 cytokine cascade, notably *IL12B* (encoding IL-12 p40), *IL12RB1* (encoding IL-12Rβ1), *IFNGR1*, *IFNGR2* (encoding IFN-γR1 and IFN-γR2 chains, respectively) or *STAT1* (encoding IFN-γR-associated STAT1). The mutational events resulted in either (i) recessive, nonfunctional null alleles, (ii) recessive, partially functional alleles, or (iii) dominant-negative alleles, causing partial functionality. Upon further analysis, these deleterious mutations appear to comprise a spectrum of genetically controlled deficiencies in which the extent of the defect correlates with the severity of the clinical, immunological, and histopathological phenotypes observed.
Recent developments and findings regarding defects in the IL-12/IL-12R and IFN-γ/IFN-γR/STAT1 systems respectively will be discussed.[12,13] These novel defects reveal essential molecular and cellular mechanisms of protective cell-mediated immunity against intracellular pathogens. The intriguing observation that these patients appear to be selectively susceptible to otherwise poorly pathogenic mycobacteria and *Salmonella* species will be discussed as well. Finally, comparison of the different clinical, immunological and cellular phenotypes in the various types of genetic defects reveals a spectrum of genetic disorders, which establishes genotype-phenotype relationships but also elucidates additional host- or environment-dependent variability in disease manifestations.

REFERENCES

1. Hill AVS. The immunogenetics of human infectious disease. Annu Rev Immunol 1998;16:593-617.
2. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature 1996;383:787-93.
3. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. Nature 2001;406:782-7.
4. Nakanishi K., Yoshimoto T, Tsutsui H, Okamura H. Interleukin-18 regulates both Th1 and Th2 responses. Annu Rev Immunol 2001;19:423-74.
5. Oppmann B, Lesley R, Blom B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 2000;13:715-25.
6. Chen Q, Ghilardi N, Wang H, et al. Development of Th1-type immune responses requires the type 1 cytokine receptor TCCR. Nature 2000;407:916-20.
7. Zou W, Borvak J, Marches F, et al. Macrophage-derived dendritic cells have strong Th1-polarizing potential mediated by β-chemokines rather than IL-12. J. Immunol 2000;165:4388-96.
8. Flynn J-L, Chan J. Immunology of Tuberculosis. Annu Rev Immunol 2001;19:93-129.
9. Denis M. Killing of Mycobacterium tuberculosis within human monocytes: activation by cytokines and calcitriol. Clin Exp Immunol 1991;84:200-6.
10. Bonecini-Almeida MG, Chitale S, Boutsikakis I, et al. Induction of *in vitro* Human Macrophage Anti-Mycobacterium tuberculosis Activity: Requirement for IFN-g and Primed Lymphocytes. J Immunol 1998;160:4490-9.
11. Stenger S, Rosat J-P, Bloom BR, Krensky AM, Modlin RL. Granylysin: a lethal weapon of cytolytic T cells. Immunol Today 1999;20:390-4.
12. Dupuis S, Döffinger R, Picard C, et al. Human interferon-α-mediated immunity is a genetically controlled continuous trait that determines the outcome of mycobacterial invasion. Immunol Rev 2000;178:129-37.
13. Ottenhoff THM, Kumararatne D, Casanova JL. Novel human immunodeficiencies reveal the essential role of type-1 cytokines in immunity to intracellular bacteria. Immunol Today 1998;19:491-4.

Discussion following lecture by T.H.M. Ottenhoff

Van der Meer: Regarding the interaction between the T lymphocyte and the macrophage, you did not point to defects of IL-18. The question is, of course, is IL-18 an irrelevant molecule, didn't we look carefully enough, or could it be that the IL-23/IL-12 combination takes out enough of the redundancy to cause trouble.

Ottenhoff: We have not been able to find any defects in IL-18 production and IL-18 receptor expression in those patients we have analysed. Other laboratories did not find any mutations or defects in that system either. In my view IL-18 is mainly an amplifier of IL-12 and perhaps also IL-23. Maybe IL-18 deficiency is not sufficient to cause a dramatically enhanced disease susceptibility to mycobacteria.

Netea: You have also investigated the patients with receptor defects for IL-12 and interferon-γ and found no difference in IL-18 receptor expression?

Ottenhoff: Correct.

Netea: One of the mechanisms for the synergism between IL-12 and IL-18 could be that IL-12 actually induces expression of IL-18, and IL-18 would do a lot of the work.

Ottenhoff: If you activate the cells with phytohaemagglutinin (PHA), you see normal expression of the IL-18 receptor α component. So that certainly is not IL-12-dependent in that system, or at least not IL-12-receptor-β1-dependent.

Netea: But I don't know whether PHA is a relevant stimulus. Could it be going to the T cell directly without the need of a macrophage?

Ottenhoff: PHA is a good system to screen for major defects, but physiologically IL-18 actually could contribute.

Netea: Have you looked at the IL-18 receptor in these patients with IL-12 receptor defects?

Ottenhoff: Yes, we looked at it, but in these patients there is no defect in IL-18 receptor α expression.

Netea: That would imply that those who are saying that IL-12 is working through induction of IL-18 receptor are probably wrong?

Ottenhoff: That is true. We have published this.[1] If you activate T cells in patients with IL12R-β1 defects, there is a small residual IL-12 response. This is of course β1 STAT4-independent, and most likely works through MAP kinase. Perhaps that pathway would be sufficient to induce IL-18 receptor expression in these patients.

Van Furth: Do you have an explanation for the occurrence of tuberculosis, which only arose in three patients, and the other mycobacterial infections, while activation of macrophages is only mildly affected? In contrast, AIDS patients develop tuberculosis early on, and when the CD4 count is really low infections with other mycobacteria develop.

Ottenhoff: One explanation for this paradox is that there is no exposure of these patients, who are mainly from Western countries, to *M. tuberculosis*. Another explanation is that in endemic areas these patients die prematurely and a genetic diagnosis is never made. A third option is that once a patient presents with TB, a genetic defect is not considered. In the three TB cases that have been reported so far this was more or less discovered by coincidence. These defects may be more common in tuberculosis. A fourth, less likely and more academic option is that *M. tuberculosis* inhibits, at least in part, signalling through the interferon-γ receptor STAT1 pathway in macrophages. Because *M. tuberculosis* does this, the macrophage may be less sensitive to interferon-γ, and therefore in TB such defects would have less impact than for instance in BCG or *M. avium*.

De Jongh: Sarcoidosis has many similarities with tuberculosis. Are there defects in patients with sarcoidosis?

Ottenhoff: We have tested about ten sarcoidosis patients, but it is too early to say.

Kuijpers: Interferon-γ receptor-deficient patients may have an increased susceptibility to viral diseases.[2] It is a very weak association, but isn't it the biggest surprise that it is so monogenic?

Ottenhoff: I agree. That is why I concluded that quantitative ligation of the interferon-γ receptor is important. It indicates how important full-blown activation through the interferon-γ receptor pathway is for protection. I think in the case of viral infections or toxoplasmosis, there may be sufficient backup pathways to compensate for these defects, but in the case of mycobacterial and salmonella infection these are not sufficient.

McAdam: What about the ages of the patients with the defects? You suggested the poor life expectancy in the tropics. Certainly we have screened for type-1 pathway defects in

our adult TB patients and have not found any, but that probably means that the others died of viral infections or something else much younger.

Ottenhoff: Patients with complete interferon-α receptor 1 and 2 defects usually present during early life and quite often die before the age of ten. So that is really a severe, often fatal syndrome. IL-12 receptor deficiencies are a little less severe. Those patients can present at an early age but even at 30 years and older. They often survive quite long.

De Groot: Do you have data on the genetics of parents, grandparents and other family members, and have you gone back some generations to see if there were comparable diseases?

Ottenhoff: Yes, all the families we have analysed from Turkey are consanguineous. They can be traced back to one precedent generation. The parents are always heterozygous for the mutations. We performed extensive family analyses and showed that these really segregated recessive traits, as you would expect. At the moment we are investigating whether there are founder effects in Turkey, if certain alleles are more common in the population than other alleles, and if these alleles are maintained in the population and sometimes cause major disease in these families.

De Groot: How would this fit in with the theories on HLA and genetic susceptibility at population level? By changing the HLA make-up over generations apparently disease becomes modified? How does this relate to these cellular defects?

Ottenhoff: That is a difficult question. The defects I described work at the level of innate immunity as well as of the adaptive immunity: first there will be a major deficiency in innate immunity, and this will later on also lead to a major defect in acquired immunity. At that stage there is interaction with HLA leading to enhancement or a decreased susceptibility. These genes I have discussed have a major impact, especially on the early, innate phase, at that stage of course apart from HLA.

De Vries: Also, such a rare mutant, which results in a rather severe phenotype, works out in a recessive way. The genetic situation is completely different when you are talking about HLA alleles which are polymorphic at relatively high frequencies, and which are working in a dominant or co-dominant way. This situation is open to selection.

Netea: Regarding IL-12 p40, could it be, for example, that there are TB patients who produce too much p40 and in that way would be blocking the normal p40/p35 heterodimer action?

Ottenhoff: There is great variation in p40 production between different individuals, that I know, but I am not aware that this is related to tuberculosis.

Appelmelk: I am curious, how are you planning to go on?

Ottenhoff: It is difficult to decide. Should we opt for a candidate gene approach? That is something we are doing. One of these is the interleukin-10 gene. If you want a totally unbiased approach you should go for a genome scan and do linkage analysis and then try to positionally clone the genes involved. That is not easy. It would be a good idea to start using micro-arrays and compare genetic expression profiles of tuberculosis patients with exposed but protected individuals, and try to find which pathways light up in susceptible *vs* protected individuals and to define the different cascades.

Van Agtmael: Has this pathway been evaluated in the patients with *Legionella*, for instance during the recent outbreak in Bovenkarspel in the Netherlands?

Speelman: I can answer that: with Professor van Dissel we are investigating this. So far the results have not been very exciting.

REFERENCES

1. Verhagen et al. J Exp Med 2000;192:157.
2. Donnay et al. J Pediatr 1999;135:640.

NRAMP1 (SLC11A1) and vitamin D receptor genes: disease associations

K.P.W.J. McAdam

Medical Research Council Laboratories, Banjul, The Gambia

ABSTRACT

Resistance to infectious diseases is complex, being regulated by multiple genes, and so far no one locus has been shown to dominate the susceptibility in outbred human populations. Multi-case families have contributed greatly to defining important host immune response pathways using a candidate gene approach. It is likely that genome scans will identify novel genes for resistance to disease that could help define new approaches to drug and vaccine development.

INTRODUCTION

Susceptibility to infectious diseases is under polygenic control, as exemplified by genetic susceptibility to malaria. Severe *Plasmodium falciparum* malaria exerts enormous selective pressure on populations living in malaria-endemic areas. In the era before the advent of antimalarial medicines, those who could not survive malaria and develop effective immunity were wiped out at a young age and only those who survived were able to pass on their genes to the next generation. The sickle-cell haemoglobin mutation (S) confers protection against malaria in its single-dose heterozygote form (AS) and provides a selective advantage, despite the disadvantage conferred by the two-dose (SS) form in homozygotes.[1] Many genes have now been described as providing resistance against malaria, including various haemoglobin variant genes giving rise to thalassaemia[2] and glucose-6-dehydrogenase deficiency, as well as class I and II HLA molecules. HLA B53 is found in 30% of the Gambian population whereas it is uncommon in Europe. The nine-amino-acid peptide that fits best in the groove of HLA-B*53 codes for a portion of the malarial liver-specific antigen LSA-1, presumably demonstrating that the selective advantage of B*53 identifies a vulnerable target in the parasite life cycle. Following on from this work in The Gambia, new malaria vaccine constructs have been designed to include this and other liver-stage antigens not previously considered as vaccine candidates. The promise that identification of susceptibility/resistance alleles can lead to the development of new approaches to vaccines or therapeutic agents has encouraged us in The Gambia to pursue studies of the genetics of disease susceptibility. We have focused on important endemic infectious diseases in Africa including malaria, tuberculosis, hepatitis B, chlamydia and HIV. This paper briefly summarises our efforts on two candidate genes, NRAMP 1 and vitamin D receptor, to define susceptibility to tuberculosis.

GENETIC SUSCEPTIBILITY TO TUBERCULOSIS

Heritability of tuberculosis has been suggested by various studies in twins,[3] by racial differences in resistance to TB[4,5] and by animal studies in rabbits and inbred strains of mice.[6,7] Families expressing a high rate of mycobacterial disease have been important in defining various Mendelian susceptibilities in the interferon-γ and IL-12 pathways.[8-11]

Most people who breathe in aerosol droplets containing *M. tuberculosis* become infected. However, only 5 to 10% of close contacts of a case of open pulmonary TB go on to develop tuberculosis, and it has been assumed that this susceptibility is related to the effectiveness of the immune system. Many factors including genetic background influence the innate and acquired arms of the immune response. Poor nutrition, alcoholism and immunosuppression due to drugs or other infections, including HIV and measles, have been implicated in an increased susceptibility to TB.

An increasing number of genes have been implicated in susceptibility to tuberculosis. HLA is perhaps the best documented, with conflicting positive and negative associations reported. An increased frequency of HLA-DR2 is the most consistent association but has not been found in all populations examined, nor was there clear linkage to the MHC in a genome-wide screen carried out on patients from The Gambia and South Africa.[12]

When the Scottish missionary Dr David Livingstone travelled through Central Africa in the mid 19th century he did not see a lot of TB, though he would have observed plenty in Britain. Senegalese troops who fought in the 1914-18 war contracted TB at a higher rate than European soldiers, suggesting that they were nonimmune and had not been exposed to *M. tuberculosis* previously, or perhaps were more susceptible genetically. Stead reported that black Americans in Arkansas nursing homes had twice the risk of acquiring *M. tuberculosis* infection compared with white subjects.[4] Crowle confirmed a similar trend *in vitro*, showing that macrophages from blacks were more easily infected and less activated by vitamin D.[5]

VITAMIN D RECEPTOR AND TUBERCULOSIS

Historically, there has been circumstantial evidence for an association between vitamin D and tuberculosis. Prior to effective chemotherapy, patients were sent off to sanatoria where they were exposed to fresh air and sunlight, which was thought to provide the best environment for recovery. Patients with skin TB or lupus vulgaris responded well to vitamin D therapy but some patients with pulmonary tuberculosis fared badly. Rook showed that macrophages stimulated with vitamin D were activated, providing a rationale for these anecdotal clinical observations.[13] The active form of vitamin D, 1,25 dihydrocholecalciferol (1,25D3), binds the vitamin D receptor (VDR) in the cytoplasm of macrophages and activated lymphocytes. VDR is a zinc-finger DNA-binding transcription factor which migrates to the nucleus, resulting in the expression of a number of hormone-sensitive genes.

