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Genetic Susceptibility in Infectious Diseases

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Introduction

Even though exposure to infectious diseases in the developed world has decreased over the last 50 years, infection still remains a significant cause of morbidity and mortality, whilst in the developing world infectious diseases are still the primary healthcare problem. Naturally, the majority of infections are self-limiting and confer no significant morbidity on the host. Few infections are universally fatal, so the infections which are of primary interest here are those where there is significant variation in outcome between individuals. There are a number of these infections but, for the purposes of this article, I will concentrate on hepatitis B virus (HBV) and hepatitis C virus (HCV).

There are a number of factors which may influence the outcome of infection and these can, for convenience, be divided into host, pathogen and environment. Environmental factors might include nutrition, environmental toxins, and age at which the individual is exposed. Pathogen-derived factors generally imply virulence factors, which either increase the colonization ability of the organism, or increase the pathogenicity through expression of a toxin. Factors attributed to the host principally imply the host genetic make-up, though the outcome of infections is rarely inherited in a typical Mendelian pattern. Thus, any specific outcome from exposure to an infectious pathogen may be considered, genetically speaking, as a complex trait.

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Abbreviations used: HBV, hepatitis B virus; HCV, hepatitis C virus; MHC, major histocompatability complex; HLA, human leukocyte antigen; TDT, transmission disequilibrium test; DNA, deoxyribose nucleic acid; DGGE, denaturing gradient gel electrophoresis; TGGE, temperature gradient gel electrophoresis; SSCP, single strand conformational polymorphism analysis; DHPLC, denaturing high-performance liquid chromatography; CCM, chemical cleavage of mismatch; TNFα, tumour necrosis factor-α; CTL, cytotoxic T lymphocytes; APC, antigen presenting cells; Th, T helper lymphocytes; TAP I, transporter associated with antigen processing; HFE, haemochromatosis gene; YFV, Yellow Fever virus; RNA, ribose nucleic acid.

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Outcomes in infectious diseases

HBV is one of the world's most important pathogens; there are estimated to be 300 million people worldwide with chronic HBV infection, and over one million deaths a year from the long-term sequelae. HBV is also important because it has a clearly dichotomous outcome; infection may be acute and self-limiting, or it may be persistent. There are, broadly speaking, three patterns of natural history in HBV infection. In China and South East Asia, the virus is passed from mother to child at birth; a process known as vertical transmission. Infection acquired in this way leads invariably to persistent infection in the new host. In sub-Saharan Africa and around the Mediterranean littoral, HBV is passed from child to child within the first five years of life by mechanisms that have not been clearly elucidated. This process, known as 'horizontal' transmission, results in 5–15% of individuals becoming persistently infected, whilst the majority will have an asymptomatic self-limiting infection. In Northern Europe and North America, HBV infection is usually acquired during adult life as a result of high-risk behaviours such as promiscuous sexuality and intravenous drug abuse. In this context, persistent HBV infection occurs in less than 5% of individuals, unless they are otherwise immunocompromised, by co-infection with HIV, for example. Persistent HBV infection begins with a phase of high level viral replication during which liver cirrhosis and/or hepatocellular carcinoma may develop. However, many individuals move from the high-level replication phase into a carrier state with low level replication without suffering any significant liver injury.

Chronic HCV infection affects more than 150 million people worldwide and the geographical distribution, unlike HBV, is more focused on the developed rather than the developing world. HCV is transmitted parenterally. Therefore, the most common modes of transmission are through blood product transfusion and through intravenous drug abuse. In addition, in certain areas iatrogenic transmission through vaccination with unsterilized needles appears to have been responsible for high prevalence rates.

Following exposure to HCV, 20–30% of individuals will spontaneously eliminate the virus, often during an asymptomatic hepatitis. However, unlike HBV, the majority of individuals will develop a chronic infection with varying degrees of liver inflammation and fibrosis. Infection with HCV may lead to liver cirrhosis after an average of 20 years, and hepatocellular carcinoma after an average of 28 years (Tong *et al.*, 1995). Fortunately, only 20–30% of chronically infected individuals are likely to develop these long-term sequelae. The principal phenotypic variation between individuals is the rate at which the fibrosis develops (Poynard *et al.*, 1997).

