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Gene polymorphisms, inflammatory diseases and cancer

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Genes whose products play a critical role in regulation of the immune response include the human leucocyte antigen (HLA) and cytokine families of genes. The HLA genes are the most polymorphic found in the human genome, and the bulk of this polymorphism results in functional differences in expressed HLA molecules, resulting in inter-individual differences in presentation of peptide antigens to T-cells. In addition, a considerable number of cytokine-associated gene polymorphisms have been identified, the bulk of which occur in the upstream promoter sequences of these genes, which in many cases results in differential *in vitro* expression of the respective pro- or anti-inflammatory gene product. Particular HLA polymorphisms result in well-defined associations with a large number of immunologically-mediated diseases, including some diseases with known dietary risk factors. For example, individuals of HLA-DQA1*0501, DQB1*0201 genotype have a greater than 200-fold increased risk of developing intolerance to dietary wheat gluten (coeliac disease), and additional HLA-related factors may influence the development of malignant lymphoma within pre-existing coeliac disease. Similarly, HLA-DRB1 alleles sharing a common sequence motif constitute the primary known genetic risk factor for rheumatoid arthritis. The influence of polymorphisms associated with differential cytokine expression on disease susceptibility is currently of much interest. Most attention has been focused on associations with susceptibility to benign immunologically-mediated diseases, including a number of gut diseases. However, recent work from our laboratory indicates that cytokine polymorphisms may influence susceptibility to and prognosis in a number of different cancers, including malignant melanoma skin cancer and solid tumours which may be influenced by diet, such as prostate cancer (collaboration with the CRC/BPG UK Familial Prostate Cancer study). In addition, preliminary work suggests that dietary modulation of expression levels of certain cytokines in healthy human subjects may be genotype dependent.

Résumé

Parmi les gènes dont les produits jouent un rôle critique dans la régulation de la réponse immune, on trouve les familles de gènes de l'antigène leucocyte humain (ALH) et de la cytokine. Les gènes ALH sont les plus polymorphes du génome humain, et ce polymorphisme entraîne des différences fonctionnelles des molécules ALH exprimées, qui se traduit par des différences individuelles dans la manière dont des peptides antigéniques se présentent aux cellules T. De plus, un nombre considérable de polymorphismes des gènes associés à la cytokine ont été identifiés, la plupart d'entre eux se produisent au niveau du promoteur de ces gènes, ce que dans de nombreux cas se traduit par l'expression différentielle *in vitro* de leurs respectifs produits génétiques pro- ou anti-inflammatoire. Des polymorphismes ALH particuliers aboutissent à des associations bien définies avec un grand nombre de maladies immunologiquement médiées, parmi lesquelles quelques-unes

Abbreviations: CMM, cutaneous malignant melanoma; HLA, human leucocyte antigen; IL, interleukin; LT, lymphotoxin; PC, prostate cancer; PCR, polymerase chain reaction; RA, rheumatoid arthritis; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism; SSOP, sequence-specific oligonucleotide probe; TNF, tumour necrosis factor.

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avec des facteurs de risque diététique. Par exemple, des individus de génotype HLA-DQA1*0501, DQB1*0201 ont un risque deux cent fois plus grand de développer une intolérance au gluten alimentaire (maladie céliaque), et des facteurs additionnels en relation avec l'ALH peuvent influencer le développement d'un lymphome maligne dans une maladie céliaque existante. De même, des allèles HLA-DRB1 qui partagent une séquence motive commune constituent le premier facteur de risque génétique de l'arthrite rhumatoïde. L'influence de polymorphismes associés à une expression de cytokine différentiel sur la susceptibilité aux maladies présent dans ce moment un grand intérêt. La plupart de l'attention a été centré les associations avec la susceptibilité de maladies médicamenteuses immunologiquement bénignes, parmi eux un grand nombre de maladies de l'intestin. Toutefois, un travail récent dans notre laboratoire indique que les polymorphismes de la cytokine peuvent influencer la susceptibilité à certains cancers (ainsi que son pronostic), entre eux le mélanome de peau maligne et des tumeurs solides qui peuvent être affectés par l'alimentation, comme le cancer de prostate (collaboration avec le CRC/BPG étude familial du cancer de prostate Royaume-Uni). De plus, des travaux préliminaires suggèrent que la modulation par l'alimentation des niveaux d'expression de certaines cytokines dans des sujets humains peut être dépendent du génotype.