VDR maps to chromosome 12q13-14, and belongs to the steroid/thyroid hormone receptor family with 11 exons. Polymorphisms within the VDR gene include a Taq I site at codon 352, an Apa I site and a Bsm I site in intron 8 and a Fok I site in exon 2 which leads to an alternative transcription site. Although the association between VDR polymorphisms and bone mineral density has been hotly debated,[14] the homozygous Taq I genotype 'tt' has been associated with decreased bone mineral density (osteoporosis). Bellamy *et al.* found this genotype less commonly in 400 TB cases in The Gambia than in the 400 ethnically matched controls (odds ratio 0.53, 95% confidence limits 0.31-0.88, p=0.01).[15] Wilkinson *et al.* undertook a case-control study of Gujarati Indians in West London, demonstrating an association between TB and a combination of VDR genotype and serum 1,25D3 concentration.[16] Another study in India did not confirm an association with VDR, though the TT genotype was overrepresented in female cases compared with controls.[17] Vitamin D receptor polymorphism has been shown to be relevant to susceptibility to leprosy in Calcutta, where 'tt' was overrepresented in tuberculoid cases and 'TT' was associated with lepromatous disease.[18] Interestingly, 'tt' has also been associated with enhanced clearance of Hepatitis B virus, suggesting that 1,25D3 and VDR may play a more central role in regulating immune responses against infections.[15]

NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN 1

Natural resistance associated macrophage protein 1 (NRAMP1) has recently been renamed SLC11A1 (Solute Carrier family11 member1). Originally described in inbred mouse strains, this locus was confirmed to be a single gene on chromosome 1 when a knockout mouse proved NRAMP1 was responsible for resistance to several intracellular infections.[19] A single amino acid change on the 4th transmembrane domain of the protein resulted in an absence of the protein in susceptible mice.[7,20] This naturally occurring mutation led to a Gly-Asp substitution at amino acid 169, which enhanced susceptibility in mice to intracellular infections with leishmania, salmonella, toxoplasma, candida and mycobacteria.[19]

In humans the NRAMP1 gene is located on chromosome 2q 35[21] and has been associated/linked to various infectious diseases (tuberculosis, leprosy, [?meningococcal meningitis], leishmaniasis and HIV) and autoimmune diseases (rheumatoid arthritis, diabetes, sarcoidosis, and Crohn's disease).[22-26]

In 400 cases and controls in The Gambia, Bellamy and colleagues have shown that four variants of the NRAMP1 gene are significantly associated with an increased risk of developing cavitary pulmonary tuberculosis.[23] In the patient group there was an increased risk (odds ratio 4.08, 95% confidence interval 1.86-9.12, p<0.001) of TB in individuals heterozygous for both a single nucleotide polymorphism (SNP) in intron 4 and a 'TGTG' deletion in the 3'untranslated region (3'UTR).[23] A family-based study from Guinea-Conakry confirmed the association with the intron 4 polymorphism[27] and a case-control study has confirmed the association with the 3'UTR deletion in Koreans.[28] Gao *et al.* have found associations with a 5' promoter region polymorphism in

Japanese patients.[29] Linkage to a chromosome 2 region, which includes the NRAMP1 gene, has been documented in a Canadian aboriginal family.[30] However, the results from a linkage study in a Brazilian population showed no evidence of NRAMP1 involvement in susceptibility to TB.[22] Moreover, a genome-wide screen demonstrates that NRAMP1 is not a major susceptibility gene in Gambians, in whom only a weakly positive linkage was shown to 2q 35.[12]

Slc11a1 is found in late endosomes and lysosomes but not in early endosomal membranes. It is unclear how Slc11a1 functions in the maturation of the phagolysome nor in the reduced acidification found in mycobacterium-containing lysosomes. Slc11a1 is a proton/divalent cation transporter (Fe, Zn, Mn) which can move divalent cations in either direction against a proton gradient. Thus cations can be delivered from the cytosol to the acidic compartment of late endosomes/lysosomes, where they catalyse the production of reactive oxygen intermediates (ROI) including hydroxyl radicals. Killing of mycobacteria probably involves both ROIs as well as reactive nitrogen intermediates (RNI) generated by nitric oxide synthetase NOS2, and by apoptosis of macrophages induced by ATP through the P2X7 receptor.[31]

Blackwell has defined four alleles containing different numbers of DNA repeats at positions (i)203, (ii)201, (iii)199 and (iv)189, with allele frequencies of (ii) and (iii) being 0.25 and 0.75 respectively.[26] Using a reporter gene system, LPS drives five to eightfold higher gene expression by allele (iii) and significant reduction in expression by allele (ii). They proposed that the higher expressing allele (iii) with (GT)n polymorphism would be associated with autoimmune disease and the low-expressing allele (ii) with infection. Allele frequencies for SLC11A1 allele(ii) are in the range 0.14 to 0.20 in West and Southern Africa, compared with 0.25 to 0.29 in Northern Europe and 0.36 in Brazil. Individuals homozygous for iii/iii produced higher concentrations of TNF and were at significantly higher risk of severe clinical meningococcal disease (Hibbard, Levin & Blackwell).

REFERENCES

1. Allison AC. Protection afforded by sickle cell trait against subtertian malarial infection. BMJ 1954;1:190.
2. Clegg JB, Weatherall DJ. Thalassemia and malaria: New insights into an old problem. Proc Assoc Am Physicians 1999;111:278-82.
3. Comstock GW. Tuberculosis in Twins: A re-analysis of the Prophit Survey. Am Rev Resp Dis 1978;117:621-4.
4. Stead W, Senner J, Reddick W, Lofgren J. Racial Differences in susceptibility to Infection by Mycobacterium tuberculosis. N Engl J Med 1990;322:422-7.
5. Crowle A, Elkins N. Relative permissiveness of macrophages from Black and White people for virulent tubercle bacilli. Infect Immun 1990;58:632-8.
6. Lurie MB, Zappasodi P, Dannenberg AMJ, Weiss GH. On the mechanism of genetic resistance to tuberculosis and its mode of inheritance. Am J Hum Genet 1952;4:302-14.
7. Bushman E, Skamene E. From bcg/lsh/ity to Nramp1: Three decades of search and research. Drug Metab Dispos 2001;29:471-3.
8. Newport MJ, Huxley CM, Huston S, et al. A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. N Engl J Med 1996;335(26):1941-9.
9. Altare F, Durandy A, Lammas D, et al. Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. Science 1998;280(5368):1432-5.
10. Dorman SE, Holland SM. Mutation in the signal-transducing chain of the interferon-gamma receptor and susceptibility to mycobacterial infection. J Clin Invest 1998;101(11):2364-9.
11. Doffinger R, Altare F, Casanova JL. Genetic heterogeneity of Mendelian susceptibility to mycobacterial infection. Microbes Infect 2000;2(13):1553-7.
12. Bellamy R, Beyers N, McAdam KP, et al. Genetic susceptibility to tuberculosis in Africans: a genome-wide scan. Proc. Natl Acad Sci USA 2000;97(14):8005-9.
13. Rook GA, Hernandez-Pando R. The pathogenesis of tuberculosis. Annu Rev Microbiol 1996;50:259-84
14. Audi L, Garcia-Ramirez M, Carrascosa A. Genetic determinants of Bone Mass. Horm Res 1999;51:105-23
15. Bellamy R, Ruwende C, Corrah T, et al. Tuberculosis and chronic hepatitis B virus infection in Africans and variation in the vitamin D receptor gene. J Infect Dis 1999;179(3):721-4.
16. Wilkinson RJ, Llewelyn M, Toossi Z, et al. Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in West London: a case study. Lancet 2000;355:618-21.
17. Selvaraj P, Narayanan PR, Reetha AM. Association of vitamin D receptor genotypes with susceptibility to pulmonary tuberculosis in female patients and resistance in female contacts. Indian J Med Res 2000;111:172-9.
18. Roy S, Frodsham A, Saha B, Hazra SK, Mascie-Taylor CG, Hill AV. Association of vitamin D receptor genotype with leprosy type. J Infect Dis 1999;179:187-91.
19. Vidal S, Tremlay ML, Govoni G, et al. The Ity/Lsh/Bcg Locus: Natural Resistance to Infection with Intracellular Parasites is Abrogated by Disruption of the Nramp1 Gene. J Exp Med 1995;182:655-66.
20. Buu N, Sanchez F, Schurr E. The Bcg host-resistance gene. Clin Infect Dis 2000;31(suppl 3):S81-5.
21. Liu J, Fujiwara TM, Buu NT, et al. Identification of polymorphisms and sequence variants in the human homologue of the mouse natural resistance-associated macrophage protein gene. Am J Hum Genet 1995;56(4):845-53.
22. Shaw MA, Collins A, Peacock CS, et al. Evidence that genetic susceptibility to Mycobacterium tuberculosis in a Brazilian population is under oligogenic control: linkage study of the candidate genes NRAMP1 and TNFA. J Infect Dis 2000;82(5):1473-8.
23. Bellamy R, Ruwende C, Corrah T, McAdam KP, Whittle HC, Hill AV. Variations in the NRAMP1 gene and susceptibility to tuberculosis in West Africans. N Engl J Med 1998;338(10):640-4.
24. Blackwell JM. Genetics of host resistance and susceptibility to intra-macrophage pathogens: a study of multicase families of tuberculosis, leprosy and leishmaniasis in north-eastern Brazil. Int J Parasitol 1998;28(1):21-8.

25. Abel L, Sanchez FO, Oberti J, et al. Susceptibility to leprosy is linked to the human NRAMP1 gene. J Infect Dis 1998;177(1):133-45.
26. Blackwell JM, Goswami T, Evans CAW, et al. SLC11A1 (formerly NRAMP1) and disease resistance. Cell Microbiol 2001;3(12):773-84
27. Cervino AC, Lakiss S, Sow O, Hill AV. Allelic association between the NRAMP1 gene and susceptibility to tuberculosis in Guinea-Conakry. Ann Hum Genet 2000;64(Pt6):507-12.
28. Ryu S, Park YK, Bai GH, Kim SJ, Park SN, Kang S. 3'UTR polymorphisms in the NRAMP1 gene are associated with susceptibility to tuberculosis in Koreans. Int J Tuberc Lung Dis 2000;4(6):577-80.
29. Gao PS, Fujishima S, Mao XQ, et al. Genetic variant of NRAMP1 and active tuberculosis in Japanese populations. Clin Genet 2000;58(I):74-6.
30. Greenwood CM, Fujiwara TM, Boothroyd LJ, et al. Linkage of tuberculosis to chromosome 2q35 loci, including NRAMP1, in a large aboriginal Canadian family. Am J Hum Genet 2000;67(2):405-16.
31. Lammas DA, Stober C, Harvey CJ, Kendrick N, Panchalingam S, Kumararatne DS. ATP-induced killing of mycobacteria by human macrophages is mediated by purinergic P2Z(P2X7) receptors. Immunity 1997;7(3):433-44.

Discussion following lecture by K.P.W.J. McAdam

Fenton: Are there any data suggesting that similar susceptibility loci are involved in other populations and racial groups?

McAdam: Quite a large number of different places have been looked at, for instance for HLA. The NRAMP work has been done in South America, the USA and in Canadian aboriginals.

De Vries: For the known four associations – HLA, the vitamin D receptor, the NRAMP1 and interleukin-1 – there are several ways to get an idea to what extent they explain the genetic variability. Has that been studied?

McAdam: My understanding is that the heritability has been derived from the twin studies. In terms of the individual genes, not one of those comes up on the whole genome scan as a susceptibility area for linkage. So I suspect the contribution is quite small.

Ottenhoff: There was a recent paper by Adrian Hill's group showing that there is a major locus at chromosome 10 estimated to contribute up to about 40% of the total genetic component in leprosy.[1] So that is actually quite a large contribution for a single locus.

De Groot: The paper by Wilkinson *et al*. on West Gujarati Indians clearly showed the gene-environment interaction in an infectious disease.[2] Do you know what happened in the follow-up of this population? Are they prescribing vitamin D for these people, and does it have an effect on the susceptibility to tuberculosis?

McAdam: I am told that the authors are not using vitamin D therapy or suggesting any intervention. I would actually like to know whether anyone else is considering vitamin D therapy and how this would be tested. Vitamin D has been used historically and in the book by Dubos & Dubos it is recorded that quite a lot of people did worse with vitamin D therapy.[3] They seemed to lyse their lungs. So I'm not sure about this intervention, except for skin tuberculosis – there vitamin D is very effective.

Van der Meer: We would rather use vitamin A and zinc supplementation in view of a placebo-controlled study in Indonesia in which we were involved.[4] After two months the supplement had a distinct effect on both wellbeing and the X-ray signs of the tuberculosis. This effect was lost after six months, probably because there was no resistant tuberculosis among the patients, and all of them responded well to the antituberculous drugs which were given concomitantly.

McAdam: And the people on isoniazide, did they do as well as the people on isoniazide plus zinc and vitamin A?

Van der Meer: The latter group did better. All this has still to be confirmed in a larger independent study.

McAdam: How are we, as a clinical investigating community, going to respond to genes that are going to be accumulative in terms of their effect? If you select patients with a particular vitamin D receptor genotype, you do not actually know – unless you type their genes – what other susceptibility loci they have. Ottenhoff's suggestion of using the array technology to develop a haplotype might have to be followed before one can actually look at functional genomics.

El-Omar: Quite a number of populations that are predisposed to tuberculosis also have a high incidence of starvation, malnutrition, malabsorption enteropathies and so forth.

To what extent do you think these environmental factors are modifying the genetic constitution in terms of expression of the disease, either towards susceptibility or towards responding to the infectious agent?

McAdam: That is a very pertinent question, to which I do not know the answer. Common sense says it must make a huge difference, and if you look at animal studies, it does make a dramatic difference. When you look at guinea pigs that are fed a protein-deficient diet and then are challenged with aerosol tuberculosis they die very rapidly, whereas protein-sufficient animals do not. Of course, many of these patients, by the time you see them, have a nutritional problem. With tuberculosis they have lost weight, they have had fever, they have got lots of inflammatory cytokines on board. We are doing a prospective case-contact study at the moment trying to analyse that, but it is not easy to investigate.

Appelmelk: I am confused about how vitamin D works, if it works. You stated that it causes macrophage activation and then bacteria are killed, but at the same time, as you said, it causes a shift from Th1 to Th2, meaning the opposite.

McAdam: It originates from studies done by Graham Rook and the supposition is that vitamin D modulates the cytokine profile, leading to proinflammatory cytokine production.[5]

Netea: I wonder whether we are on the right track if we are not taking into account that susceptibility to mycobacteria may vary depending on the strain. This would hold for *M. tuberculosis* versus atypical mycobacteria but perhaps also within *M. tuberculosis* isolates. I am thinking for instance of the Beijing strains.

REFERENCES

1. Siddiqui MR, Meisner S, Tosh K, et al. A major susceptibility locus for leprosy in India maps to chromosome 10p13. Nat Genet 2001;27:439-441.
2. Wilkinson RJ, Llewelyn M, Toossi Z, et al. Influence of Vitamin D deficiency and Vitamin D receptor polymorphisms on susceptibility to tuberculosis among Gujarati Asians in west London: a case-control study. Lancet 2000;355:618-21.
3. Dubos R, Dubos J. The white plague: Tuberculosis, man and society. Boston: Little, Brown & Co., 1952.
4. Karyadi E, West CE, Schultink W, et al. A double-blind, placebo-controlled study of vitamin A and zinc supplementation in persons with tuberculosis in Indonesia: effects on clinical response and nutritional status. Am J Clin Nutrition 2002;75:720-7.
5. Rook GAW, Taverne J, Leveton C, Steele J. The role of gamma interferon, vitamin D3 metabolites and tumor necrosis factor in the pathogenesis of tuberculosis. Immunology 1987;62:229-34.