Evidence for genetic influences

Before initiating research to identify the genes responsible for the different outcomes of infectious diseases, it is important to examine the evidence that the host genetic make-up makes a significant contribution to the outcome of the infection. Such evidence may be provided by three sources; family studies, twin studies and, where a suitable model exists, inbred strains of animal models.

Family studies identify an increased incidence or prevalence of a disease within a family. Typically, these studies attempt to define the ratio of incidence in first degree relatives (usually siblings) to the incidence in the general population and may be

expressed as λ_s. These studies have been useful in the context of complex traits, which are not linked to infectious pathogens; eg diabetes and coeliac disease. However, as exposure to pathogens, infectious dose, and pathogen virulence factors may all contribute to the outcome of infection, family studies are not sufficiently rigorous to provide evidence for a significant genetic determinant.

Twin studies overcome the problem found in family studies by comparing the incidence of disease within the same family. In these studies, the incidence of a disease is compared between monozygotic and dizygotic twins. A simple χ^2 test comparing the twin concordance rates will provide evidence for genetic determinants and the contribution of host genetics to the outcome can be estimated. More sophisticated studies which compare concordance rates in twins who have been reared apart can provide quantitative estimates for the contribution of environmental factors. Concordance rates for chronic HBV infection in monozygotic twins are nearly twice the rate for dizygotic twins (Lin *et al.*, 1989).

A number of infectious diseases have a suitable animal model, which may provide supporting evidence for genetic determinants in humans. The reliability of the evidence depends, to a large extent, on how close the model is to human disease. Inbred strains of rodents may show large variations in the outcome of infection, where the strain of the infectious organism, the dose, site of infection, and age at which the infection was acquired are all controlled. In this situation, only genetic differences can account for the variation in disease outcome. These models have a further advantage in that, by generating interspecific crosses, the individual genetic determinants may be mapped and identified (Lander and Schork, 1994).

Methods for identifying susceptibility genes

Three effective methods for identifying disease susceptibility genes are widely used; disease association studies, allele sharing, and interspecific crosses in rodent models. These methods are not mutually exclusive but, for logistical reasons, they are not all applicable to each infectious disease.

The most familiar of the three methods is the disease association study. In this method, a candidate polymorphic gene is identified and allele frequencies are compared between a disease group and a suitable control population. Although this sounds relatively straightforward, there are a number of issues, which must be addressed in order to generate reliable and reproducible results. Firstly, the selection of candidate polymorphic genes: there should be plausible biological reasons for selecting the candidate. Some authorities believe that the functional significance of the allelelic variation should also be fully characterized before a polymorphic gene is selected as a candidate, whereas, in reality, this information is available on very few relevant polymorphisms.

Disease groups should be defined by a robust and clear phenotype. Rigorous definitions, with clear clinical implications, are available for many infectious diseases, such as the Blantyre criteria for cerebral malaria, or the definition of chronic HBV infection.

Controversy surrounds the choice of control groups. Many disease association studies have used healthy, 'normal' populations as the control group following, presumably, from autoimmune disease studies and other complex traits where no

other suitable alternative was available. However, with infectious diseases such as HBV, HCV, and malaria, the outcomes can be dichotomized; acute versus chronic for HBV and HCV, or mild versus cerebral malaria. In order to identify the genes, which influence the outcome of infection in those who have been exposed, it can be argued that the most suitable control group is individuals who have the opposite outcome. Difficulty may arise where the outcome is more of a spectrum or a continuous variable, as is found in the rate of fibrosis progression in patients with chronic HCV infection. The difficulty may be overcome by selecting phenotypic extremes with clear areas of separation between disease and control groups.