Gene polymorphisms: Inflammatory diseases: Cancer: Human leucocyte antigen genes: Cytokine genes

Presentation of processed antigenic peptide to T-cell receptor molecules expressed on T-cells by cell-surface human leucocyte antigen (HLA) molecules is the critical initiating event in the T-cell immune response. In the presence of appropriate co-stimulatory molecules, responding T-cells secrete a range of soluble cytokine mediators, which act to regulate both T- and B-cell immune responses, as well as acting on other cellular components of the immune response, such as macrophages and natural killer cells. In the present article, the function, extent and identification of naturally-occurring polymorphisms of the HLA loci will be reviewed, and the role of HLA polymorphism in predisposition to immune-mediated diseases will be considered. Coeliac disease and rheumatoid arthritis (RA) will be considered as exemplars, since the HLA associations concerned are particularly well described and both diseases have dietary risk factors. Immunogenetic evidence linking development of enteropathic lymphoma with coeliac disease will also be presented. Polymorphism in coding sequences of T-cell receptor genes is much more limited in extent (Kay, 1996) and will not be reviewed here.

In addition, an increasing number of polymorphisms associated with differential production of cytokine gene expression *in vitro* has been described, many of which show associations with susceptibility to a number of immune-mediated diseases. The nature of these polymorphisms and their laboratory identification will be summarised. Recent work from a number of laboratories, including our own, suggests that certain of these polymorphisms are associated with susceptibility to and/or prognosis in a number of cancers. These cancers may include those with dietary links, such as prostate cancer (PC). Finally, evidence has been obtained indicating that dietary modulation of expression levels of certain cytokines in healthy human subjects may be genotype dependent.

Human leucocyte antigen molecules and gene polymorphisms

Cell-surface HLA molecules function to present processed antigenic peptide, held within a groove in the HLA molecule, to T-cell receptor molecules expressed on the surface of T-cells. HLA class I A, B and C molecules present intracellularly processed peptides (eight to ten amino acids in length), largely derived from endogenous antigens (both self and viral antigens), to CD8+ T-cells, the majority of which are of cytotoxic phenotype. HLA class II DR, DQ and DP molecules present processed peptides (fourteen to twenty-five amino acids in length), largely derived from exogenous antigens, to CD4+ T-cells, which are mainly of 'helper' phenotype. While HLA class I molecules are expressed on virtually all nucleated cells and platelets, HLA class II expression is more limited, being restricted to B-cells, professional antigen-presenting cells and activated T lymphocytes, but is also interferon- γ -inducible, and up-regulated HLA class II expression is often observed in inflammatory and autoimmune diseases.

The genes encoding the HLA molecules, within the major histocompatibility complex (chromosome 6p21.3), are the most polymorphic within the human genome, with at least 225 HLA-A, 444 HLA-B, 111 HLA-C 2 DRA, 350 DRB, 22 DQA1, 47 DQB1, 20 DPA1 and 96 DPB1 alleles currently recognised (Robinson *et al.* 2001). The antigen-binding groove of class I molecules is encoded by exons 2 and 3 of the gene, while the class II antigen-binding groove is encoded by exon 2 of the respective A and B genes for that subregion of the major histocompatibility complex. The vast majority of coding polymorphisms is contained within these exons. In addition, polymorphism is clustered within a limited number of hypervariable regions within each exon (e.g. typically six hypervariable regions for exon 2 of an HLA class II gene such as DPB1). Polymorphic differences

in the hypervariable regions of these exons are rarely allele-specific. Rather, hypervariable sequence motifs are often shared between a number of alleles, and so a unique allele sequence is composed of a unique combination of sequence motifs at these hypervariable regions. The situation with respect to HLA class I genes is more complex, not only because polymorphism is spread over two exons, but because sequence motifs at the hypervariable regions are not only shared between different alleles of the same locus, but also can be shared between alleles of more than one class I gene or pseudogene.

The overwhelming majority of HLA polymorphisms result in functional amino acid substitutions in the expressed HLA molecules, with the bulk of these functional substitutions occurring with the peptide-binding grooves of the molecules. This process results in expressed HLA molecules encoded by different alleles exhibiting different peptide-binding specificities. In this way, genetic polymorphism within the HLA-encoding genes can modulate the immune response to a vast range of antigens, with inter-individual differences in immune responses to complex or simple antigens resulting from differing efficiencies of peptide binding to particular expressed HLA molecules. Due to this exuberant HLA polymorphism and its central role in the immune response, characterisation of this polymorphism within the HLA genes and encoded molecules is of critical importance in the clinical laboratory in both bone marrow and solid organ transplant patient-donor matching and in HLA-disease association studies (Bidwell & Navarrete, 2000). In our laboratory, characterisation of HLA polymorphism has also provided a tool for determination of individual identity during the investigation of potentially-contaminated or mislabelled surgical biopsy specimens (Bateman *et al.* 1994, 1996, 1997, 1999).