Netherlands
The Journal of Medicine

The role of interleukin-1 beta in *Helicobacter pylori*-associated disease

E.M. El-Omar

Aberdeen University, Department of Medicine and Therapeutics, Aberdeen, Scotland

ABSTRACT

Helicobacter pylori **infects half the world's population and is associated with serious human diseases including peptic ulcer disease and gastric neoplasia. The clinical outcome is largely dependent on the severity and distribution of the *H. pylori*-induced gastritis. The reasons for such variable clinical outcomes remain poorly understood. Bacterial virulence factors contribute to the pathogenicity but do not explain the divergent outcomes. There is emerging evidence that host genetic factors play a key role in determining the clinical outcome to *H. pylori* infection. In particular, proinflammatory genotypes of the interleukin-1 beta (IL-1β) gene are associated with increased risk of gastric cancer and its precursors. The effects are most likely mediated through induction of hypochlorhydria and severe corpus gastritis with subsequent development of gastric atrophy. In this article we discuss the role of IL-1β and other host genetic factors in the pathogenesis of *H. pylori*-related disease.**

INTRODUCTION

It has long been established that human susceptibility to infectious agents is at least partly under genetic control. Several observations from twin, adoptee, pedigree, and candidate gene studies point to host genetic factors as key determinants of this susceptibility.[1,2] The recent explosion in genetic knowledge, accelerated by the human genome project, is helping to unravel the molecular pathways that mediate genetic susceptibility to human diseases, including infections and cancer.[3-5] While genetic susceptibility may apply to the risk of acquisition of an infectious agent, it is becoming increasingly recognised that host genes also influence the pathophysiological response to infections, which ultimately determines the clinical outcome.

The role of infectious agents in carcinogenesis has commanded significant scientific interest culminating in five Nobel prizes in the 20th century.[6,7] Infections can cause cancer by a variety of mechanisms including direct transformation of cells, induction of immunosuppression with consequent reduced cancer immunosurveillance, or by causing chronic inflammation. The last-mentioned is becoming increasingly recognised as an essential component of many epithelial cancers by virtue of its combined effects of generating genotoxic by-products and increased cellular proliferation, thus maximising the potential for DNA damage.

In this article, I hope to demonstrate how interactions between an infectious agent, host genetic makeup, and environmental factors could influence the pathogenesis of a cancer. The infectious agent in question is *Helicobacter pylori*, the world's commonest chronic bacterial infection, and the malignancy is gastric cancer, second only to lung cancer in its global incidence and impact. We demonstrate how this gastric infection could be utilised as a paradigm for gene-environment interactions in human disease, one that could help unravel a multitude of other microbially induced malignancies.

HELICOBACTER PYLORI INFECTION

The *Helicobacter* genus consists of at least 24 species found in the GI tracts of animals and humans.[8] One of these species is *Helicobacter pylori (H. pylori)*, a Gram-negative, spiral-shaped, micro-aerophilic, urease-positive bacillus, which is known to chronically infect the stomachs of over half the world's population. The infection is acquired during childhood, most probably via the faecal/oral or gastric/oral routes, and if not treated with antibiotics, will persist throughout life.[9] The organism is 2.5 to 5 micrometers long

with four to six unipolar flagellae. These flagellae are thought to allow it to move through the mucus layer within the stomach and come to reside between this layer and the gastric epithelium. Eighty percent of the bacilli are free; however the remainder are attached to epithelial cells. Once attached, the organism induces ultra-structural changes in the gastric epithelial cells. Although the bacteria mainly reside on the surface mucus gel layer with little invasion of the gastric glands, the host responds with an impressive humoral and cell-mediated immune response. This immune response is largely ineffective, however, as most infections become chronically established with little evidence that spontaneous clearance occurs.

H. pylori can only proliferate on gastric epithelial cell surfaces and in the overlying mucus layer. Thus *H. pylori* has to survive in one of the harshest and least hospitable niches in the human body. Gastric acidity acts as a formidable first line of defence against food-borne pathogens, and the constant outpouring of gastric secretions, coupled with regular peristalsis, ensure that gastric contents, including microbial agents, are constantly flushed away. Despite this, *H. pylori* seems well equipped and adapted for habitation within this harsh environment. Recent studies show that *H. pylori* maintains its periplasmic pH within viable limits through possession of an acid-induced urea channel that regulates intra-bacterial urease activity.[10,11] Essential nourishment for *H. pylori* is drawn from host gastric tissue through the inflammatory exudate it induces.[12]

Since its original description and culture by Marshall and Warren in 1982,[13] *H. pylori* has been causally implicated in a variety of gastric diseases including simple gastritis, gastric and duodenal ulcer disease, and gastric cancer. While the link between *H. pylori* and peptic ulcer disease was established soon after successful culture of the bacterium, the association with gastric cancer lagged almost a decade before credible evidence was presented. The major reason for this delay was inability to demonstrate the presence of active infection in gastric tissue of cancer patients. In order to understand how this bacterium can predispose to such variable clinical outcomes, it is necessary to understand the basic pathophysiological consequences of its presence within the human stomach.

H. PYLORI AND CHRONIC GASTRIC INFLAMMATION

The key pathophysiological event in *H. pylori* infection is the initiation of an inflammatory response.[14] This response is most probably triggered by the bacterium's lipopolysaccharide, urease, and/or cytotoxins and is mediated by cytokines. Cytokines, including the interleukins, are soluble peptide molecules that mediate the interaction between immunocompetent and haematopoietic cells and between the immune and neuroendocrine systems.[15] They are produced by a variety of activated cells and exert their biological effects through binding to specific receptors on target cells. The cytokine repertoire comprises a multitude of pro- and anti-inflammatory mediators whose function is to coordinate an effective immune/inflammatory response against invading pathogens without causing undue damage to the host.

In addition to their pro- or anti-inflammatory properties, some *H. pylori*-induced cytokines have direct effects on gastric epithelial cells that have a profound effect on gastric physiology. For example, the proinflammatory cytokine interleukin-1 beta (IL-1β) is the most potent of known agents that are gastric cytoprotective, antiulcer, antisecretory, and inhibitory of gastric emptying.[16] Wolfe and Nompleggi estimated that on a molar basis, IL-1β is 100 times more potent than both prostaglandins and the proton pump inhibitor omeprazole and 6000 times more potent than cimetidine in inhibiting acid secretion.[17] Another important proinflammatory cytokine that is upregulated by *H. pylori* infection is tumour necrosis factor alpha (TNF-α), which also inhibits gastric acid secretion, but to a lesser extent than IL-1β.

In physiological terms, the stomach could be divided into two main compartments: an acidic proximal corpus that contains the acid-producing parietal cells, and a less acidic distal antrum that does not have parietal cells but contains the endocrine cells that control acid secretion.[18] *H. pylori* infection is first established in parts of the stomach that have a higher pH such as the antrum. This is most likely due to the bacterium's attempt at energy preservation, for although *H. pylori* is well equipped for survival at low pH, this is achieved at a high cost of energy expenditure. Thus, high acid production by the parietal cells probably protects the corpus mucosa from initial colonisation. Both animal and human ingestion studies suggest that successful colonisation of the gastric mucosa is best achieved with the aid of acid suppression.[19-21] Furthermore, pharmacological inhibition of acid secretion in infected subjects leads to redistribution of the infection and its associated gastritis from an antral to a corpus-predominant pattern.[22-24] Thus lack of gastric acid extends the area of colonisation and also maximises the tissue damage resultant from this colonisation.

H. PYLORI INFECTION AND THE DIVERGENT CLINICAL OUTCOMES

H. pylori infection is associated with divergent clinical outcomes that range from simple asymptomatic gastritis

to more serious conditions such as peptic ulcer disease and gastric neoplasia. The extent of this remarkable divergence is made more striking by the observation that certain outcomes of the infection, such as duodenal ulcer disease, are actually protective against others, such as gastric cancer.[25] The key determinants of these outcomes are the severity and distribution of the *H. pylori*-induced gastritis. There are three main gastric phenotypes that result from chronic *H. pylori* infection: (1) the commonest by far is a mild pangastritis that does not affect gastric physiology and is not associated with significant human disease, (2) a corpus-predominant gastritis associated with gastric atrophy, hypochlorhydria and increased risk of gastric cancer,[26] and (3) an antral-predominant gastritis associated with high gastric acid secretion and increased risk of duodenal ulcer disease.[27] The association of *H. pylori* with such variable outcomes poses a most fascinating scientific challenge, the unravelling of which will not only explain how ulcers and gastric cancer develop, but will also act as a paradigm for gene-environment interactions in most human diseases.

H. PYLORI INDUCES VARIABLE GASTRIC PHENOTYPES: THE GASTRIC CANCER VERSUS DUODENAL ULCER PHENOTYPES

There is accumulating evidence that acid secretory capacity is crucial in determining the distribution and natural history of *H. pylori* infection.[18] In hosts with low secretory capacity (genetically determined or secondary to pharmacological inhibition) the organism is capable of colonising a wider niche than would be possible in the presence of high volumes of acid. Colonisation of a wider niche, including the corpus mucosa, leads to corpus gastritis with resultant functional inhibition of acid secretion. This inhibition is mediated by *H. pylori*-induced inflammatory cytokines (such as IL-1β and TNF-α) and the net effect is the establishment of a more aggressive gastritis that accelerates the development of gastric atrophy. Once atrophy develops, acid secretion is not only attenuated by the functional inhibition caused by inflammatory mediators but by a more permanent morphological change that is harder to reverse. This situation is very relevant to the subgroup of humans who develop the gastric cancer phenotype in the presence of chronic *H. pylori* infection.

In contrast to subjects who have an increased risk of gastric cancer, subjects who develop duodenal ulcer disease are known to have a large parietal cell mass that is relatively free of *H. pylori*-induced inflammatory activity. This pattern of antral-predominant gastritis with high acid output characterises the duodenal ulcer diathesis. The high acid output is associated with the development of duodenal gastric metaplasia (DGM), a protective mechanism against the persistent delivery of an increased acid load to the duodenum. The presence of gastric epithelium (DGM) in the duodenum is an invitation for antral *H. pylori* infection to colonise this new niche. The ensuing gastritis with the production of proinflammatory cytokines such as IL-1β and TNF-α greatly weakens the resistance of this mucosa, and in the presence of large volumes of acid, and a reduction in duodenal mucosal bicarbonate production,[28] ulcers develop.

As mentioned above, the effect of acid secretion on changing the distribution of *H. pylori* colonisation and gastritis is most markedly exposed in subjects in whom acid secretion is manipulated by pharmacological means. Thus *H. pylori*-infected subjects on long-term proton pump inhibitors undergo a shift in the pattern of gastritis from antral- to corpus-predominant, and they have a higher risk of developing gastric atrophy, a precursor lesion for gastric neoplasia.[23] This observation provided a clue as to the role of potential endogenous substances that could also inhibit acid secretion, such as IL-1β and TNF-α. As will be discussed later, these two cytokines were prime candidates as host genetic factors that may increase risk of gastric cancer. IL-1β is the archetypal pleiotropic cytokine being produced by many cells and exerting its biological effects on almost all cell types.[29] IL-1β is a very potent proinflammatory cytokine and is involved in the host's response to many antigenic challenges.

GENETIC POLYMORPHISMS IN THE *IL-1* GENE CLUSTER INCREASE THE RISK OF GASTRIC CANCER AND ITS PRECURSORS

The key question in *H. pylori* research is how this infection could be associated with such divergent clinical outcomes as gastric cancer and duodenal ulcer disease. A large volume of research has focused on the role of bacterial virulence factors in the pathogenesis of these diseases, and although these factors undoubtedly contribute to the degree of tissue damage, they do not distinguish between the two key outcomes.[30] This prompted us to concentrate on the host genetic factors that may be relevant to this process. The search for the appropriate candidate genes had to stem from a profound understanding of gastric physiology and how it is disrupted by *H. pylori* infection. Since *H. pylori* achieves most of its damage through induction of chronic inflammation, it was reasonable to consider genes that control this process as appropriate candidates.

The *IL-1* gene cluster on chromosome 2q contains three related genes within a 430 kb region, *IL-1A*, *IL-1B* and *IL-1RN*, which encode for the proinflammatory cytokines

IL-1α and IL-1β as well as their endogenous receptor antagonist IL-1ra, respectively.[31] IL-1β is upregulated in the presence of *H. pylori* and plays a central role in initiating and amplifying the inflammatory response to this infection.[32-34] IL-1β is also an extremely potent inhibitor of gastric acid secretion;[35,36] on a molar basis it is estimated to be 100-fold more potent than proton pump inhibitors and 6000-fold more potent than H_2-antagonists.[37] Three diallelic polymorphisms in *IL-1B* have been reported, all representing C-T transitions, at positions -511, -31, and +3954 bp from the transcriptional start site.[38] There are conflicting data regarding the functional effects of these polymorphisms on IL-1β production.[39,40] The *IL-1RN* gene has a penta-allelic 86 bp tandem repeat (VNTR) in intron 2, of which the less common allele 2 (*IL-1RN*2*) is associated with a wide range of chronic inflammatory and autoimmune conditions.[38] *IL-1RN*2* is associated with enhanced IL-1β production *in vitro*,[40] but data regarding its effects on IL-1ra production are contradictory.[41-43] The gene also has a number of functionally relevant polymorphisms that could be correlated with high or low IL-1β production. This provided an ideal opportunity to design the appropriate hypothesis-driven epidemiological studies.

We first studied the correlation of these high IL-1β genotypes (two polymorphisms in the *IL-1B* and *IL-1RN* genes) with hypochlorhydria and gastric atrophy in a Caucasian population of gastric cancer relatives. These relatives are known to be at increased risk of developing the same cancer and have a higher prevalence of the precancerous abnormalities, but only in the presence of *H. pylori* infection. We found that the high IL-1β genetic markers significantly increase the risk of these precancerous conditions. In a logistic regression model including both genotypes, the estimated age-adjusted odds ratios for *IL-1B-511/-31)*2+* and *IL-1RN*2/*2* were 7.5 (95% CI: 1.8-31) and 2.1% (95% CI: 0.7-6.3 respectively).[44] We proceeded to examine the association between the same IL-1β genetic polymorphisms and gastric cancer itself utilising another Caucasian case-control study comprising 366 gastric cancer patients and 429 population controls. We confirmed the same positive association between these genotypes and gastric cancer. In a logistic regression model including both genotypes, the estimated odds ratios for *IL-1B-511/-31T+* and *IL-1RN*2/*2* were 1.6 (95% CI: 1.2-2.2) and 2.9 (95% CI: 1.9-4.4) respectively.[44]

Although IL-1β was the perfect candidate gene, other genes involved in the *H. pylori*-induced gastritis cascade are also legitimate targets. Our most recent search has confirmed a positive but weaker role for polymorphisms in the *TNF-A* gene that correlate with high TNF-α levels (El-Omar *et al.*, unpublished data). The TNF-α polymorphism increases the risk of gastric cancer and its precursors in a similar fashion to the IL-1β polymorphisms. This proinflammatory cytokine is also upregulated in *H. pylori* infection and has acid-inhibitory properties, albeit weaker than IL-β. So it is clear that the targeted and hypothesis-driven search for these host genetic factors will aid in unravelling the pathogenesis of *H. pylori*-related diseases.