A number of early disease association studies reported amazingly strong associations, which have never been reproduced. The underlying explanation of these results is that disease and association groups were not sampled from the same ethnic groups. Ethnic stratification is the strongest criticism of disease association studies, but a number of steps can be taken to overcome the problem. The problem has been eloquently illustrated in the chopstick dexterity test in which a disease association study is conducted in San Francisco with the MHC class I locus as the candidate polymorphism. If the disease phenotype were the ability to use chopsticks well, then on a random selection of residents in San Francisco one would find a strong association between HLA-A1 and chopstick dexterity. This is not because variation in this antigen presentation molecule confers remarkable fingertip coordination, but the correct explanation is that HLA-A1 is far more common in the Oriental population than in other ethnic groups. Ethnic stratification is best avoided by selecting both disease and control groups from the same ethnic background. However, latent stratification can still give rise to false positive disease associations, and other techniques are available for confirming the validity of an association. Reproducing associations in disease and control groups collected in geographically and ethnically distinct areas is perhaps the most accessible supportive evidence, but it is more rigorous to use a transmission disequilibrium test (TDT) (Lander and Schork, 1994). TDT requires a disease group and a heterozygous biological parent for each member of the group, which is why, from a logistical perspective, the test, is difficult to conduct. The rate of transmission of the suspected disease allele from parents to affected children is compared with the rate of transmission of the other allele(s) at the same locus. An alternative method, though less widely applied, is to compare the rate of transmission of disease alleles to affected children with the rate of transmission to unaffected children.

In favour of the disease association study, it is statistically powerful, allowing identification of relatively weak associations in small sample populations. Furthermore, in practice this has been the most productive method for identifying disease susceptibility genes in infectious diseases.

The major limitation to disease association studies, apart from those addressed above, is that they are currently limited to the relatively short list of known candidate polymorphic genes. As we enter the 'post-genomic era', this list will rapidly increase but, at present, whilst it is most expeditious to select a polymorphic gene from the list, it is more scientifically rigorous to select a gene on the basis of the function of its product and the known pathogenesis of the specific disease and then look to see if there is allelic variation in the gene.

Detection of new polymorphisms is resource intensive, but a number of short cuts

and new technology may speed up the process. Ultimately, DNA sequencing is required to accurately characterize variants but, as human genes are frequently as large as 50 kb, sequencing of the whole gene may be impractical. An alternative approach is to use screening technologies to identify which part of the gene is polymorphic. A number of such techniques are available, including denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single strand conformational polymorphism analysis (SSCP), double strand conformational polymorphism analysis (DSCP) and denaturing high-performance liquid chromatography (DHPLC) (Muller *et al.*, 1998; Arguello *et al.*, 1998; Chiarelli *et al.*, 1998; Freson *et al.*, 1998; Taylor and Deeble, 1999; Van Orsouw and Vijg, 1999; Muniappan and Thilly, 1999). Each of these techniques relies on the changes in physico-chemical properties created by nucleotide substitutions, insertions or deletions. The chemical cleavage of mismatch detects nucleotide mismatches in double stranded DNA (CCM). In this technique, candidate DNA fragments are hybridized to reference sequences and chemically or enzymatically cleaved at the site of any mismatches. Detection of cleavage products may be achieved by fluorescentally labelling the DNA samples by using labelled primers in the polymerase chain reaction amplification procedure. It is claimed that DGGE, DSCP and DHPLC are sufficiently sensitive to detect single nucleotide substitutions in 1 kb fragments but, in reality, the sensitivity depends on the site and nature of the substitution and decreases with increasing fragment lengths.

In general, the allelic variations which influence the outcome of non-fatal infectious diseases are not likely to cause gross phenotypic changes in people who are not exposed to the infection and are therefore likely to have relatively subtle effects on the function of the gene product. It has therefore been argued that single nucleotide polymorphisms, or small deletion/insertion mutations, are likely to be found in the regulatory region of a gene rather than within the coding region of the gene (Mitchison, 1997). Evidence supporting this hypothesis is available by reviewing the current literature; polymorphisms in the promoter region of cytokine genes have been associated with variation in the outcome of HBV, HCV and malaria, but a mutation in the coding region of the interferon-γ receptor is linked to fatal infection with atypical mycobacteria (Newport *et al.*, 1996; McGuire *et al.*, 1994; Hohler *et al.*, 1998). It is therefore imperative to sequence as much of the regulatory region as possible.