Molecular identification of human leucocyte antigen polymorphisms

Early methods for HLA typing were based on the detection of expressed HLA molecules on the surface of separated T-cells (HLA class I products) and B-cells (HLA class II products) using panels of antisera, usually obtained from multiparous women in a complement-dependent cytotoxicity test (Mittal *et al.* 1968). This technique has limited powers of resolution, especially for HLA class II alleles, and is dependent on isolation of live lymphocytes from the individuals to be genotyped.

For the past decade, DNA-based methods have been the principal tools for HLA typing in both research and clinical laboratories. The first comprehensive DNA-based HLA class II DR/DQ typing system was based on restriction fragment length polymorphism (RFLP; Bidwell *et al.* 1988). DNA-RFLP typing has now been almost entirely superseded by the more rapid polymerase chain reaction (PCR)-based DNA-amplification methods. When combined with initial PCR amplification of the gene or exon in question, restriction enzyme cutting sites can be identified within PCR amplicons (PCR-RFLP; for example, see Uryu *et al.* 1990). The principle of the widely-used PCR-sequence-specific oligonucleotide probe (SSOP) typing approach is that individual alleles or allele groups can be discriminated

by hybridization of immobilized PCR product containing the hypervariable regions to be typed with appropriately labelled SSOP (Krausa & Browning, 1996). PCR-sequence-specific primer methods are based on the specificity of a PCR being dependent on precise matching of the terminal 3' end of a PCR primer and its target DNA sequence (Olerup & Zetterquist, 1992). PCR-sequence-specific conformation polymorphism relies on single-stranded form PCR products containing different sequence polymorphisms that will adopt different conformations and will therefore differ in mobility in electrophoresis (Blasczyk *et al.* 1995). Heteroduplex analysis is another conformation-based technique that relies on the formation of mismatched heteroduplexes between closely similar but non-identical complementary DNA strands during PCR. This approach has recently been developed into a reliable method for allelic-level HLA typing, termed reference-strand-mediated conformation analysis (Arguello *et al.* 1998).

Despite their tremendous utility, PCR-SSOP and PCR-sequence-specific primer-based methods can only test for known HLA polymorphisms, although aberrant typing results may provide preliminary evidence for potential new alleles (Hemmatpour *et al.* 1998). Direct DNA sequencing is needed to identify and characterize such alleles (Ross, 2000). A more detailed review of DNA-based HLA typing methods is given by Howell & Poole (2002).

Human leucocyte antigen polymorphism and disease predisposition

Due to the critical role of the HLA system in regulating the immune response, combined with the extensive polymorphism of both HLA class I and class II genes, it is perhaps unsurprising that particular HLA polymorphisms have been linked to susceptibility to a large number of immunologically-mediated diseases, including skin, gut, endocrine and joint diseases (for reviews, see Thorsby, 1997; Lechler & Warrens, 2000). A non-exhaustive

Table 1. Human leucocyte antigen (HLA)-associated non-neoplastic diseases

Disease	Associated HLA allele	Relative risk*
Ankylosing spondylitis	B27	>150
Reiter's disease	B27	>40
Anterior uveitis	B27	>20
Narcolepsy	DQ6	>38
Grave's disease	DR3	4
Myasthenia gravis	DR3	2
Addison's disease	DR3	5
Rheumatoid arthritis	DR4	9
Juvenile rheumatoid arthritis	DR8	8
Coeliac disease †	DQ2	250
Multiple sclerosis	DR2, DQ6	12
Type I diabetes mellitus‡	DQ8	14
	DQ6	0.02

*The relative risk is a measure of how much more frequently the disease in question occurs in individuals possessing the particular HLA allele, compared with those not carrying the allele.

†The most common HLA association with coeliac disease is with the DQA1 *0501, DQB1 *0201 alleles.

‡Complex HLA associations exist with type I diabetes mellitus.

summary is given in Table 1. Some HLA–disease associations occur with only a single or related group of HLA alleles within a particular HLA gene (e.g. ankylosing spondylitis and molecular subtypes of the HLA-B27 group of alleles; Sorrentino & Tosi, 1997), while others are rather complex (e.g. type 1 diabetes mellitus which is associated with a certain combination of the HLA-DQB1 alleles inherited either on the same or on different chromosomes; Nepom, 2000). These associations are believed to occur due to inter-individual differences in the efficiency of presentation of antigens important in the disease pathogenesis, to T-cells.