But how do these IL-1β/TNF-α polymorphisms explain the divergent outcome to *H. pylori* infection? We speculate that the effect of these polymorphisms operates early in the disease process and requires the presence of *H. pylori* infection. When *H. pylori* infection challenges the gastric mucosa, a vigorous inflammatory response with a high IL-1β/TNF-α component may appear to be beneficial, but it has the unfortunate side effect of switching acid secretion off, thus allowing the infection to extend its colonisation and damaging inflammation to the corpus mucosa, an area that is usually well protected by secretion of acid. A decreased flow of acid will also undermine attempts to flush out these toxic substances, causing further damage to the mucosa. More inflammation in the corpus leads to more inhibition of acid secretion and a continuing cycle that accelerates glandular loss and onset of gastric atrophy. It is apparent that this vicious cycle ultimately succeeds in driving the infection out, but at a very high price for the host. This is amply demonstrated by the finding that *H. pylori* density becomes progressively lower with the progression from mild gastritis through severe gastritis, atrophy and intestinal metaplasia. Indeed, by the time gastric cancer develops, it is extremely hard to demonstrate any evidence of the infection.[45]

ROLE OF ENVIRONMENTAL FACTORS IN GASTRIC CARCINOGENESIS

A very obvious question at this juncture is why only a few *H. pylori*-infected subjects with these polymorphisms develop gastric cancer. Why isn't everyone with such a genetic makeup at risk of this outcome? The answer lies in the polygenic and multifactorial nature of most complex human diseases. These genetic factors operate only in the presence of an infectious agent and lead to the development of an atrophic phenotype. Progression of atrophy towards cancer depends on other components of the host genetic constitution acting epistatically, as well as dietary and other factors in the environment. While *H. pylori* infection and host genetics interact to initiate a hypochlorhydric and atrophic phenotype, environmental co-factors may mediate subsequent neoplastic transformation, even after disappearance of the infection. Diet may be particularly relevant, with greater consumption of fresh fruits and vegetables shown to protect against the risk of gastric as well as several other cancers. Dietary vitamin C reduces the formation of

N-nitroso-compounds and scavenges mutagenic reactive oxygen metabolites generated by gastric inflammation,[46] and supplemental vitamin C is associated with significantly lower risk of noncardia gastric cancer.[47] Furthermore, vitamin C concentrations and bioavailability are reduced in the presence of *H. pylori* infection.[48,49] Another important co-factor is cigarette smoking, which was found to nearly double the risk of transition from atrophic gastritis to dysplasia in a high-risk population.[50] Thus, cytokine gene polymorphisms represent only one component of a complex interplay among host, pathogen, and environmental factors involved in gastric carcinogenesis.

These proinflammatory polymorphisms, therefore, can distinguish between subjects who will develop the hypochlorhydric atrophic phenotype in response to *H. pylori* infection and those who will manage to limit the infection to a smaller area and offer relatively better protection of their corpus function. Another valid question is whether these proinflammatory polymorphisms actually offer protection against the other extreme clinical outcome, namely, duodenal ulcers. Could it be that a low IL-1β/TNF-α response to *H. pylori* infection, and a consequently lower inhibition of acid secretion, is the determinant of the antral-predominant, corpus-sparing gastritis pattern seen in duodenal ulcer patients? To date, no reports have been published addressing this specific question. It would be tempting to speculate that this would be the case, but my gut feeling is that the large parietal cell mass frequently seen in duodenal ulcer patients is determined by other genetic factors, hitherto undescribed, that relate to parietal cell development and the endocrine receptors they express. This genetically determined capacity to secrete large volumes of gastric acid will probably neutralise any subtle contribution of a genetic polymorphism in a cytokine gene.

CONCLUSIONS AND A LOOK TO THE FUTURE

IL-1β is a very important proinflammatory cytokine with profound effects on gastric physiology. Its acid-inhibitory properties uniquely qualify it as a major player in the host's response to *H. pylori* infection and the diseases associated with it. Polymorphisms in the gene for IL-1β that correlate with higher levels of this cytokine have been found to increase the risks of hypochlorhydria and gastric atrophy in response to *H. pylori* infection and to increase the risk of gastric cancer itself. These host genetic factors that affect IL-1β may determine why some individuals infected with *H. pylori* develop gastric cancer while others do not. Future research should focus on identifying the molecular pathways that mediate this increased risk. The search for other host genetic factors that contribute to the pathogenesis of the disease should continue, particularly in view of the wonderful new opportunities made possible by the human genome project. A special effort should be directed at understanding these host genetic factors in non-Caucasian populations, and in populations with high and low incidences of gastric cancer. We should also target other upper GI diseases that may be linked indirectly to these genetic polymorphisms that alter gastric physiology. Prime amongst these are oesophageal cancers and gastro-oesophageal reflux disease.

REFERENCES

1. Hill AV. The genomics and genetics of human infectious disease susceptibility. Annu Rev Genomics Hum Genet 2001;2:373-400.
2. Hill AV. The immunogenetics of human infectious diseases. Annu Rev Immunol 1998;16:593-617.
3. Subramanian G, Adams MD, Venter JC, Broder S. Implications of the human genome for understanding human biology and medicine. JAMA 2001;286(18):2296-307.
4. Subramanian G, Mural R, Hoffman SL, Venter JC, Broder S. Microbial disease in humans: A genomic perspective. Mol Diagn 2001;6(4):243-52.
5. Ponder BA. Cancer genetics. Nature 2001;411(6835):336-41.
6. Kuper H, Adami HO, Trichopoulos D. Infections as a major preventable cause of human cancer. J Intern Med 2000;248(3):171-83.
7. Parsonnet J. Introduction. In: Parsonnet J (ed). Microbes and Malignancy. New York: Oxford University Press, 1999:3-15.
8. Fox JG. The non-H pylori helicobacters: their expanding role in gastrointestinal and systemic diseases. Gut 2002;50(2):273-83.
9. Everhart JE. Recent developments in the epidemiology of Helicobacter pylori. Gastroenterol Clin North Am 2000;29(3):559-78.
10. Scott DR, Weeks D, Hong C, et al. The role of internal urease in acid resistance of Helicobacter pylori. Gastroenterology 1998;114(1):58-70.
11. Sachs G, Scott D, Weeks D, Melchers K. Gastric habitation by Helicobacter pylori: insights into acid adaptation. Trends Pharmacol Sci 2000;21(11):413-6.
12. Blaser MJ, Berg DE. Helicobacter pylori genetic diversity and risk of human disease. J Clin Invest 2001;107(7):767-73.
13. Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet 1984;1(8390):1311-5.
14. Israel DA, Peek RM. pathogenesis of Helicobacter pylori-induced gastric inflammation. Aliment Pharmacol Ther 2001;15(9):1271-90.
15. Fridman WH, Tartour E. Cytokines and cell regulation. Mol Aspects Med 1997;18:1-90.
16. Robert A, Olafsson AS, Lancaster C, Zhang WR. Interleukin-1 is cytoprotective, antisecretory, stimulates PGE2 synthesis by the stomach, and retards gastric emptying. Life Sci 1991;48(2):123-34.
17. Wolfe MM, Nompleggi DJ. Cytokine inhibition of gastric acid secretion – a little goes a long way. Gastroenterology 1992;102(6):2177-8.
18. McColl KE, El-Omar E, Gillen D. Helicobacter pylori gastritis and gastric physiology. Gastroenterol Clin North Am 2000;29(3):687-703.
19. Marshall BJ, Armstrong JA, McGechie DB, Glancy RJ. Attempt to fulfil Koch's postulates for pyloric Campylobacter. Med J Aust 1985;142(8):436-9.

20. Morris A, Nicholson G. Ingestion of Campylobacter pyloridis causes gastritis and raised fasting gastric pH. Am J Gastroenterol 1987;82(3):192-9.
21. Danon SJ, O'Rourke JL, Moss ND, Lee A. The importance of local acid production in the distribution of Helicobacter felis in the mouse stomach. Gastroenterology 1995;108(5):1386-95.
22. Kuipers EJ, Uyterlinde AM, Pena AS, et al. Increase of Helicobacter pylori-associated corpus gastritis during acid suppressive therapy: implications for long-term safety. Am J Gastroenterol 1995;90(9):1401-6.
23. Kuipers EJ, Lundell L, Klinkenberg-Knol EC, et al. Atrophic gastritis and Helicobacter pylori infection in patients with reflux esophagitis treated with omeprazole or fundoplication. N Engl J Med 1996;334(16):1018-22.
24. Kuipers EJ, Lee A, Klinkenberg-Knol EC, Meuwissen SG. Review article: the development of atrophic gastritis—Helicobacter pylori and the effects of acid suppressive therapy. Aliment Pharmacol Ther 1995;9(4):331-40.
25. Hansson LE, Nyren O, Hsing AW, et al. The risk of stomach cancer in patients with gastric or duodenal ulcer disease. N Engl J Med 1996;335(4):242-9.
26. El-Omar EM, Oien K, El Nujumi A, et al. Helicobacter pylori infection and chronic gastric acid hyposecretion. Gastroenterology 1997;113(1):15-24.
27. El-Omar EM, Penman ID, Ardill JE, et al. Helicobacter pylori infection and abnormalities of acid secretion in patients with duodenal ulcer disease. Gastroenterology 1995;109(3):681-91.
28. Hogan DL, Rapier RC, Dreilinger A, et al. Duodenal bicarbonate secretion: eradication of Helicobacter pylori and duodenal structure and function in humans. Gastroenterology 1996;110(3):705-16.
29. Dinarello CA. Biologic basis for interleukin-1 in disease. Blood 1996;87(6):2095-147.
30. Graham DY, Yamaoka Y. Disease-specific Helicobacter pylori virulence factors: the unfulfilled promise. Helicobacter 2000;5(suppl 1):S3-9.
31. Galbraith GM, Palesch Y, Gore EA, Pandey JP. Contribution of interleukin 1beta and KM loci to alopecia areata. Hum Hered 1999;49(2):85-9.
32. Nemetz A, Nosti-Escanilla MP, Molnar T, et al. IL1B gene polymorphisms influence the course and severity of inflammatory bowel disease. Immunogenetics 1999;49(6):527-31.
33. Wilkinson RJ, Patel P, Llewelyn M, et al. Influence of polymorphism in the genes for the interleukin (IL)-1 receptor antagonist and IL-1beta on tuberculosis. J Exp Med 1999;189(12):1863-74.
34. Sciacca FL, Ferri C, Vandenbroeck K, et al. Relevance of interleukin 1 receptor antagonist intron 2 polymorphism in Italian MS patients. Neurology 1999;52(9):1896-8.
35. Cox A, Camp NJ, Cannings C, et al. Combined sib-TDT and TDT provide evidence for linkage of the interleukin-1 gene cluster to erosive rheumatoid arthritis. Hum Mol Genet 1999;8(9):1707-13.
36. Engebretson SP, Lamster IB, Herrera-Abreu M, et al. The influence of interleukin gene polymorphism on expression of interleukin-1beta and tumor necrosis factor-alpha in periodontal tissue and gingival crevicular fluid. J Periodontol 1999;70(6):567-73.
37. Gonzalez SR, Araoz P, Rodriguez R, et al. [Polymorphism of the IL1RN gene in Spanish patients with ulcerative colitis]. Med Clin (Barc) 1999;112(20):778-9.
38. Cantagrel A, Navaux F, Loubet-Lescoulie P, et al. Interleukin-1beta, interleukin-1 receptor antagonist, interleukin-4, and interleukin-10 gene polymorphisms: relationship to occurrence and severity of rheumatoid arthritis. Arthritis Rheum 1999;42(6):1093-100.
39. Papo M, Quer JC, Gutierrez C, et al. Genetic heterogeneity within ulcerative colitis determined by an interleukin-1 receptor antagonist gene polymorphism and antineutrophil cytoplasmic antibodies [see comments]. Eur J Gastroenterol Hepatol 1999;11(4):413-20.
40. Donn RP, Farhan AJ, Barrett JH, et al. Absence of association between interleukin 1 alpha and oligoarticular juvenile chronic arthritis in UK patients. Rheumatology (Oxford) 1999;38(2):171-5.
41. Francis SE, Camp NJ, Dewberry RM, et al. Interleukin-1 receptor antagonist gene polymorphism and coronary artery disease [see comments]. Circulation 1999;99(7):861-6.
42. Cork MJ, Crane AM, Duff GW. Genetic control of cytokines. Cytokine gene polymorphisms in alopecia areata. Dermatol Clin 1996;14(4):671-8.
43. Perrier S, Coussediere C, Dubost JJ, Albuisson E, Sauvezie B. IL-1 receptor antagonist (IL-1RA) gene polymorphism in Sjogren's syndrome and rheumatoid arthritis. Clin Immunol Immunopathol 1998;87(3):309-13.
44. El-Omar EM, Carrington M, Chow WH, et al. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature 2000;404(6776):398-402.
45. Kuipers EJ. Review article: exploring the link between Helicobacter pylori and gastric cancer. Aliment Pharmacol Ther 1999;13(suppl 1):3-11.
46. Correa P. Human gastric carcinogenesis: a multistep and multifactorial process – First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. Cancer Res 1992;52(24):6735-40.
47. Mayne ST, Risch HA, Dubrow R, et al. Nutrient intake and risk of subtypes of esophageal and gastric cancer. Cancer Epidemiol Biomarkers Prev 2001;10(10):1055-62.
48. Banerjee S, Hawksby C, Miller S, et al. Effect of Helicobacter pylori and its eradication on gastric juice ascorbic acid. Gut 1994;35(3):317-22.
49. Woodward M, Tunstall-Pedoe H, McColl K. Helicobacter pylori infection reduces systemic availability of dietary vitamin C. Eur J Gastroenterol Hepatol 2001;13(3):233-7.
50. Kneller RW, You WC, Chang YS, et al. Cigarette smoking and other risk factors for progression of precancerous stomach lesions. J Natl Cancer Inst 1992;84(16):1261-6.

Discussion following lecture by E.M. El-Omar

Van der Meer: Being a person who performs research with interleukin-1, I would be interested to hear why you left interleukin-1α out. Conceptually, IL-1α would also be an interesting candidate. I wonder if you did consider that and if you have data on it?

El-Omar: It is a very good point. We did not ignore interleukin-1α. IL-1α does not have any effect whatsoever on this particular model.

Kimman: Your genetic data appear very convincing, but are they supported by physiological data? Do people with a proinflammatory genotype really have higher IL-1β levels in their gastric lesions than people with a less inflammatory genotype?

El-Omar: Polymorphism has to be functional. There are some data showing these proinflammatory genotypes to be associated with higher IL-1β levels, but it is pretty weak. The IL-1 receptor antagonist is probably the strongest. Allele 2 of that VNTR polymorphism is probably the strongest that we have. It is actually very difficult to measure the cytokine content and correlate that with genetic polymorphisms. In the presence of the infection, depending on the severity, it may be quite difficult to correct for the degree of inflammation in that particular locus. We lack tools to address this question appropriately and we are lagging behind in terms of explaining the physiological effects of these polymorphisms, but the epidemiological data are pretty powerful.

Netea: IL-1 receptor antagonist is approved for treatment of rheumatoid arthritis and anti-TNF treatments have already been in use for a couple of years. Is there anything known about whether this pattern of gastric pathology is changing in some of the patients who also have *Helicobacter* infections?

El-Omar: A very interesting question. We have not been studying this. Rheumatologists are probably not going to look for changes in the stomach. It is a very worthy experiment to perform, perhaps in an animal model.

Netea: Would patients with a duodenal ulcer, who receive anti-TNF for rheumatoid arthritis, get worse as a result of the treatment?

El-Omar: Nothing is known about that, but people with rheumatoid arthritis who get ulcers usually get them because of nonsteroidal anti-inflammatory drugs. So you are dealing with two different issues, which makes it difficult.