The next most popular method for identifying disease susceptibility genes is the affected sibling-pair/genome scanning technique. This is an allele-sharing method where the observed ratio of shared alleles for two siblings is compared with that expected from simple Mendelian inheritance laws (ie allele inherited by neither sibling – 25%, by one sibling – 50%, by both siblings – 25%). Affected sibling pairs and their parents are genotyped at three or four hundred polymorphic dinucleotide repeat markers spread evenly across the human genome and the results are analysed statistically to give maximum likelihood ratios or lod scores (Copeman *et al.*, 1995; Reed *et al.*, 1994; Weber and May, 1989). It is generally accepted that a lod score of 3.2 or greater is significant, taking into account the multiple concurrent comparisons (Kong and Cox, 1997). However, this figure is arbitrary and, in practical terms, the ability to reproduce positive data on the same chromosomal region in two or more independent studies is more convincing than a single high lod value.

The method has the advantage of not being constrained by the list of available polymorphic gene candidates, or by the list of known genes. In addition, the method is not confounded by ethnic stratification. However, from a statistical point of view, the method is relatively weak and, in practical terms, affected sib-pair/genome scanning techniques have not yet successfully identified a novel disease susceptibility gene. The statistical power is dependent on λ_s, the number of loci involved in genetic susceptibility to the particular disease trait, and on the relative contribution of each gene. In the type of infectious disease/complex traits with which we are dealing there are virtually no estimates available for any of these parameters. The only method for improving the power of an individual study is to increase the number of affected sib-pairs. Pooling subjects from a number of centres is one potential solution, but caution should be exercised in maintaining a clear disease phenotype. As an example, it would not be logical to pool subjects with chronic HBV infection from China with subjects with chronic HBV in Europe. Although the phenotypic definition is the same, it is clear that the natural history is entirely different and it is therefore unlikely that the genes influencing the outcome of HBV in Europe are the same as those in China.

There is an intrinsic drawback to the technique because it depends on the large chromosomal regions of DNA shared between siblings. On the one hand, this should potentially identify linkages between non-functional DNA markers and a disease susceptibility gene, which may be many centimorgans away. However, as a result, the chromosomal region of interest may be very large and not readily amenable to physical mapping.

Animal models are not only useful in the confirmation of the genetic effect on disease outcome, but can also be used to identify the disease susceptibility genes (Darvasi, 1998). Inbred strains of rodents are phenotyped after an infectious challenge to provide suitable phenotypic extremes. Creating an F1 cross of the strains will provide evidence of whether the particular phenotype is inherited dominantly or recessively. F1 crosses are, by definition, heterozygotes with one of each chromosome derived from each of the parental strains, and their disease phenotype should be homogeneous. F1 crosses can be used to generate an F2 cross by brother-sister matings or a backcross to one of the parental strains with the aim of producing strains with varied phenotype and varied genotype as a result of chromosomal recombination. As with human sib-pair studies, linkage of polymorphic dinucleotide repeat markers across the complete genome may be sought.

Interspecific crosses clearly have major advantages and disadvantages. The principal drawback is that few human infectious diseases have suitable rodent models. Furthermore, even when a model does exist, it will not be clear how closely the human pathogenesis is replicated. The key advantage to this technique is that statistical power can easily be increased by increasing the number of rodents. In practice, interspecific crosses have successfully identified mouse genes which influence the outcome of mycobacterial infection and the human homologue which influences the outcome of tuberculosis (Bellamy *et al.*, 1998; Schurr *et al.*, 1989).

Once a disease susceptibility gene has been identified, the logical next step is to dissect the mechanism by which the allelic variation influences the outcome of infection. Remarkably, in complex traits there are few examples where the disease association has been complimented by the mechanistic explanation. This failure may be explained by the subtlety of functional changes that arise from allelic variation. A

prime example of this problem is the single nucleotide polymorphism at position -308 in the tumour necrosis factor-α (TNFα) gene promoter. Although this polymorphism was originally described 6 years ago, the functional consequences remain controversial (Kroeger *et al.*, 1997; Louis *et al.*, 1998; Wilson *et al.*, 1997; Wilson *et al.*, 1993).