The link between HLA genetic polymorphism and the precise three-dimensional structure and peptide-binding specificity of the antigen-binding groove of the encoded HLA molecules suggests that it should be possible to determine the antigen, or derived antigenic peptide, associated with the aberrant immune response that may represent a key event in the development of the disease in question. Despite this link, the antigens that may be responsible for initiating autoimmune and other HLA-associated inflammatory diseases are not known for the majority of diseases. In only a minority of diseases have the molecular mechanisms underlying the HLA–disease association been elucidated. A comprehensive overview of HLA and disease associations will not be given here, since excellent overviews occur elsewhere (for example, for a detailed overview, see Lechler & Warrens, 2000; for brief reviews, see Thorsby, 1997; Klein & Sato, 2000). However, two of the best-described examples comprise diseases with known dietary risk factors, i.e. coeliac disease and RA, and these two examples will be considered later. In addition, we believe that HLA polymorphism may also act as a risk factor for the development of enteropathy-associated T-cell lymphoma within coeliac disease, suggesting that HLA polymorphisms can, directly or indirectly, influence the development of neoplastic disease within pre-existing benign conditions.

Human leucocyte antigen, coeliac disease and enteropathy-associated T-cell lymphoma

Coeliac disease is a malabsorptive disorder of the small intestine which is precipitated by ingestion of wheat gluten and related proteins from other cereals (principally barley, rye and possibly oats). The mucosal lesions are characterized by villous atrophy, hyperplastic crypts and T-cell infiltration in the lamina propria. The disease has long been thought to result from an abnormal immune response to gluten, initiated by activation of T-cells in the lamina propria to gluten-derived peptides (Scott *et al.* 1997). In addition, HLA-associated predisposition to coeliac disease is very strong, with DQA1*0501, DQB1*0201 genotypes conferring a relative risk of approximately 250-fold (Sollid & Thorsby, 1993). Elegant experiments have subsequently shown that the pathological intestinal T lymphocyte response to the α -gliadin component of gluten in adult coeliac disease is focused on two immunodominant DQA1*0501, DQB1*0201-restricted peptides that overlap by a seven-residue fragment of gliadin. These peptides contain a glutamic acid residue (produced by the action

of tissue transglutaminase on native antigen) which is critical for HLA-DQA1*0501, DQB1*0201 binding and T-cell recognition. These peptides cannot bind to HLA-DQ molecules expressed by individuals of other HLA-DQ genotypes, who are therefore tolerant of gluten in their diet (Arentz-Hansen *et al.* 2000).

HLA associations with neoplastic diseases may provide additional evidence for a pathogenic relationship between these conditions and non-neoplastic conditions from which they may arise. In the context of coeliac disease and enteropathy-associated T-cell lymphoma (a high-grade T-cell non-Hodgkin's lymphoma), we have shown that the DQA1*0501, DQB1*0201 alleles are also increased in frequency among patients with enteropathy-associated T-cell lymphoma, suggesting that the two conditions are genetically related (Howell *et al.* 1995). Furthermore, patients with uncomplicated coeliac disease, particularly of early onset, show an increased frequency of DQB1*0201 homozygosity compared with those with enteropathy-associated T-cell lymphoma. These results suggest that among individuals who possess the HLA-DQA1*0501, DQB1*0201 genotype, HLA-DQB1*0201 homozygosity predisposes to early-onset more-severe disease, which is more likely to be diagnosed and the patient placed on a gluten-free diet. Conversely, individuals possessing the HLA-DQA1*0501, DQB1*0201 genotype in the absence of DQB1*0201 homozygosity have a delayed clinical presentation, resulting in a longer period of subclinical disease, and therefore an extended period of gluten-derived antigen stimulation, with an increased risk of emergence of a neoplastic T-cell population (Howell *et al.* 1995).

Human leucocyte antigen and other gastrointestinal diseases

While the HLA-DQA1, DQB1 association with coeliac disease is the strongest and most clear-cut HLA–disease association, HLA associations with other gastrointestinal diseases have also been reported, but findings are variable, with respect to both ulcerative colitis and Crohn's disease. Relative risks, both predisposing and protective, are generally weak. Several studies have reported a slight increase in HLA-DRB1*15 alleles in patients with ulcerative colitis, and there are also several reports of a reduced frequency of HLA-DRB1*04, which may be associated with more extensive disease (for review, see Sollid *et al.* 2000). Conversely, there are few reports of significant HLA associations in Crohn's disease, and most studies conclude that this disease is not HLA-associated (for example, see Bouma *et al.* 1997).