Van der Meer: The nonsteroidal anti-inflammatory drugs should inhibit prostaglandins and thereby upregulate cytokine production. So you get into complicated counter-regulatory mechanisms.

El-Omar: And the link between the effect of NSAIDs and *H. pylori* is very controversial.

Van Deuren: It is even more complicated, because IL-1 upregulates cortisol. High IL-1 producers may have higher cortisol levels and therefore a higher acid secretion. On the other hand IL-1 would decrease acid secretion. Physiological tests would be decisive, I think.

El-Omar: I do not think experiments have been conducted that address these questions.

Van Deuren: We are often considering whether we should perform a repetitive gastroscopy in patients with atrophic gastritis and if so, how many times – once every three years, once or twice a year? Do you envisage that in the future, genetic make-up will be decisive in the scheme for endoscopic control?

El-Omar: It is of course much simpler to test for HP infection and if you find it, get rid of it. The genetic make-up becomes unimportant. The value of these genetic studies and genetic markers, from my point of view, is in understanding the pathogenesis.

Kusters: Clearly there are markers for severity and for susceptibility. Could it be that your population is biased regarding the susceptibility to infection with *H. pylori* and how would that affect your data?

El-Omar: What I have shown is that these markers influence the severity and the outcome of the infection, not necessarily the susceptibility. We looked at susceptibility originally and found that high IL-10 genotypes will increase the risk of contracting a chronic *H. pylori* infection. This suggests that an anti-inflammatory Th2-driven response allows this infection to persist. We found this in two populations in two studies, but in a third, much larger study I could not reproduce the same data, and that made me reluctant to claim that high IL-10 genotypes increase the risk of acquiring the infection. However, mice that consecutively produce more IL-10 have persistent *Helicobacter* infection, while mice with a vigorous Th1-driven cytokine response clear the infection.

Appelmelk: I can understand how inflammation shuts down acid production, but I am puzzled how a shutdown of acid would act the other way around. In your view, the cause is the shift of the bacteria from antrum to corpus, but what is the evidence? In the mouse colonisation is exclusively localised in the antrum. Yet there is strong atrophy in the corpus. Could you envisage a model where the bacteria are not needed and still an acid-induced increased inflammation occurs in the corpus?

El-Omar: There are only two experiments where people have either ingested or acquired acute *H. pylori* infection. When Marshall and Morris just took a preparation and swallowed it, there was colonisation only when they took acid inhibitors. So it looks as though you have to switch off the acid secretion to allow the bacteria to take hold in the stomach. The other fact is that when *H. pylori* is first acquired, it tends to settle in the less acidic parts of the stomach. That is why you get maximal colonisation in the antrum. Although *Helicobacter* is unique in surviving in an acidic environment, it is at the cost of high energy expenditure. It has to use a lot of its machinery to try and protect itself from the acid exposure. Acid secretion seems to determine how extensive the colonisation in the stomach will be. Now, if interleukin-1β is poured out to fight this infection and at the same time acid secretion is switched off, the infection does extend from the antrum to the corpus leading to a more severe corpus gastritis. But the counts are probably even slightly reduced in both areas. What is happening here is a pouring out of acid from the glands trying to flush out all the toxins and all the mediators. If the acid secretion is switched off, you have basically got a dry part of the stomach where maximal concentrations of these damaging genotoxic substances can exert their effect.

McAdam: In West Africa the babies seem to be getting infected at weaning or before. By the age of one, using noninvasive tests, we have a *Helicobacter* infection rate of approximately 80%. This has been ascribed to all sorts of things, but certainly to malnutrition. Around the age of weaning these children start losing weight. What is the natural history when *Helicobacter* infects people from the age of one for the rest of their lives?

El-Omar: Theoretically, if the infection is acquired at a much earlier age, at the time when the stomach is not fully developed in terms of its acid secretion, the phenotype should be more in keeping with the gastric atrophy and low acid secretion and that should increase the risk of developing gastric cancer. In addition, the Africans tend to have a so-called proinflammatory genetic make-up. Yet we do not see such a dramatic increase in gastric cancer. Whether they do not live long enough to develop it, or whether there are other conditions that are modifying the inflammatory response, has not been addressed. One very interesting experiment came from Fox *et al.*[1] They looked at the role of co-infection with helminths. It showed that in an animal model, if you co-infect with a helminth the system tries to switch the immune response from a Th1 to a Th2, which attenuates your inflammatory response and prevents gastric atrophy. It may be that in these populations, where you have such a high prevalence of *H. pylori*, yet you have very little clinical disease, there are other factors, other bacteria that may be modulating this inflammatory response.

Peña: There is a great difference between duodenal ulcer and gastric ulcer. There are families that either develop gastric ulcers or duodenal ulcers. Did you study this kind of polymorphism?

El-Omar: It is fascinating that in the West duodenal ulcer and gastric cancer so seldom both strike in one patient. Classically, the people who develop gastric cancer are the ones in whom there has been manipulation of acid secretion: they either have had a vagotomy or surgery that basically attenuates their acid production. Some people would argue that inhibition of gastric acid over many years could be the reason why these people basically switch from being protected towards being at risk. Some fascinating data from Japan[2] recently showed that regarding the proinflammatory genotype, this genetic constitution in people who have DU disease at the beginning, at an early age, when they are in their teens or in their early twenties, if you follow them long enough, you will see very few people with this proinflammatory genotype actually having duodenal ulcer disease or a relapse of duodenal ulcer disease. So it may increase the risk at an early age, but it burns out, and the reason it burns out is presumably because of the effects on the corpus mucosa, discontinued inflammation, the onset of atrophy, and eventually they basically become less protected against gastric cancer and more likely to show the hypochlorhydria atrophic phenotype. The question of looking at gastric ulcers *vs* duodenal ulcers has not been addressed properly and there is one study looking at the effect of these polymorphisms on peptic ulcer disease.[3]

REFERENCES

1. Fox JG, Beck P, Dangler CA, el al. Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces helicobacter-induced gastric atrophy. Nat Med 2000;6:536-42.
2. Furuta T, El-Omar EM, Xiao F, Shirai N, Takashima M, Sugimurra H. Effect of genetic polymorphism in Inteleukin-1β on gastritis, gastric juice pH, and recurrence of peptic ulcer disease in Japanese subjects. Gastroenterology 2002;123:92-105.
3. Lanas A, Garcia-Gonzalez MA, Santolaria S, et al. TNF and LTA gene polymorphisms reveal different risk in gastric and duodenal ulcer patients. Genes Immun 2001;2:415-21.

Netherlands
The Journal of Medicine

The role of polymorphisms in chemokines and chemokine receptors in the clinical course of HIV-1 infection

H. Schuitemaker, R. van Rij

Sanguin Research at CLB, Clinical Viro-Immunology, Amsterdam, the Netherlands

ABSTRACT

The outcome of HIV-1 infection is highly variable: not all individuals exposed to HIV-1 will become infected, and among individuals who do become infected the time from infection to clinical AIDS is highly variable. This variability is thought to reflect the complex interactions between virus and host. An important role for host genetic factors in the pathogenesis of HIV-1 infection is increasingly being appreciated. Many novel genetic polymorphisms have been identified and analysed for their role in HIV-1 transmission and disease progression. Here an overview is provided of polymorphisms in chemokines and chemokine receptors that influence HIV-1 disease.

INTRODUCTION

The clinical course of HIV infection

HIV-1 infection is characterised by a gradual and progressive loss of $CD4^+$ T cells, ultimately leading to the acquired immunodeficiency syndrome (AIDS), which is characterised by opportunistic infections, neurological symptoms and malignancies. Among patients, the clinical course of HIV-1 infection is highly variable. Before antiretroviral therapy became available, the asymptomatic phase of infection could take only months (rapid progressors) or last for more than 15 years (long-term asymptomatics, LTA). Between these extremes, the median time from infection to AIDS diagnosis is eight to ten years.[1]

The course of HIV infection may be influenced by both viral and host factors. The host fights infection by generating HIV-specific cytotoxic T cells and antibodies. The error-prone nature of HIV-1 reverse transcriptase results in the generation of a spectrum of HIV-1 mutants in each replication cycle (viral quasi-species). The growth advantage of mutants with altered antigenic structures (escape mutants) allows the virus to escape from the suppressive action of the immune system. The variable nature of HIV-1 also results in the evolution of other biological characteristics, such as replicative capacity, cytopathicity and cellular tropism.

Though the significance of the humoral immune system to protect against AIDS remains controversial, cytotoxic CD8+ T cells have been shown to signifcantly influence HIV-1 disease progression. Depletion of CD8+ T cells in simian immunodeficiency virus (SIV)-infected macaques leads to a rapid increase in viral replication.[2] In humans, the presence of CTL has been associated with reduced viral load and a more benign disease course.[3-5]

The importance of the viral phenotype for HIV-1 pathogenesis is supported by multiple studies: experimental infection of macaques with late-stage SIV variants resulted in a more rapid disease progression than infection with early isolates;[6] extremely slow progression of disease was observed in haemophiliacs who were infected with an attenuated virus that lacked the regulatory viral gene nef[7]; and long-term nonprogressors more often harbour virus variants with slow *in vitro* replication kinetics.[8]

Another aspect of the HIV-1 phenotype is the so-called syncytium-inducing (SI) capacity. SI HIV-1 variants first got their name as they are able to infect and induce fusion of cells into multinucleate cells (syncytia) in T-cell lines, due to their ability to use chemokine receptor CXCR4 as a viral coreceptor (therefore SI HIV variants are also referred to as X4 HIV-1 variants).[9] Nonsyncytium-inducing (NSI)

HIV-1 variants are in general restricted to CCR5 usage (R5 HIV-1 variants). Whereas HIV-1 infection is in general established by a homogenous population of macrophage tropic NSI / R5 HIV-1 variants. SI / X4 HIV-1 variants may evolve from these R5 variants in approximately half of the HIV-1 infected patients. The emergence of these X4 variants in patients is associated with a more rapid loss of $CD4^+$ T cell numbers and a more rapid progression to AIDS.[10,11]

Chemokines, chemokine receptors and HIV-1

Chemokines are small, structurally-related molecules involved in chemotaxis of a large variety of cell types via interaction with G protein coupled 7-transmembrane spanning receptors. Chemokines play a role in a variety of biological processes, such as lymphocyte migration to sites of inflammation, migration through various lymphoid organs during lymphocyte development and in angiogenesis.[12-14] In 1996, chemokine receptors were identified as coreceptors for entry of HIV-1 (for references see *table 1*). Viral entry is a multistep mechanism, in which the envelope protein gp120 in succession binds to the CD4 molecule and a chemokine receptor. This results in a series of conformational changes, which eventually leads to fusion of the viral and cellular membrane. Several members of the chemokine receptor gene family have been identified as HIV-1 coreceptors (*table 1*). Of these, CCR5 and CXCR4 are thought to be the most relevant *in vivo*, whereas the *in vivo* role of the additional chemokine receptors remains to be established. Cellular tropism of HIV-1 variants is primarily determined by coreceptor expression on the cell surface and coreceptor preference of the virus. Thus, CCR5 expressing cells, such as macrophages and memory $CD4^+$ T cells, can be infected by R5 variants, whereas CXCR4 expressing cells, such as memory and naive $CD4^+$ T cells, can be infected by X4 variants.[15,16] Since naive cells are crucial in the process of T-cell renewal, the capacity of X4 HIV-1 variants to infect and eventually kill these cells may explain the more rapid $CD4^+$ T-cell decline associated with the presence of X4 HIV-1 variants. It should be noted that post-entry restrictions on HIV-1 replication may also influence tropism, as shown for macrophages and resting T cells, that may not efficiently support HIV-1 replication despite expression of the appropriate coreceptors.[17,18]

The natural ligands of the HIV-1 coreceptors, MIP1a, MIP1b, RANTES (ligands of CCR5) and SDF-1 (ligand of CXCR4), have been shown to inhibit virus replication *in vitro*[19-23] and enhanced *in vitro* chemokine production by patient peripheral blood mononuclear cells (PBMC) has been associated with slow disease progression.[24,25] Furthermore, expression levels of CCR5 have been shown to influence infectibility *in vitro*.[26,27] Therefore, it can be expected that genetic differences that influence the pattern and level of expression of chemokines and chemokine receptors may have a major impact on the course of HIV-1 disease.

Table 1

Chemokine receptors and structurally related molecules shown to mediate entry of HIV-1 into CD4+ cells

CHEMOKINE RECEPTOR FAMILY[a]	RECEPTOR	LIGAND	NEW NOMENCLATURE FOR LIGAND[b]	REFERENCE[c]
CC	CCR2	MCP1-4	CCL2, CCL8, CCL7, CCL13	105
	CCR3	Eotaxin, Eotaxin-2, RANTES, MIP1a	CCL11, CCL24, CCL5, CCL3	105,106
	CCR5	MIP 1a MIP 1b RANTES	CCL3, CCL4, CCL5	21,105-108
	CCR8	I-309	CCL1	109,110
	CCR9	TECK	CCL25	111
CXC	CXCR4	SDF-1	CXCL12	112
	CXCR6/BONZO/ STRL33[d]		CXCL16	113,114
CX3C	CX3CR1	Fractalkine	CX3CL1	109,115
Orphan	BOB/GPR15	Unknown		113,116
	GPR1	Unknown		116
	APJ	Unknown		111,117
	ChemR23	Unknown		118
Chemoattractant receptor	BLTR	LTB4		119
Virally encoded[e]	US28	Broad spectrum of CC Chemokines, fractalkine		109,120

[a]Chemokines can be divided into four families on the basis of the spacing between two N-terminal cysteines. Chemokine receptors are named according to the family of chemokines they bind. [b]A new nomenclature for chemokines based on chemokine family names, which consists of family name (C, CC, CXC, CX3C), L (for ligand) and the numbering of the respective gene, has recently been proposed.[13,121] [c]References for the papers first to describe the molecule as an HIV-1 coreceptor are cited. [d]Given the recent identification of a ligand and assignment of a systematic name for this molecule, alternative naming is also given. [e]This chemokine receptor is encoded by human cytomegalovirus.

POLYMORPHISMS IN CHEMOKINE RECEPTORS AND HIV-1 DISEASE PROGRESSION

CCR5 D32

Soon after the identification of chemokine receptors as the coreceptors for entry of HIV-1 into human $CD4^+$ T cells, individuals were identified who had frequently been exposed to HIV-1, and yet remained uninfected due to a homozygous genotype for an inactivating deletion of 32 base-pairs in the CCR5 gene.[28-30] This polymorphism in the CCR5 gene (CCR5 D32 leads to a premature frameshift and a nonfunctional protein that is not expressed on the cell surface. As with other polymorphisms, a large racial variation in the prevalence of the CCR5 D32 allele is observed. It is common among Caucasians, whereas it is virtually absent in African-Americans and Africans (*table 2*). The effects of CCR5 D32 on the course of HIV-1 disease have been widely studied, though primarily in cohorts of subtype B-infected homosexual men.[31-34] In these studies, heterozygosity for CCR5 D32 has been associated with a delayed progression to AIDS. In an international meta-analysis of individual patient data from ten well-characterised cohorts of seroconverters, a relative hazard of 0.74 for progression to AIDS was obtained for CCR5 D32 heterozygosity.[35] The mechanism of protection most likely involves a reduction of the number of CCR5 positive cells and hence the number of potential target cells for HIV-1, which may result in reduced virus replication already during primary infection and subsequently a lower viral set-point.[36,37]

In HIV-1-infected intravenous drug users, haemophiliacs and recipients of contaminated blood[28,38,39] no effect of CCR5 D32 on disease progression was observed, whereas a protective effect was observed among HIV-1-infected children.[40,41] It remains to be established whether this is due to study design or whether the effect of CCR5 D32 is indeed dependent on risk group and route of transmission.