Results

A number of candidate polymorphisms have been tested in the outcome of HBV infection. These include the MHC class I loci the MHC class II loci, the TNFα promoter and the vitamin D receptor gene.

MHC class I genes encode glycoproteins which bind viral peptides for presentation to CD8+ cytotoxic T lymphocytes (CTL). On recognition of antigenic peptides, CTLs induce lysis or apoptosis of the infected hepatocytes. CTLs are readily detectable in the peripheral blood of patients with acute HBV infection and are found to be polyclonal and multispecific. On the other hand, CTLs are rarely detectable in patients with persistent HBV infection, which gives rise to the hypothesis that MHC class I polymorphisms may influence the outcome of infection. Although a number of reports have examined MHC class I polymorphisms, there has not been any consistent or reproducible results (Giani *et al.*, 1979; Mota *et al.*, 1987; Lepage *et al.*, 1981; van Hattum *et al.*, 1987).

MHC class II genes encode heterodimeric proteins, which are expressed on the surface of antigen presenting cells (APC). Exogenously derived antigens taken up by APCs are processed and presented to CD4+ T helper lymphocytes (Th) which may respond by proliferating and secreting cytokines that influence the function of CTLs and B lymphocytes. Th proliferative responses in acute HBV infection are significantly more vigorous than those seen in persistent HBV infection. This gives rise to the hypothesis that MHC class II polymorphisms influence susceptibility to persistent infection. The published studies are more consistent on this issue in that the MHC class II allele HLA-DRB1*1302 has been identified in association with HBV clearance in two independent studies (Hohler *et al.*, 1997; Thursz *et al.*, 1995). It remains to be determined what is the exact mechanism by which this allele exerts its beneficial effect.

TNFα is a pro-inflammatory, anti-viral cytokine, which has been shown in transgenic mice to inhibit the replication of HBV. There are two point mutations in the promoter region of the gene, which are thought to influence the level of TNFα secretion. In The Gambian population, the TNFα -308 polymorphism has been shown to weakly influence susceptibility to persistent HBV infection, whereas in a European population the TNFα -238 polymorphism was shown to influence the outcome (Hohler *et al.*, 1998). These apparently conflicting data will require further evaluation in independent cohorts, but one potential explanation may be that neither polymorphism exerts a direct effect and that another gene in linkage disequilibrium is responsible for variation in outcome.

There is a polymorphism in the vitamin D receptor gene that appears to influence the level of transcription. Originally, this polymorphism was associated with osteoporosis in certain populations (Morrison *et al.*, 1994). However, vitamin D is more diffuse in its effects than just those involved with calcium metabolism. Acting through the vitamin D receptor, it activates monocytes and macrophages and is

thought to influence the balance of Th phenotypes between predominantly interferon-γ producing (Th1) to interleukin-4 producing (Th2) (Rook *et al.*, 1987). It has recently been shown that the vitamin D receptor polymorphism influences susceptibility to persistent HBV infection in The Gambian population (Bellamy *et al.*, 1999).

The analysis of genetic determinants in HCV infection has progressed in a similar way to that of HBV. In our own study of MHC class II loci, associations of self-limiting HCV infection with HLA-DRB1*1101 and HLA-DQB1*0301, which have been independently reported by other groups, were confirmed (Cramp *et al.*, 1998; Thursz *et al.*, 1999; Minton *et al.*, 1998; Alric *et al.*, 1997). These two sets of alleles are in tight linkage disequilibrium and it was not clear from our data which was responsible for the observed association. However, in a French study, self-limiting infection was associated with DRB1*1101 at an odds ratio of 4.1 and with DQB1*0301 at an odds ratio of 2.7, suggesting that the HLA-DRB1 allele was dominant in determining the outcome of infection (Alric *et al.*, 1997).

There have been no studies published for the association of MHC class I alleles with the outcome of HCV, but a Japanese study has established a potential association of the TAP genes with chronic HCV infection. TAP I and II are peptide transporters that transfer peptide fragments into the endoplasmic reticulum from the cell cytoplasm, after digestion in the proteasome. The peptide fragments are loaded into the binding cleft in MHC class I molecules prior to display on the cell surface.