Human leucocyte antigen and rheumatoid arthritis

RA is one of the most important autoimmune disorders, with a worldwide distribution and a prevalence in Caucasoid populations of approximately 1%. The underlying pathogenesis of the disease is multifactorial, with both genetic and environmental factors, which may include diet, playing important roles. Considerable evidence points to the importance of the immune system in joint destruction and

systemic disease (Fox, 1997), with T-cell responses important in at least the disease initiation process.

Multiple genes are likely to be involved in susceptibility to RA. However, the principal known locus contributing to disease susceptibility is HLA-DRB1. Several HLA-DRB1 alleles contribute to RA susceptibility, but each of these alleles (DRB1*0401, 0404, 0405, 0101, 1402) contains identical nucleotide sequences (except DRB1*0401, which is near-identical) encoding amino acids 67–74 of the expressed DR β 1 chain. This so-called ‘shared epitope’ hypothesis accounts for HLA associations with RA in various ethnic groups: DRB1*0401, 0404 and 0101 in Caucasian populations, DRB1*0405 in Asians and DRB1*1402 in native Americans (Ollier & Thomson, 1992). In addition, most studies which stratify patients with RA according to disease severity indicate that the DRB1*04-associated susceptibility alleles (DRB1*0401, 0404 and 0405) are associated with more severe forms of erosive disease (Nepom *et al.* 1996). The amino acids comprising the ‘shared epitope’ form two of the pockets in the peptide-binding cleft of the expressed HLA-DR molecule, and therefore may favour binding and presentation of certain ‘arthritogenic’ peptides, which have yet to be identified. Alternatively, the susceptibility-conferring HLA-DR molecules contain an amino acid sequence that also occurs in glycoprotein B of the Epstein-Barr virus and of the heat-shock protein DnaJ of *Escherichia coli*. Thus, it is possible that infections with these organisms may lead to the development of antibodies that recognise these self HLA molecules. This mechanism of induction of autoimmune disease has been termed ‘molecular mimicry’ (Baum *et al.* 1996).

Human leucocyte antigen polymorphisms and cancer

Several studies have indicated that the HLA gene complex may mediate susceptibility to a number of haematological malignancies, including Hodgkin’s disease (Bodmer *et al.* 1992) and childhood common acute lymphoblastic leukaemia (Taylor *et al.* 1998), although the HLA-associated relative risks are modest (Table 2).

Table 2. Human leucocyte antigen (HLA)-associated neoplastic diseases (for review, see Bateman & Howell, 1999)

Disease	Associated HLA allele
Hodgkin’s disease*	DPB1 *0301, DPB1 *0401, DPB1 *0901
Acute lymphoblastic leukaemia	DPB1 *02, DPB1 *05
Cervical intraepithelial neoplasia	DQB1 *03, DQB1 *0602
Cervical squamous cell carcinoma	DQB1 *0301, 0303, 0602
Kaposi’s sarcoma	DR5
Burkitt’s lymphoma	A1, B12, DR7
Colo-rectal carcinoma†	DQB1 *0301
EATL	DQB1 *03, DQA1 *0501, 0201
Cutaneous malignant melanoma	DQB1 *0301, 0303

EATL, enteropathy-associated T-cell lymphoma.

*DPB1 *0401 is associated with a protective effect for Hodgkin’s disease in Orientals, while DPB1 *0901 is associated with a shorter duration of disease remission in Japanese patients.

†DQB1 *0301 may be associated with less-advanced tumours.

HLA associations with non-lymphoreticular malignancies have also been reported, including cervical cancer, and both melanoma and non-melanoma skin cancers (Bateman *et al.* 1998; Bateman & Howell, 1999). In such cases, the precise role of tumour-associated inflammatory cell infiltrates is unclear and any such association is likely to be complex. For example, an inflammatory cell infiltrate may represent an immune response against the tumour, resulting in a better disease prognosis, as seen in cutaneous malignant melanoma (Clark *et al.* 1989). Alternatively, inflammatory cells may promote or inhibit tumour growth via secondary effects on other molecular processes such as angiogenesis (Leek *et al.* 1996). The latter hypothesis provides a potential link between HLA molecules and cytokines in the control of the immune response in both inflammatory and neoplastic diseases.