CCR5 promoter

The CCR5 5'untranslated region (UTR) consists of three exons and two introns. In this region, 12 single nucleotide polymorphisms (SNPs) have been described,[42-45] which may, in part, explain differences in basal expression levels of CCR5 among individuals homozygous for the wild-type non-deleted CCR5 gene. To standardise the different numbering systems for the CCR5 promoter in literature, a numbering system was recently proposed in which the first nucleotide of the translation start site is designated as position 1, the nucleotide immediately upstream of this position as position -1, and so on.[46] In the paragraph below, we will use this numbering system and show the alternative nomenclature between brackets.

Among SNPs in the promoter region of CCR5, a high degree of linkage disequilibrium exists, allowing the identification of four common haplotypes (P1 to P4) and six rare haplotypes (P5 to P10), consisting of different combinations of 10 SNPs.[45] The P1 haplotype, including T-2135C (alternative nomenclature T627C, T59353C), was associated with a more rapid course of disease. SNP G-2459A (alternative nomenclature G59029A or G303A) was independently described to be associated with a more rapid disease course.[44] This SNP is in complete linkage disequilibrium with the T-2135C[47-49] and is now considered to be part of the P1 haplotype. The association of these SNPs with enhanced disease progression was observed in different risk groups, such as homosexuals, haemophiliacs and perinatally-infected children.[44,45,47-49] It is likely that these SNPs or linked mutations are involved in the regulation of transcription of CCR5, but results from reporter assays have been inconsistent thus far.[44,45]

CCR2 64I

A valine-to-isoleucine transition in the second transmembrane region of CCR2 (CCR2 64I) has been associated with a delayed progression to AIDS.[50] The protective effect of CCR2 64I is similar to the effect of CCR5 D32 (RH of 0.76 in meta-analysis of combined cohorts,[35] results from individual cohorts).[31,32,34] Though the effect of CCR2 64I on disease progression is obvious, the mechanism is still not understood. CCR2 is rarely used as a coreceptor and the mutated CCR2 molecule does not alter *in vitro* infectibility of cells,[51,52] therefore it is unlikely that the polymorphism directly influences infection. The mutation in CCR2 is in strong linkage disequilibrium with a single-nucleotide polymorphism in the promoter of the CCR5 gene, C-1835T (alternative nomenclature C927T or C59653T)[42,43] and may thus indirectly be involved in the regulation of expression of CCR5. However, neither basal expression levels of CCR5 nor transcription levels in primary lymphocytes were reduced in CCR2 64I heterozygotes.[51,52] An effect on CCR5 expression has been suggested by the finding that re-expression of CCR5 after internalisation by N-terminal modified RANTES was less rapid in two out of three CCR2 64I heterozygotes.[53] An alternative explanation for the effect of CCR2 64I was provided by Mellado *et al.*, who showed that CCR2 64I protein was able to form dimers with CXCR4 after sensitisation with the cognate chemokines, whereas the normal CCR2 protein was unable to do so.[54] This capacity may thus reduce the amount of CXCR4 available on the cell surface among CCR2 64I carriers. This, however, does not explain the finding that CCR2 64I already affects the viral load early in infection, when in general only NSI / R5 variants are present.[55]

CX3CR1 249I 280M

Although CX3CR1 is only used by a minority of HIV-1 variants as a coreceptor, an enhanced progression to AIDS was observed among patients homozygous for CX3CR1 variant V249I T280M. These two amino acid substitutions result in a reduced capacity to bind the cognate ligand

Table 2

Common polymorphisms described in chemokines and chemokine receptor genes, reported to significantly influence HIV-1 disease progression in prospective cohort studies, for details see text

GENE	POLYMORPHISM (NAME)	REFERENCES[a]	ALLELIC FREQUENCY[b]	EFFECT ON GENE PRODUCT	EFFECT ON HIV-1 DISEASE PROGRESSION	DISEASE MODIFYING GENOTYPE *VS* REFERENCE GROUP	REFERENCES
CCR5[c]	Deletion of 32 basepair (D32)	28-30	C: 0.08 A: 0.017	Defective protein, no expression on cell surface	Delayed, RH 0.76[d]	D32/+ *vs* +/+	31-34
CCR2	Val to Ile in first trans-membrane region (64I)	50	C: 0.098 A: 0.151 H: 0.172	Amino acid substitution	Delayed, RH 0.76[d]	64I/+ and 64I/64I *vs* +/+	31,32,34
CCR5	SNP in promoter region G-2459A[e,f]	44	C: 0.57 A: 0.43 H: 0.68	Enhanced promoter function[f]	More rapid, RH 1.74	A/A *vs* G/G	47-49
CCR5	10 haplotypes of 10 SNP in promoter region (P1 to P10)[f]	45	C: 0.560 A: 0.431g	Unknown[f]	More rapid, RH 1.53	P1/P1 *vs* all other genotypes	47-49
CX3CR1	Two amino acid substitutions (V249I T280M)	56	C: 0.135	Reduced ligand binding	Conflicting results	280 M/M *vs* T/T	56,57
SDF-1	SNP in 3' untranslated region (3'A)	62	C: 0.211 A: 0.057 H: 0.160	Unknown	Conflicting results, RH 0.99[d]	3A/3A *vs* +/+	42,62-64
RANTES	Two SNP in promoter region (C-28G, G-403A)[h,i]	58	C: 0.148h A: 0.357 H: 0.217	Increased promoter activity[58]	Delayed, RH 0.65[i]	II/I vs I/I[h]	
RANTES	Intron variant (In1.1C)[i,j]	61	n.a.[j]	Reduced promoter activity	More rapid[i]	n.a.[j]	

[a]Reference for the study in which the polymorphism was originally described. [b]Allelic frequencies of the polymorphisms may vary between different races. Here, allelic frequency of Caucasians (C), Hispanics (H) and African Americans (A) are shown, if available. [c]22 additional mutations leading to an amino acid substitution have been described in the coding region of CCR5.[46,122] Of these, only one allele was associated with reduced entry of HIV-1, and three with a limited ability to bind MIP1a.[122] Due to the low prevalence of the mutations, effects on disease progression can not be established in a cohort study. [d]Relative hazard as obtained in meta-analysis of individual cohorts.[35] [e]According to recently proposed nomenclature. [f]G-2459 is now considered a part of the P1 haplotype. As the study population in both studies is partially overlapping, the results obtained should not be considered confirmatory. [g]Allelic frequency of the disease modifying haplotype (P1) is shown. [h]Due to the linkage disequilibrium between these SNPs, three haplotypes were identified (haplotype I: -403G -28C, II: -403A -28C and III: -403A -28G). Prolonged survival was observed among individuals carrying haplotype I and II as compared with homozygotes for haplotype I. Allelic frequency for haplotype I is given. [i]A strong linkage disequilibrium between intron variant In1.1C and SNPs at position -28 -403 of the RANTES promoter is observed. In1.1C almost always occur in conjunction with -403A. Therefore, results from these survival studies should be considered as conflicting. [j]This study is not available in print yet, therefore, further details are missing.

fractalkine.[56] The effect on the course of HIV-1 infection could, however, not be confirmed in three US-based cohort studies.[57]

POLYMORPHISMS IN CHEMOKINES AND HIV-1 INFECTION

RANTES promoter

Beta-chemokines can block HIV-1 infection via CCR5 *in vitro*[19-21] and high production levels of these chemokines have been associated with less rapid disease course.[24,25] *In vitro* RANTES production levels can vary widely among PBMC from different individuals, which in part may be due to differences in the genetic make-up of the RANTES gene. Two SNPs were identified within the RANTES promoter region (C-28G and G-403A)[58-60] and recently a variant in intron 1 (In1.1C) was identified.[61] These variants are in strong linkage disequilibrium: almost all subjects who carry In1.1C also carry -403A, whereas -28G always occurs in combination with -403A / In1.1C. Both promoter SNPs display increased promoter activity,[58,60] whereas In1.1C is associated with a strong downregulation of promoter activity.[61] In a cohort of Caucasian homosexuals the -403A -28C haplotype was associated with a reduced progression of disease,[59] which could not be confirmed in an analysis of five US-based cohorts.[61] In the latter study, In1.1C was associated with more rapid disease progression in both Caucasians and African Americans.

SDF-1 3'A

Initially, a very strong protective effect was reported for homozygosity for a G-to-A mutation in the 3' untranslated region of the SDF-1 gene (SDF-1 3'A),[62] encoding the ligand for CXCR4. This effect could not be confirmed in other studies,[42,63-67] including an international meta-analysis of individual cohorts (RH=0.99).[35]

CHEMOKINE RECEPTOR POLYMORPHISMS AND THE ACQUISITION OF SI/X4 HIV-1 VARIANTS

The development of X4 / SI HIV-1 variants is a hallmark of disease progression, and their appearance has invariably been associated with a more rapid progression to AIDS.[10,11,68,69] It is still not understood why X4 variants develop in some patients and not in others. Several factors have been suggested to influence the development of X4 variants, including structural restrictions[70] and loss of fitness during the adaptive process of gp120,[71] levels of proteins that bind to CXCR4, such as SDF-1[23] and HIV-1 tat protein[72], and immune control.[73] Recently it was shown that host genetic factors may play a role in the appearance of X4 HIV-1 variants.

As compared with the CCR5 WT genotypic group, the acquisition of SI variants was delayed in the group of CCR5 D32 heterozygotes. An unexpected finding was the association of the CCR2 64I allele with an increased conversion rate toward X4 variants.[55,74,75] As this mutation is linked to the promoter mutation in CCR5, enhanced X4 conversion may be due to altered levels or patterns of CCR5 expression.

CHEMOKINE RECEPTOR POLYMORPHISMS AND HIV-1 TRANSMISSION

HIV-1 may be transmitted from mother to child, via sexual contact, needle sharing, or exposure to contaminated blood products. Exposure to HIV-1 does not invariably lead to persistent infection. A multitude of factors influence transmission rates, such as frequency and magnitude of exposure, inoculum size, disease stage, $CD4^+$ T cell numbers and immune response of the patient.[76-79] Early in infection, a homogenous population of mainly macrophage tropic, NSI / R5 virus variants can be found, suggesting a strong selection pressure with regard to virus phenotype in acute infection. Indeed, susceptibility of cells from the exposed individual to R5 HIV-1 variants has been correlated with transmission.[80,81] One of the most prominent determinants for transmission is the viral load in the donor, irrespective of whether it involves homosexual, heterosexual, parenteral or perinatal transmission.[40,81-85]

The role of host genetic factors in viral transmission is typically studied in a case-control setting, in which the prevalence of a genetic marker in a population of HIV-1-infected patients is compared with the prevalence of this marker in an HIV-1-negative control group or, more extremely, with a group of individuals who are known to have been exposed to HIV-1, yet remain uninfected (exposed uninfected). There has been considerable debate about the role of host genetic factors in protection against transmission of HIV-1. Part of these conflicting results may be due to differences in the composition of the study population and selection of the HIV-1-negative control group. Diverging results have indeed been reported upon selection of highly exposed uninfected individuals or nonexposed HIV-1-negative individuals as a control group.[62,86] Furthermore, confounding factors, such as viral load in donor, should preferentially be taken into account in transmission studies.

Considerable efforts have been undertaken to study the role of CCR5 D32 in transmission of HIV-1. Homozygosity for CCR5 D32 has been associated with protection from transmission in all risk groups studied.[39,87-90] This indicates an absolute requirement for CCR5 in the establishment of infection, irrespective of the route of entry. Despite the near complete resistance, the few case reports of infected CCR5 D32 homozygotes[91-95] and the identification of laboratory workers accidentally infected

with T cell line adapted, X4 restricted virus variants,[96] indicate that transmission via X4 variants can occur in selected cases. The role of CCR5 D32 heterozygosity in protection from transmission has been more controversial. Though protective effects of CCR5 D32 heterozygosity have been reported,[30,97] the majority of studies fail to show a protective effect of CCR5 D32 heterozygosity on transmission.[28,37,40,87,88,90,98-100]

Polymorphisms in RANTES (-G403A, -C28G, In1.1C), the ligand for CCR5, were shown to be associated with increased risk of homosexual transmission.[59,61] This fits the finding that $CD4^+$ T cells from exposed uninfected individuals express higher levels of MIP1a, MIP1b and RANTES upon *in vitro* stimulation.[20,101]

Kostrikis *et al.* reported a significant increase of HIV-1 transmission to African-American infants homozygous for a promoter allele CCR5 C-2132T (or CCR5 C59356T, C630T).[99] This mutation was rare in Caucasians and Hispanics, and a potential role for this allele could not be assessed in these children. John *et al.* showed that maternal SDF 3'A heterozygosity was associated with an increased risk in transmission, which was more pronounced when transmission occurred via breastfeeding.[102]

CONCLUDING REMARKS

Our insights into the role of host genetic factors in the course of HIV-1 infection are growing. However, it is important to note that the majority of genetic factors described so far only have a relatively mild influence on the course of disease and can only partly explain differences in disease course among patients. Of note, only a minority of long-term nonprogressing HIV-1-infected individuals carry known protective alleles and, conversely, the presence of a protective allele does not warrant a benign disease course. Furthermore, the majority of frequently exposed but uninfected individuals do not contain CCR5 D32 homozygous or other protective genotypes, and therefore other mechanisms, such as a potent CTL response or reduced infectibility of $CD4^+$ T-lymphocytes, may contribute to the resistance to infection in these individuals.[79,103,104] Without doubt, the identification of novel HIV-1 disease-modifying genetic factors will be ongoing in the coming years, yielding further insights into the complex interplay between virus and host and the relative role of host genetic factors therein. Besides expanding our understanding of the pathogenesis of HIV-1 infection, this will hopefully lead to the identification of critical targets for therapeutic interventions.