MHC class II polymorphisms may also influence the severity of liver fibrosis in HCV infection, although the data is not so clear-cut. Studies in Caucasians found that histological severity was reduced in subjects with HLA-DQB1*0301 or have found no association (Czaja *et al.*, 1996). In Japanese populations, a number of alleles have been associated with histological severity but the results are inconsistent. One of these associations appears to be particularly strong: HLA-DR13 was associated with mild fibrosis (odds ratio 10.9, $P < 0.003$) (Kuzushita *et al.*, 1996).

One other gene has been reported to influence the severity of liver disease in HCV infection: the haemochromatosis gene (HFE) (Smith *et al.*, 1998). A cysteine to tyrosine amino acid substitution at codon 282 in the HFE gene has been identified in patients with the iron storage disease haemochromatosis (Feder *et al.*, 1996) . One group has reported that heterozygotes for this mutation are found more frequently in patients with severe fibrosis than amongst those with mild liver disease. This result is currently controversial and needs to be reproduced before it becomes accepted.

There are no suitable rodent models for HBV infection. Therefore, an approach using the interspecific cross technique is not practicable. However, there is a sib-pair/genome scan currently underway which should be published in the next 12 months.

In HCV infection, the rate of exposure to the virus is much lower than with HBV and exposure usually occurs during adult life. The number of identifiable affected sibling pairs is extremely small. In order to be most informative, sib-pair studies normally take the rare disease phenotype as the affected state, which in the case of HCV would be spontaneous viral clearance. This affected state is difficult to identify as elimination of the virus is usually followed by loss of detectable antibody. Sib-pair studies are therefore not practical in HCV due to logistical reasons.

HCV is a flavivirus, a family of single stranded RNA viruses, which also includes Dengue, West Nile virus and Yellow Fever virus (YFV). HCV will not infect rodents but YFV and other flaviviridae do. Most inbred strains of mice are susceptible to YFV

infection, which is fatal within a few days, whereas outbred mice are resistant to YFV. Susceptible mice have high titres of virus in the brain, whereas the resistant mice have viral titres that are significantly lower (Brinton *et al.*, 1985; Brinton, 1982). An inbred strain of mouse C3H/RV has been developed which is resistant to YFV infection, and interspecific crosses using this strain and susceptible strains have been used to map a flavivirus resistance locus to a 0.9 centiMorgan region on mouse chromosome 5 (Urosevic *et al.*, 1995). Although the gene has not yet been identified, phenotypic differences between the mice strains have been observed which suggest that this locus may be pertinent to the human HCV infection. Flaviviridae have a highly conserved stem-loop structure in the 3' untranslated region of the negative strand RNA. Generation of the positive strand genome requires binding of this stem-loop structure to a replication complex consisting of the viral polymerase and a number of unknown host proteins (Shi *et al.*, 1996; Blackwell and Brinton, 1995). *In vitro* studies suggest that the dissociation constant for at least one of the proteins is much higher in the resistant strain of mice leading to a lower rate of viral replication (Shi *et al.*, 1996). As the stem-loop structure in the 3' untranslated region of the negative strand RNA is probably conserved in HCV, as with the other flaviviridae, the mouse model may identify an important disease susceptibility gene in humans.

Summary

The outcome of infectious disease varies tremendously between individuals due to a number of factors and may therefore be viewed by the geneticist as complex traits. The identification of genes which influence disease outcome is, at present, a resource-intensive project and therefore should not be undertaken without clear evidence, preferably from twin studies, that the genetic contribution is significant. Although three principal techniques are available for the identification of disease susceptibility alleles, they are not applicable to all infectious diseases for logistical reasons. Whether a candidate polymorphic gene is identified through allele sharing studies, from interspecific crosses or taken from the currently available candidate list, the final evaluation will require carefully conducted disease association studies. As we move into the post genomic era, the identification of candidate polymorphisms and the characterization of their functional significance will rapidly increase, which will make the analysis of disease susceptibility in infectious diseases steadily more tractable.

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PART 3

Microbial Biotechnology