Cytokine polymorphisms and the immune response

Cytokines are generally small molecules secreted by one cell to alter the behaviour of itself or another cell, generally within the haematopoietic system. Cytokines act on target cells by binding to specific receptor ligands, initiating signal transduction and second messenger pathways within the target cell. Cytokines function as players in a highly-coordinated network in which they induce or repress their own synthesis as well as that of other cytokines and cytokine receptors. This complexity is compounded by the fact that there is often considerable overlap and redundancy between the functions of individual cytokines. Production of numerous cytokines by the cells of the immune system, in response to both antigen-specific and non-specific stimuli, plays a critical role in the generation of both pro- and anti-inflammatory immune responses. For example, cytokines such as tumour necrosis factor (TNF) α (encoded within the major histocompatibility complex at chromosome 6p21.3) and interleukin (IL)1 (chromosome 2q14) are usually regarded as key pro-inflammatory cytokines, while cytokines such as IL-10 (chromosome 1q31–32) are anti-inflammatory, with strong immunosuppressive properties.

A considerable body of research undertaken in recent years (for review, see Bidwell *et al.* 1999) has shown that polymorphisms occur in the upstream regulatory (‘promoter’) regions of many cytokine genes, which may be functionally relevant in that these polymorphisms may influence the level of expression of these genes. Many studies support this hypothesis for several cytokine genes, e.g. TNF- α (Allen, 1999), IL-10 (Turner *et al.* 1997b), although *in vivo* the situation is almost certainly more complex, and genotype *v.* expression correlations may be influenced both by stimulus and cell type (Kroeger *et al.* 2000). Due to the documented role of up-regulation or dys-regulation of expression of key pro- and anti-inflammatory cytokines in inflammatory lesions and in a large number of autoimmune diseases and malignancies, there has been considerable interest in determining whether the cytokine genetic profile of an individual results in qualitative or quantitative programming of his or her inflammatory response. This factor may result in cytokine gene polymorphisms mediating predisposition to particular immunologically-

mediated diseases, or influencing their clinical course. Accordingly, development of robust molecular genotyping techniques for identifying known and novel cytokine polymorphisms is crucial for research in this area.

Molecular identification of cytokine polymorphisms

Unlike HLA polymorphisms, which consist of multiple polymorphic sites within a single gene, with clusters of hypervariable sequence motifs resulting in multiple expressed alleles, most cytokine coding and non-coding coding sequence polymorphisms are single nucleotide polymorphisms (SNP), usually bi-allelic in nature. Despite this fundamental difference, many of the methods used to type for HLA polymorphisms can also be used to type for cytokine SNP. Screening for novel cytokine promoter SNP is often undertaken using the PCR–sequence-specific conformation polymorphism approach (for example, see Fishman *et al.* 1998), with subsequent confirmation by DNA sequencing. Genotyping for known polymorphisms can then be performed by a number of approaches, principally PCR–RFLP, PCR–SSOP and amplification refractory mutation system–PCR, the latter being synonymous with PCR–sequence-specific primer methodology.

Certain cytokine SNP lead to substitutions in the recognition sites of particular restriction endonucleases e.g. the TNF- β (lymphotoxin (LT)- α) +252 intron polymorphism results in two alleles, the DNA of one allele being sensitive to digestion by the *Nco* I restriction enzyme, while DNA from the second allele is not (Messer *et al.* 1991). Polymorphisms such as these are detectable by the PCR–RFLP approach. Further examples of such polymorphisms are TNF- α –308 (*Bsm*F I) and IL-6 –174 (*Nco* I). Numerous other cytokine SNP correspond to restriction endonuclease cutting sites (for review, see Bidwell *et al.* 1999).

In principle, all cytokine SNP are detectable using PCR–SSOP, using separate oligonucleotide probes for each SNP (Perrey *et al.* 1998). Amplification refractory mutation system–PCR approaches have also been developed for numerous cytokine SNP, an area in which our laboratory has a special interest (Howell *et al.* 2001, 2002*b*; Poole *et al.* 2001). While standard amplification refractory mutation system–PCR techniques for bi-allelic SNP utilise two PCR per SNP, i.e. one for each allele, it is possible to modify the methodology such that both alleles are typable in a single PCR, using the so-called ‘tetraprimer approach’ (Karhukorpi & Karttunen, 2001; Ye *et al.* 2001).