REFERENCES

1. Hendriks JC, et al. The treatment-free incubation period of AIDS in a cohort of homosexual men. AIDS 1993;7:231-9.
2. Jin X, et al. Dramatic rise in plasma viremia after CD8+ T cell depletion in simian immunodeficiency virus-infected macaques. J Exp Med 1999;189:991-8.
3. Ogg GS, et al. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. Science 1998;279:2103-6.
4. Koup RA, et al. Temporal associations of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol 1994;68:4650-5.
5. Klein MR, et al. Kinetics of Gag-specific CTL responses during the clinical course of HIV-1 infection: A longitudinal analysis of rapid progressors and long-term asymptomatics. J Exp Med 1995;181:1365-72.
6. Kimata JT, et al. Emerging cytopathic and antigenic simian immunodeficiency virus variants influence AIDS progression. Nat Med 1999;5:535-41.
7. Learmont JC, et al. Immunological and virological status after 14 to 18 years of infection with an attenuated strain of HIV-1. A report from the Sydney Blood Bank Cohort. N Engl J Med 1999;340:1756-7.
8. Blaak H, et al. In vitro replication kinetics of HIV-1 variants in relation to viral load in long-term survivors of HIV-1 infection. J Infect Dis 1998;177:600-10.
9. Berger EA, et al. A new classification for HIV-1. Nature 1998;391:240.
10. Koot M, et al. Prognostic value of human immunodeficiency virus type 1 biological phenotype for rate of CD4+ cell depletion and progression to AIDS. Ann Intern Med 1993;118:681-8.
11. Connor RI, et al. Increased viral burden and cytopathicity correlate temporally with CD4+ T-lymphocyte decline and clinical progression in human immunodeficiency virus type 1 infected individuals. J Virol 1993;67:1772-7.
12. Ward SG, Bacon K, Westwick J. Chemokines and T lymphocytes: More than an attraction. Immunity 1998;9:1-11.
13. Loetscher P, Moser B, Baggiolini M. Chemokines and their receptors in lymphocyte traffick and HIV infection. Adv Immunol 2000,74:127-80.
14. Cyster JG. Chemokines and cell migration in secondary lymphoid organs. Science 1999,286:2098-102.
15. Rij RP van, et al. Differential coreceptor expression allows for independent evolution of non-syncytium-inducing and syncytium-inducing HIV-1. J Clin Invest 2000;160:1039-52.
16. Blaak H, et al. in vivo HIV-1 infection of $CD45RA^+CD4^+$ T cells is established primarily by syncytium-inducing variants and correlates with the rate of CD4+ T cell decline. Proc Natl Acad Sci USA 2000;97:1269-74.
17. Fouchier RAM, et al. HIV-1 macrophage-tropism is determined at multiple steps of the viral replication cycle. J Clin Invest 1994;94:1806-14.
18. Zack JA, et al. Incompletely reverse-transcribed human immunodeficiency virus type 1 genomes in quiescent cells can function as intermediates in the retroviral life cycle. J Virol 1992;66:1717-25.
19. Cocchi F, et al. Identification of RANTES, MIP-1a, and MIP-1b as the major HIV-suppressive factors produced by CD8+ T cells. Science 1995;270:1811-5.
20. Paxton WA, et al. Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposures. Nat Med 1996;2:412-7.
21. Deng HK, et al. Identification of the major co-receptor for primary isolates of HIV-1. Nature 1996;381:661-6.

22. Bleul CC, et al. The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. Nature 1996;382:829-32.
23. Oberlin E, et al. The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T cell-line adapted HIV-1. Nature 1996;382:833-5.
24. Garzino-Demo A, et al. Spontaneous and antigen-induced production of HIV-inhibitory β-chemokines are associated with AIDS-free status. Proc Natl Acad Sci USA 1999;96:11986-91.
25. Cocchi F, et al. Higher macrophage inflammatory protein (MIP)-1a and MIP-1b levels from CD8+ T cells are associated with asymptomatic HIV-1 infection. Proc Natl Acad Sci USA 2000;97:13812-7.
26. Paxton WA, et al. Reduced HIV-1 infectability of CD4+ lymphocytes from exposed-uninfected individuals: Association with low expression of CCR5 and high production of β-chemokines. Virology 1998;244:66-73.
27. Wu L, et al. CCR5 levels and expression pattern correlate with infectability by macrophage-tropic HIV-1, in vitro. J Exp Med 1997;185:1681-91.
28. Dean M, et al. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Science 1996;273:1856-62.
29. Liu R, et al. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell 1996;86:1-20.
30. Samson M, et al. Resistance to HIV-1 infection in Caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nat 1996;382:722-5.
31. Rij RP van, Schuitemaker H. The role of HIV-1 biological phenotype and patient genotype in anti-retroviral treatment decisions. Curr Opin Anti-infect Invest Drugs 1999;1:155-65.
32. Hoffman TL, Doms RW. Chemokines and coreceptors in HIV/SIV-host interactions. AIDS 1998;12:S17-26.
33. Paxton WA, Kang S, Koup RA. The HIV type 1 coreceptor CCR5 and its role in viral transmission and disease progression. AIDS Res Hum Retroviruses 1998;14:S89-91.
34. Ioannidis JPA, et al. Genetic effects on HIV disease progression. Nature Med 1998;4:536.
35. Ioannidis JPA, et al. Effects of CCR5 D32, CCR2 64I and SDF-1 3'A polymorphisms on HIV disease progression: an international meta-analysis of individual-patient data. Ann Intern Med 2001;135:782-95.
36. Meyer L, et al. Early protective effect of CCR5 D32 heterozygosity on HIV-1 disease progression: relationship with viral load. AIDS 1997;11:F73-8.
37. Huang Y, et al. The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. Nat Med 1996;2:1240-13.
38. Schinkel J, et al. No evidence for an effect of the CCR5 D32/+ and the CCR2b 64I/+ mutations on Human Immunodeficiency Virus type 1 (HIV-1) disease progression disease progression among HIV-1 infected injecting drug users. J Infect Dis 1999;179:825-31.
39. Wilkinson DA, et al. A 32-bp deletion within the CCR5 locus protects against transmission of parenterally acquired Human Immunodeficiency Virus but does not affect progression to AIDS defining illness. J Infect Dis 1998;178:1163-6.
40. Misrahi M, et al. CCR5 chemokine receptor variant in HIV-1 mother-to-child transmission and disease progression in children. JAMA 1998;279:277-80.
41. Buseyne F, et al. Impact of heterozygosity for the chemokine receptor CCR5 32-bp deleted allele on plasma virus load and CD4 T lymphocytes in perinatally Human Immunodeficiency Virus-infected children at 8 years of age. J Infect Dis 1998;178:1019-23.
42. Mummidi S, et al. Genealogy of the CCR5 locus and chemokine system gene variants associated with altered rates of HIV-1 disease progression. Nat Med 1998;4:786-93.
43. Kostrikis LG, et al. A chemokine receptor CCR2 allele delays HIV-1 disease progression and is associated with a CCR5 promotor mutation. Nat Med 1998;4:350-3.
44. McDermott DH, et al. CCR5 promotor polymorphism and HIV-1 disease progression. Lancet 1998;352:866-70.
45. Martin MP, et al. Genetic acceleration of AIDS progression by a promotor variant of CCR5. Science 1998;282:1907-11.
46. Carrington M, et al. Genetics of HIV-1 infection: chemokine receptor CCR5 polymorphism and its consequences. Hum Mol Gen 1999;8:1939-45.
47. Clegg AO, et al. CCR5 promotor polymorphisms, CCR5 59029A and CCR5 59353C, are under represented in HIV-1-infected long-term non-progressors. AIDS 2000;14:103-8.
48. An P, et al. Influence of CCR5 promotor haplotypes on AIDS progression in African-Americans. AIDS 2000;14:2117-22.
49. Ometto L, et al. Polymorphisms in the CCR5 promotor region influence disease progression in perinatally Human Immunodeficiency Virus type 1-infected children. J Infect Dis 2001;183:814-8.
50. Smith MW, et al. Contrasting genetic influence of CCR2 and CCR5 variants on HIV-1 infection and disease progression. Science 1997;277:959-65.
51. Mariani R, et al. CCR2-64I polymorphism is not associated with altered CCR5 expression or coreceptor function. J Virol 1999;73:2450-9.
52. Lee B, et al. Influence of the CCR2-V64I polymorphism on human immunodeficiency virus type 1 coreceptor activity and on chemokine receptor function of CCR2b, CCR3, CCR5 and CXCR4. J Virol 1998;72:7450-8.
53. Sabbe R, et al. Donor- and ligand-dependent differences in C-C chemokine receptor 5 reexpression. J Virol 2001;75:661-71.
54. Mellado M, et al. Chemokine control of HIV-1 infection. Nature 1999;400:723-4.
55. Rij RP van, et al. Role of CCR2 genotype in the clinical course of syncytium-inducing (SI) or non-SI human immunodeficiency virus type 1 infection and in the time to conversion to SI virus variants. J Infect Dis 1998;178:1806-11.
56. Faure S, et al. Rapid progression to AIDS in HIV+ individuals with a structural variant of the chemokine receptor CX3CR1. Science 2000;287:2274-7.
57. McDermott DH, et al. Genetic Polymorphism in CX3CR1 and risk of HIV disease. Science 2000;290:2031.
58. Liu H, et al. Polymorphism in RANTES chemokine promotor affects HIV-1 disease progression. Proc Natl Acad Sci USA 1999;96:4581-5.
59. McDermott DH, et al. Chemokine RANTES promotor polymorphism affects risk of both HIV infection and disease progression in the Multicenter AIDS cohort study. AIDS 2000;14:2671-8.
60. Nickel RG, et al. Atopic dermatitis is associated with a functional mutation in the promotor of the C-C chemokine RANTES. J Immunol 2000;164:1612-6.
61. Winkler C, et al. Interacting RANTES variants modulate HIV infection and progression. Keystone Symposia, HIV Pathogenesis 2001.
62. Winkler C, et al. Genetic restriction of AIDS pathogenesis by an SDF-1 chemokine gene variant. Science 1998;279:389-93.
63. Rij RP van, et al. The role of a stromal cell-derived factor-1 chemokine gene variant in the clinical course of HIV-1 infection. AIDS 1998;12:F85-90.

64. Magierowska M, et al. Combined genotypes of CCR5, CCR2, SDF1 and HLA genes can predict the long-term nonprogressor status in Human Immunodeficiency Virus-1 infected individuals. Blood 1999;93:936-41.
65. Easterbrook PJ, et al. Chemokine receptor polymorphisms and Human Immunodeficiency Virus disease progression. J Infect Dis 1999;180:1096-105.
66. Balotta C, et al. Identification of two distinct subsets of long-term non-progressors with divergent viral activity by Stromal-Derived Factor 1 chemokine gene polymorphism analysis. J Infect Dis 1999;180:285-9.
67. Brambilla A, et al. Shorter survival of SDF1-3'A/3'A homozygotes linked to CD4+ T cell decrease in advanced Human Immunodeficiency Virus Type 1 infection. J Infect Dis 2000;182:311-5.
68. Koot M, et al. HIV-1 biological phenotype in long term infected individuals, evaluated with an MT-2 cocultivation assay. AIDS 1992;6:49-54.
69. Richman DD, Bozzette SA. The impact of the syncytium-inducing phenotype of human immunodeficiency virus on disease progression. J Infect Dis 1994;169:968-74.
70. Fouchier RAM, Schuitemaker H. Molecular determinants of human immunodeficiency virus type 1 phenotype variability. Eur J Clin Invest 1996;26:175-85.
71. Kuiken CL, et al. Evolution of the V3 envelope domain in proviral sequences and isolates of human immunodeficiency virus type 1 during transition of the viral biological phenotype. J Virol 1992;66:4622-7.
72. Xiao H, et al. Selective CXCR4 antagonism by Tat: Implications for in vivo expansion of coreceptor usage by HIV-1. Proc Natl Acad Sci USA 2000;97:11466-71.
73. Koot M, et al. Conversion rate towards a syncytium inducing (SI) phenotype during different stages of HIV-1 infection and prognostic value of SI phenotype for survival after AIDS diagnosis. J Infect Dis 1999;179:254-8.
74. Nakayama EE, et al. Polymorphism in the Interleukin-4 promotor affects acquisition of Human Immunodeficiency Virus Type 1 Syncytium-Inducing Phenotype. J Virol 2000;74:5452-9.
75. Vicenzi E, et al. CCR2-64I polymorphism, Syncytium-Inducing Human Immunodeficiency Virus strains and disease progression. J Infect Dis 2000;182:1578-9.
76. Caceres CF, Griensven GJP van. Male homosexual transmission of HIV-1. AIDS 1994;8:1051-61.
77. Ahmad N. Maternal-fetal transmission of human immunodeficiency virus. J Biomed Sci 1996;3:238-50.
78. Busch MP, et al. Factors influencing human immunodeficiency virus type 1 transmission by blood transfusion. J Infect Dis 1996;174:26-33.
79. Wout AB van 't, Koup RA, Paxton WA. HIV-1 Transmission. Schuitemaker H, Miedema, F. AIDS pathogenesis. In: Bird G (ed). Immunology and Medicine. Dordrecht: Kluwer Academic Publishers, 2000, 1', 1-17.
80. Ometto L, et al. Viral phenotype and host T cell susceptibility to HIV-1 infection as risk factors for mother-to-child HIV-1 transmission. AIDS 1995;9:427-34.
81. Blaak H, et al. Infectious cellular load in HIV-1-infected individuals and susceptibility of PBMC from their exposed partners to NSI HIV-1 as major determinants for HIV-1 transmission in homosexual couples. J Virol 1998;72:218-24.
82. Fang G, et al. Maternal plasma human immunodeficiency virus type 1 RNA level: a determinant and projected threshold for mother-to-child transmission. Proc Natl Acad Sci USA 1995;92:12100-4.
83. Khouri YF, et al. Vertical transmission of HIV-1. J Clin Invest 1995;95:732-7.
84. Dickover RE, et al. Identification of levels of maternal HIV-1 RNA associated with risk of perinatal transmission. JAMA 1996;275:599-605.
85. Gray RH, et al. Probability of HIV-1 transmission per coital act in monogamous, heterosexual, HIV-1 discordant couples in Rakai, Uganda. Lancet 2001;357:1149-53.
86. Shin HD, et al. Genetic restriction of HIV-1 pathogenesis to AIDS by promotor alleles of Il10. Proc Natl Acad Sci USA 2000;97:14467-72.
87. Aarons E, et al. CC Chemokine receptor 5 genotypes and *in vitro* susceptibility to AIDS of a cohort of British HIV-exposed uninfected homosexual men. AIDS 1997;11:688-9.
88. Zamarchi R, et al. Frequency of mutated CCR-5 allele (D32) among Italian healthy donors and individuals at risk of parenteral HIV infection. AIDS Res Hum Retroviruses 1999;15:337-44.
89. Kupfer B, et al. Protection against parenteral HIV-1 infection by homozygous deletion in the C-C chemokine receptor 5 gene. AIDS 1999;13:1025-8.
90. Salkowitz JR, et al. Characterization of high-risk HIV-1 seronegative hemophiliacs. Clinical Immunology 2001;98:200-11.
91. Theodorou I, et al. HIV-1 infection in an individual homozygous for CCR5 D32. Lancet 1997;346:1219-20.
92. O'Brien TR, et al. HIV-1 infection in a man homozygous for CCR5 D32. Lancet 1997;349:1219.
93. Balotta C, et al. Homozygous D32 deletion of the CCR5 chemokine receptor gene in an HIV-1 infected patient. AIDS 1997;11:F67-71.
94. Biti R, et al. HIV-1 infection in an individual homozygous for the CCR5 deletion allele. Nat Med 1997;3:252-3.
95. Michael NL, et al. Exclusive and persistent use of the entry coreceptor CXCR4 by human immunodeficiency virus type 1 from a subject homozygous for CCR5 D32. J Virol 1998;72:6040-7.
96. Sipsas NV, et al. Identification of type-specific cytotoxic T lymphocytes responses to homologous viral proteins in laboratory workers accidentally infected with HIV-1. J Clin Invest 1997;99:752-62.
97. Hoffman TL, et al. CCR5 genotypes in sexually active couples discordant for human immunodeficiency virus type 1 infection status. J Infect Dis 1997;176:1093-6.
98. Williams SB, et al. CCR5 genotype and Human Immunodeficiency Virus (HIV)-specific mucosal antibody in seronegative women at high risk for HIV infection. J Infect Dis 1999;179:1310-1.
99. Kostrikis LG, et al. A polymorphism in the regulatory region of the CC-chemokine receptor 5 gene influences perinatal transmission of Human Immunodeficiency Virus type 1 to African-American infants. J Virol 1999;73:10264-71.
100. Lockett SF, et al. Effect of chemokine receptor mutations on heterosexual Human Immunodeficiency Virus transmission. J Infect Dis 1999;180:614-20.
101. Zagury D, et al. C-C chemokines, pivotal in protection against HIV type 1 infection. Proc Natl Acad Sci USA 1998;95:3857-61.
102. John GC, et al. Maternal SDF1 3'A polymorphism is associated with increased perinatal Human Immunodeficiency Virus Type 1 transmission. J Virol 2000;74:5736-9.
103. Paxton WA, Koup RA. Mechanisms of resistance to HIV infection. Springer Semin Immunopathol 1997;18:323-40.
104. Kaul R, et al. HIV-1-specific mucosal CD8+ lymphocyte responses in the cervix of HIV-1-resistant prostitutes in Nairobi. J Immunol 2000;164(3):1602 -11.