Cytokine polymorphisms and immune-mediated diseases

In recent years a considerable effort has been expended by numerous laboratories in order to determine whether cytokine promoter polymorphisms (and indeed other coding and non-coding polymorphisms associated with differential cytokine expression *in vitro*) contribute to susceptibility to, or influence clinical course in, a number of immune-mediated diseases and in clinical transplantation. A fairly up-to-date summary is provided by Bidwell *et al.* (1999). For example, claimed associations between TNF- α –238, –308 and –376 promoter polymorphisms and RA (Brinkman *et al.*

1997), cerebral malaria (Knight *et al.* 1999), asthma (Moffatt & Cookson, 1997) and cardiac and renal transplant rejection (Turner *et al.* 1997*a*; Sankaran *et al.* 1998) have been reported. Likewise, associations between IL-10 promoter polymorphisms and systemic lupus erythematosus (Lazarus *et al.* 1997), RA (Hajeer *et al.* 1998) and asthma (Lim *et al.* 1998) have been described. Other cytokine polymorphisms, including IL-2-related polymorphisms, have been implicated in susceptibility to RA (John *et al.* 1998). However, there is still some conflict in the published literature in this field. For example, studies from our own and other laboratories do not show a significant association between recipient TNF- α genotype and renal transplant rejection, possibly due to differing patient selection criteria and/or immunosuppressive drugs used (Marshall *et al.* 2000; Poole *et al.* 2001).

Cytokine polymorphisms and cancer

A number of studies have reported associations between TNF- α and/or LT α polymorphisms and particular cancers, including chronic lymphocytic leukaemia (Demeter *et al.* 1997) non-Hodgkin’s lymphoma and breast cancer (Chouchane *et al.* 1997). In our laboratory we are investigating whether cytokine polymorphisms influence susceptibility to and/or prognosis in cutaneous malignant melanoma (CMM), since there is strong evidence for anti-tumour immune responses in CMM: early (radial growth phase) CMM tumours are characteristically associated with a T-cell infiltrate; the prognosis of more advanced and potentially metastasising (vertical growth phase) tumours is influenced by the extent of T-cell infiltration, while such T-cells have been shown to recognise melanoma antigen-derived peptides in an HLA-restricted fashion (Le Drea *et al.* 1995). These findings have been supported by variable HLA associations with susceptibility to, and prognostic markers in, CMM (for example, see Bateman *et al.* 1998). Elevated expression of IL-10 (a potent immunosuppressive cytokine) has been implicated in inhibition of the anti-tumour T-cell immune response in CMM, leading to tumour escape (for example, see Dummer *et al.* 1995). Conversely, other studies have described IL-10-associated anti-tumour activities in CMM, with IL-10 acting to inhibit angiogenesis and hence tumour growth (Huang *et al.* 1999). Work from our group has supported an anti-tumour effect of IL-10 in CMM, with genotypes associated with low IL-10 expression *in vitro* associated with disease susceptibility and with markers of more advanced, poorer prognosis disease (higher stage, vertical growth phase, thicker primary tumours), while ‘high expression’ genotypes are protective (Howell *et al.* 2001). Thus, IL-10 genotype is associated with both susceptibility to, and prognostic indicators in, CMM. This outcome may be mediated by high levels of IL-10 inhibiting expression of vascular endothelial growth factor (chromosome 6p12), a potent mediator of tumour angiogenesis. In support of this possible mechanism, further studies in our laboratory have indicated that vascular endothelial growth factor-promoter genotypes, associated with lower vascular endothelial growth factor expression, are associated with markers of less-advanced better disease prognosis (lower stage, thinner primary tumours; Howell *et al.* 2002*a*). Conversely, we have failed to demonstrate any associations between TNF- α and/or

LT α polymorphisms and CMM susceptibility or prognosis (Howell *et al.* 2002b).

Dietary factors have been reported to influence the development of numerous cancers, including a number in which immune responses to tumour may also be important. In this context, PC is of particular interest, since it is an ideal target for dietary chemoprevention, due to its long latency, high incidence, tumour marker availability and identifiable pre-neoplastic lesions and high-risk groups. There is also considerable evidence that diets rich in fibre and soyabean protein and low in animal fats are protective in PC development (for review, see Schulman *et al.* 2001). Other vitamins, especially vitamin E, minerals and trace elements such as Zn, Se and isoflavonoids are also implicated in prostate tumour inhibition (Schulman *et al.* 2001). Identification of genotypically 'at risk' individuals could further tailor this approach. Linkage studies have mapped several PC-susceptibility loci, but as yet no genes have been identified with certainty. However, there is now accumulating evidence that anti-tumour immune responses can occur in PC, as suggested by the isolation of prostate-infiltrating T-cells from normal tissue and PC lesions, responsive to prostate-specific antigens (Vesalainen *et al.* 1994). Dysregulation of cytokine expression in patients with PC has also been reported, both systemically and within tumours (for example, see Culig *et al.* 1998). A preliminary analysis of cytokine-promoter polymorphisms in our laboratory suggests that, in agreement with findings for CMM, genotypes associated with low IL-10 and high vascular endothelial growth factor production *in vitro* are risk factors for PC (McCarron *et al.* 2002). These genotypes may act to promote tumour angiogenesis, as we have hypothesised for CMM.

Diet-genotype interactions in modulating the immune response

Considerable evidence now exists that both diet (Calder, 2001) and genotype can modulate immune function, but as yet there have been virtually no studies of whether diet and genotype interact in modulating the immune response in health and disease. In our laboratory, as proof of principle, we are investigating whether diet and genotype show interactive effects on TNF- α production by peripheral blood mononuclear cells in healthy subjects.

In the immune response TNF- α expression is induced rapidly following infection and injury, with widespread effects, including stimulus of a diverse range of immunomodulatory cytokines and oxidant molecules (Grimble, 1996), creating a hostile environment for invading pathogens. However, excessive or untimely TNF- α production plays a major part in mortality and morbidity from sepsis (van der Poll & van Deventer, 1999), meningitis (Westendorp *et al.* 1997) and malaria (Knight & Kwiatowski, 1999). TNF- α also plays an important part in the pathology of inflammatory diseases such as RA (Maini & Taylor, 2000) and inflammatory bowel disease (Murch *et al.* 1991), in the development of atherosclerotic plaques (Ross, 1993) and in rejection of transplanted tissues (Kutukculer *et al.* 1995). While expression of TNF- α is normally constant in healthy males and post-menopausal females (Jacob *et al.* 1990), as

reviewed earlier, polymorphisms in both the TNF- α and LT α genes are associated with differential TNF- α and LT α expression *in vitro*. Furthermore, these polymorphisms show associations with susceptibility to a number of inflammatory and neoplastic diseases.

Fish oil, which is rich in *n*-3 polyunsaturated fatty acids has been shown to exert an anti-inflammatory influence in a number of animal models of inflammation and produces anti-inflammatory effects in RA (Kremer, 2000), Crohn's disease (Belluzzi *et al.* 1996) and psoriasis (Mayser *et al.* 1998). Fish oil may therefore provide a means of treating inflammatory disease in addition to standard drug therapy. Several (but not all) studies have also shown that dietary supplementation with fish oil reduces production of TNF- α by peripheral blood mononuclear cells, but with high levels of intra-individual variation in response to fish oil (for example, see Caughey *et al.* 1996). In order to clarify our understanding of the ability of fish oil to variably reduce expression of TNF- α , we have investigated *ex vivo* TNF- α production by peripheral blood mononuclear cells stimulated with lipopolysaccharide, before and after a 3-month period of fish oil supplementation in healthy males, and related TNF- α production to polymorphisms in the TNF- α and LT α genes of the subjects. Preliminary results indicate a complex interaction between dietary fish oil supplementation and genotype. First, differing sensitivities of the subjects to dietary supplementation with fish oil were observed, with individuals showing high inherent production of TNF- α being more sensitive to the anti-inflammatory effects of fish oil (Grimble *et al.* 2002). Second, medium and high inherent TNF- α production was associated with homozygosity for the LT α +252 (TNFB)2 allele. Third, individuals with medium or low levels of TNF- α production were more likely to experience the anti-inflammatory effects of fish oil if they were heterozygous for the LT α +252 (TNFB) alleles (Grimble *et al.* 2002). While the molecular mechanisms underlying this diet-genotype interaction in modulating TNF- α production remain uncertain, our results do suggest that investigation of the precise nature of both genetic and non-genetic determinants will enable fish oil supplementation to be used in influencing inflammation with greater precision than is currently the case. This concept could be extended to understanding the relationship between the availability of certain nutrients and the responsiveness of individuals to those nutrients with respect to predisposing to health or disease.

Concluding remarks

Immune response genes exhibit extensive polymorphism. In the case of HLA genes, the bulk of this polymorphism is functional and influences antigenic peptide presentation to T-cells, a critical step in initiation of T-cell responses to antigen. HLA polymorphism is implicated in conferring genetic susceptibility to a large number of immune-mediated diseases, including some cancers. Similarly, single nucleotide polymorphisms have been identified in the promoter regions of a number of cytokine genes, which may influence expression of these genes *in vitro* and perhaps *in vivo*. A considerable body of data indicates that particular cytokine polymorphisms, especially those

involving TNF- α and IL-10 genes, may influence susceptibility to, and in some cases prognosis in, both benign and neoplastic diseases. Very preliminary evidence suggests that cytokine genotype may interact with dietary factors in modulating pro-inflammatory immune responses.

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