105. Doranz BJ, et al. A dual-tropic primary HIV-1 isolate that uses fusin and the β-chemokine receptors CKR-5, CKR-3 and CKR-2b as fusion cofactors. Cell 1996;85:1149-58.
106. Choe H, et al. The β-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell 1996;85:1135-48.
107. Dragic T, et al. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature 1996;381:667-73.
108. Alkhatib G, et al. CC CKR5: A RANTES, MIP-1a, MIP-1b receptor as a fusion cofactor for macrophage-tropic HIV-1. Science 1996;272:1955-8.
109. Rucker J, et al. Utilization of chemokine receptors, orphan receptors, and herpesvirus-encoded receptors by diverse human and simian immunodeficiency viruses. J Virol 1997;71:8999-9007.
110. Horuk R, et al. The CC chemokine I-309 inhibits CCR8-dependent infection by diverse HIV-1 strains. J Biol Chem 1998;273:386-91.
111. Choe H, et al. The orphan seven-transmembrane receptor Apj supports the entry of primary T cell-line-tropic and dual-tropic human immunodeficiency virus type 1. J Virol 1998;72:6113-8.
112. Feng Y, et al. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science 1996;272:872-7.
113. Deng HK, et al. Expression cloning of new receptors used by simian and human immunodeficiency viruses. Nature 1997;388:296-300.
114. Liao F, et al. STRL33, a novel chemokine receptor-like protein, functions as fusion cofactor for both macrophage-tropic and T cell line-tropic HIV-1. J Exp Med 1997;185:2015-23.
115. Combadiere C, et al. Identification of CX3CR1, a chemotactic receptor for the human CX3C chemokine fractalkine and a fusion coreceptor for HIV-1. J Biol Chem 1998;273:23799-804.
116. Farzan M, et al. Two orphan seven-transmembrane segment receptors which are expressed in CD4-positive cells support simian immunodeficiency virus infection. J Exp Med 1997;186:405-11.
117. Edinger AL, et al. An orphan seven-transmembrane domain receptor expressed widely in the brain functions as a coreceptor for human immunodeficiency virus type 1 and simian immunodeficiency virus. J Virol 1998;72:7934-40.
118. Samson M, et al. ChemR23, a putative chemoattractant receptor, is expressed in monocyte-derived dendritic cells and macrophages and is a coreceptor for SIV and some primary HIV-1 strains. Eur J Immunol 2000;28:1689-700.
119. Owman C, et al. The leucotriene B4 receptor functions as a novel type of coreceptor mediating entry of primary HIV-1 isolates into CD4-positive cells. Proc Natl Acad Sci USA 1998;95:9530-4.
120. Pleskoff O, et al. Identification of a chemokine receptor encoded by human cytomegalovirus as a cofactor for HIV-1 entry. Science 1997;276:1874-8.
121. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. Immunity 2000;12:121-7.
122. Blanpain C, et al. Multiple nonfunctional alleles of CCR5 are frequent in various human populations. Blood 2001;96:1638-45.

Discussion following lecture by H. Schuitemaker

Hoepelman: The first studies with anti-CCR5 antibodies were performed in heavily pretreated AIDS patients. Surprisingly, a rapid decline in the viral load is seen in these patients. That decline persists at least 16 weeks. I would have expected there would have been a rapid shift of the virus, because many patients probably harbour R4 virus. Do you think this is due to short follow-up or is there another explanation?

Schuitemaker: When CCR5 is not properly functioning in the CCR5 D32 heterozygotes, we see a protective effect, even in individuals who have X4 viruses, so I guess you achieve benefits by blocking only the R5-using population. In really late-stage patients with very low CD4 counts, we sometimes see a natural infection which the X4 viruses do not survive, because at that time, X4 viruses mainly use naive T cells as their targets, whereas R5 viruses use memory cells and this leads to a depletion of naive T cells. So it might be by this shift in the predator-prey relation that at that time there are no longer any T cells that could serve as target cells.

Hoepelman: My second question concerns the prostitutes in Kenya who do not get infected. I thought this was due to homozygosity for the deletion, but you stated you do not see that in Africa. So what is the explanation?

Schuitemaker: These women are highly protected by cytotoxic T lymphocytes and it is assumed that when these women keep working, they are protected. There is continuous antigenic stimulation of their immune system. However, when they take a holiday, there is an increase in infection. It is completely opposite to what was expected.

De Marie: Persistent viraemia with a GB virus type C has been recently recognised as a protective factor, even in HIV patients treated with antiretroviral therapy. Did you study the presence of these viruses in relation to the CR5 gene?

Schuitemaker: We are going to do that. It is very difficult to set up a proper study, because the GBVC viraemia is normally transient, and only viraemia is associated with a protective effect.

De Groot: The disease course in perinatal infection is much more rapid with incubation time of about a year. Is there information on the expression of CCR5 on neonatal T cells?

Schuitemaker: I am not aware of those data. HIV infection being a viral disease, the loss of cells was thought to be a result of virus-mediated killing. The more recent idea is that it is chronic immune stimulation that consumes the T cells, rather than virus-mediated killing. All cells go through the process of becoming infected and dying a natural death from apoptosis. And since neonates respond more strongly to all kinds of infection, you can imagine that HIV infection may have a more dramatic course.

Verbrugh: Since AIDS is a relatively new disease, the R5 polymorphism was presumably there before the virus hit mankind and also the difference across the globe probably was there as far as the dissolution of this polymorphism is concerned. What would the role of this receptor have been in other types of disease?

Schuitemaker: When people ask me this question, I always say that CCR5 protects you from the negative effects of eating polar bears. I say this because in the northern regions like Scandinavia there is up to 40% of that genotype. SCCR5 is an activation marker and allows T cells to respond to inflammation and lead to immunopathology. For some infections it is not good to overreact. It is pure speculation, but having this polymorphism could be advantageous. It is of interest that individuals that are CCR5 D32 homozygous have no record of immune deficiency.

Kimman: Is CCR5 used as a receptor by other pathogens? I am thinking of endogenous retroviruses. Our genome harbours many retroviruses. Have they used CCR5?

Schuitemaker: Not that I am aware of.

Van Strijp: The finding of a coreceptor may turn the main receptor also into a coreceptor. Are there experiments with overexpressing one of these receptors, CD4 or CCR5, to see whether one of the receptors can do it on its own? Which is the most important one?

Schuitemaker: It is possible to change the chemokine receptor, but you need CD4 in addition. Therefore, CD4 is still assumed to be the main receptor. So in artificial systems you can use CCR5, but also CCR2 and CCR4 next to CD4, and it works. However, HIV-2 virus can become CD4-independent after passage, and also SIV, the related virus in macaques, is very frequently CD4-independent. It could very well be that the HIV was first using only chemokine receptors and, after introduction into humans, the virus adapted to using CD4 because of the proximity of CCR5 and CD4. High expression of the coreceptor does not change the susceptibility of the cell and that CD4 is always the limiting factor. That is also why macrophages are not susceptible to SI / X4 viruses, despite the presence of CXCR4 and CD4 on macrophages. When you upregulate CD4, these cells become susceptible to X4 viruses. So it seems that CD4 is the main receptor.

Kuijper: To give it a different perspective, the CCR5 D32 deletion seems also to be a susceptibility marker of asthma.[1] I also have a question: Is there a difference in proliferative potential between NSI and SI viruses? How easily do they disseminate?

Schuitemaker: Among individuals dissemination of NSI viruses is more efficient than of SI viruses. The viral load however is not very different between NSI and SI carriers, being slightly higher in SI individuals. The burst size of the viruses, however, is different and SI viruses may be able to kill cells earlier, thereby not allowing as much reproduction as NSI viruses do. This more or less compensates for the fact that SI viruses have more target cells, due to the availability of more CXCR4 CD4 positive cells than CD4 CCR5 positive cells.

Kuijpers: So you take away the disseminating potential of the NSI part? And this could perhaps explain the anti-CCR5 monoclonal effect?

Appelmelk: Where is the DC sign in your scheme with the coreceptors?

Schuitemaker: DC sign is only expressed on dendritic cells. It is still a matter of debate however whether DCs themselves really become infected via DC sign or whether this molecule only facilitates the infection of T lymphocytes. I know that people have found polymorphisms in DC sign, but considering the fact that I heard about this two years ago and that nothing has been published on a correlation between these polymorphisms and the clinical course, I assume that there are no correlations.

Appelmelk: What happens to the virus when it makes these new variants at a molecular level? Does it change its outside, maybe in the sugar chains? Because, as you know, GP120 uses a ligand to attach to the DC sign.

Schuitemaker: Looking at the envelope, the variable domain 3 (the loop that sticks out and is important for the interaction with the coreceptor) becomes more positively charged in SI viruses. There are two specific amino acid residues in the SI viruses at position 11 and 28. One or both are positively charged in SI viruses.

McAdam: You mentioned the differing use of receptors for HIV-2. Could that account for the slowness of its natural history and the low viral load?

Schuitemaker: HIV-2 is a very different story. Even when you provide an optimal situation, the virus still hardly grows, in contrast to HIV-1. I think something in the genome attenuates the virus and therefore it takes so long before the HIV-2 leads to disease. I think that it is not just due to coreceptor use.

REFERENCE

1. Sandford et al. J Allergy Clin Immunol 2001;108:69.

List of participants

M.A. van Agtmael
VU University Medical Centre
Department of Internal Medicine
De Boelelaan 1117
1081 HV Amsterdam
The Netherlands

B.J. Appelmelk
VU University Medical Centre
Department of Medical Microbiology and Infection
Van der Boechorststraat 7
1081 BT Amsterdam
The Netherlands

A. van Belkum
Erasmus Medical Centre
Department of Medical Microbiology and Infectious Diseases
Dr. Molewaterplein 40
3015 GD Rotterdam
The Netherlands

J.E. Degener
University Hospital/Faculty of Medicine Groningen
Department of Medical Microbiology
PO Box 30.001
9700 RB Groningen
The Netherlands

M. van Deuren
University Medical Centre St Radboud
Department of General Internal Medicine
PO Box 9101
6500 HB Nijmegen
The Netherlands

E.M. El-Omar
Aberdeen University
Department of Medicine and Therapeutics
Polwarth Building, Foresterhill
Aberdeen AB25 2ZD
Scotland
United Kingdom
E-mail: e.el-omar@abdn.ac.uk

J.D.A. van Embden
National Institute of Public Health and the Environment (RIVM)
PO Box 1
3720 BA Bilthoven
The Netherlands

M.J. Fenton
Boston University School of Medicine
The Pulmonary Center
80 East Concord St.
Boston, MA 02118-2394
USA

R. van Furth
Emeritus Professor Infectious Diseases
Laan van Oud Poelgeest 44
2341 NL Oegstgeest
The Netherlands

R. de Groot
Erasmus Medical Centre
Department of Paediatrics
Dr. Molewaterplein 60
3015 GJ Rotterdam
The Netherlands

P.W.M. Hermans
Erasmus University Rotterdam
Department of Paediatrics
Laboratory of Paediatrics, Room EE 15-00
PO Box 1738
3000 DR Rotterdam
The Netherlands

I.M. Hoepelman
University Medical Centre Utrecht
Department of Infectious Diseases and AIDS
PO Box 85500
3508 GA Utrecht
The Netherlands

B.M. de Jongh
St. Antonius Hospital
Department of Medical Microbiology and Immunology
Koekoekslaan 1
3435 CM Nieuwegein
The Netherlands

T.G. Kimman
National Institute of Public Health and the Environment (RIVM)
Department of Infectious Diseases and Epidemiology
PO Box 1
3720 BA Bilthoven
The Netherlands

T.W. Kuijpers
Emma Children's Hospital
AMC G8-205
Meibergdreef 9
1066 CX Amsterdam
The Netherlands
E-mail: t.w.kuijpers@amc.uva.nl

B.J. Kullberg
University Hospital Nijmegen
Department of Medicine
Division of General Internal Medicine
PO Box 9101
6500 HB Nijmegen
The Netherlands

J.G. Kusters
Erasmus Medical Centre
Department of Gastroenterology and Hepatology, L448
Dr. Molewaterplein 40
3015 GD Rotterdam
The Netherlands

S. de Marie
Erasmus Medical Centre
Department of Medical Microbiology and Infectious Diseases
Dr. Molewaterplein 40
3015 GD Rotterdam
The Netherlands

K.P.W.J. McAdam
Medical Research Council Laboratories
PO Box 273
Banjul
The Gambia

J.W.M. van der Meer
University Medical Centre St Radboud
Department of General Internal Medicine
Geert Grooteplein Zuid 8
6500 HB Nijmegen
The Netherlands

M.G. Netea
University Medical Centre St Radboud
Department of Medicine
Geert Grooteplein 8
6500 HB Nijmegen
The Netherlands

T.H.M. Ottenhoff
Leiden University Medical Centre
Department of Immunohematology and Blood Transfusion
PO Box 9600
2300 RC Leiden
The Netherlands

A.S. Peña
Laboratory of Immunogenetics
Department of Gastroenterology
PO Box 7057
1007 MB Amsterdam
The Netherlands

H. Schuitemaker
Sanguin Research at CLB
Clinical Viro-Immunology
Plesmanlaan 125
1066 CX Amsterdam
The Netherlands
E-mail: J-Schuitemaker@CLB.nl

P. Speelman
Academic Medical Centre
University of Amsterdam
Department of Infectious Diseases, F4-217
Meibergdreef 9
1105 AZ Amsterdam
The Netherlands

J.A.G. van Strijp
University Medical Centre Utrecht
Eijkman-Winkler Institute
G04-614
PO Box 85500
3508 GA Utrecht
The Netherlands

M.W. Turner
Institute of Child Health
University College London
Immunobiology Unit
30, Guilford Street
London WC1 1FH
United Kingdom

C.M.J.E. Vandenbroucke-Grauls
University Hospital Free University
Department of Internal Medicine
De Boelelaan 1117
1081 HV Amsterdam
The Netherlands

H.A. Verbrugh
Erasmus University Medical Centre
Department Medical Microbiology and Infectious Diseases
Dr. Molewaterplein 40
3015 GD Rotterdam
The Netherlands

P.E. Verweij
University Medical Centre St Radboud
Department of Medical Microbiology
PO Box 9101
6500 HB Nijmegen
The Netherlands

R.R.P. de Vries
Leiden University Medical Centre
Department of Immunohematology and Blood Transfusion
PO Box 9600
2300 RC Leiden
The Netherlands