REVIEW ARTICLE

HLA typing from Serology to Sequencing Era

Derek Middleton

Northern Ireland Histocompatibility and Immunogenetics Laboratory,
City Hospital, Belfast, Northern Ireland

ABSTRACT

The last two decades have seen a massive growth in the application of DNA technology in Histocompatibility and Immunogenetics. This review summarises the history and application of DNA methods in this area.

Keywords: DNA Typing; HLA Typing; Polymerase Chain Reaction

Serological Techniques

Traditionally HLA antigens have been defined using serological techniques. These techniques rely on obtaining viable lymphocyte preparations (for HLA class II typing, B lymphocytes are needed) and the availability of suitable antisera to recognise the HLA antigens. The advent of magnetic beads, coated with antibody used for isolation of B lymphocytes, made class II typing easier to accomplish. Reliable antisera was not available commercially and laboratories needed to screen to find their own reagents and exchange these with other such-minded laboratories. This meant a difference in the quality of reagents between laboratories and led to some laboratories producing more accurate results than others. In addition these reagents could not be replenished. Whilst serology performed adequately in typing family members, it proved unsatisfactory in typing unrelated donors for bone marrow transplantation, once the extent of polymorphism was known within "serological identical" specificities. Serological typing was also difficult in those cases of poor cell viability or poor expression and in confirming or refuting phenotypic homozygosity; by implementing a DNA technique for HLA-C alleles we have reduced the homozygosity role in our stem cell donor registry from 50% by serology to 21%. In addition typing of non-Caucasians by serological methods has always proved difficult when the majority of HLA sera available have been derived from Caucasian donors.

Corresponding Author: Derek Middleton, D.Sc, Ph.D, FRCPath; Northern Ireland Histocompatibility and Immunogenetics Laboratory, City Hospital, Belfast, BT9 7AD Northern Ireland. Tel: (+4428) 9026 3676, Fax: (+44 28) 9026 3676, E-mail: derek.middleton@bll.n-i.nhs.uk

The last twenty years has seen an exponential growth in the application of DNA technology to the field of Histocompatibility and Immunogentics (H&I). Initially this was confined to a few research laboratories. However, development and application of several different DNA methods by many laboratories has led to the situation whereby nearly every H&I laboratory performs some DNA typing for the detection of HLA alleles.

Restriction Fragment Length Polymorphism (RFLP)

In 1982, Wake et al. described restriction fragment length polymorphisms (RFLPs) using a DRβ probe.[1] Complexities of binding patterns and inter-locus cross hybridisation of probes drew attention to the drawbacks of using full length HLA class II cDNA probes. In order to overcome these problems some investigators used short or exon-specific probes.[2-4] Even at this early stage of development, there were indications that RFLP was more accurate than serology.[5] The lack of locus-specific probes limited the characterization of class I RFLP, as cloned class I gene probes cross-hybridised with all members of the class I family.[6] Some HLA-A and -B probes were constructed[7,8] and RFLPs were defined which correlated with serologically defined HLA-A, -B and -C alleles.[9-14]

In 1987, two reports using Taq I enzyme showed how short probes for DRβ, DQβ and DQα could be applied sequentially, after dehybridization, to a single membrane.[16,17] The recognition site of Taq I includes the nucleotide dimer CpG and restriction sites

containing this dimer show a higher frequency of polymorphism in human DNA than other restriction sites.[15] Not only was there an excellent correlation between RFLP and serologically determined antigens, but heterogeneity was proven in several DR and DQ specificities, especially HLA-DR6.

One of the novel ideas (and a sign of the future) was the use of 19 base pair oligonucleotides as probes based on sequence information.[18-22] As the probes also hybridised to other genomic sequences, restriction enzyme digestion and gel electrophoresis were required to separate the target sequence from the bulk of the DNA. In addition, due to the small number of available copies of the relevant DNA this approach lacked sensitivity. A refinement of this method was to use the oligonucleotides to probe total RNA, as non-specific binding was not found on Northern blots.[23,24]

The advent of DNA techniques highlighted discrepancies in HLA-DR assignment between serological and RFLP methods.[25,26] A subsequent report showed that by using RFLP and eliminating these antigen discrepancies from the determination of HLA-DR antigens, the success rate of HLA matched cadaveric transplants at one year (90%) approached that of HLA identical sibling grafts (93%).[27] As a result of this report, many laboratories strove to establish molecular methods for routine typing. The introduction of these methods helped to improve the serological results in laboratories who complemented their serological method with a DNA typing method.

The RFLP methods were not without disadvantages. They did not directly identify the polymorphic coding sequences within the second exon of DRβ, DQβ and DQβ, but relied on polymorphic restriction sites situated generally outside these exons. In addition, they required the use of DR-DQ associations to discriminate between certain DR alleles that had identical DRβ RFLP patterns. Thus, care was needed to apply the system to non-Caucasian populations. The method was cumbersome and could take up to 16 days to produce results for only 24 samples. A non-radioactive RFLP method was described[28] using digoxigenin and chemiluminescence, but by this time fundamentally different techniques were being developed. Eventually RFLPs were replaced, but not before the results from their use had acted as a stimulus for the development of better methods for DNA typing.

Availability of Genomic Sequence

As developments were taking place in restriction enzyme methods, several research groups were concentrating on sequencing various class I and II HLA genes. The first HLA class I cDNA clones were obtained in 1980[29] and 1981[6] and cDNA for HLA-DRA[30] and DRB[31,32] genes were isolated in 1982. By 1984 representative genes for all of the classical HLA loci had been identified. Isolation of complete genomic clones led to the nucleotide sequencing of the complete genes encoding HLA-A3 and -Cw3.[33,34] Thereafter much of the sequencing concentrated on the exons. As early as 1984, samples considered to be homozygous by serological methods were found to be heterozygous by sequence analysis.[35] By 1986, although only two class II molecules (DRα and DRβ) were sequenced in their entirety, 30 different complete or almost complete amino acid sequences for class II genes were reported.[36] In 1987 a summary of sequence diversity data generated from studies on reference cell lines for the first domain of DR and DQ was reported.[37]

Polymerase Chain Reaction (PCR)

The advent of PCR[38,39] was a seminal discovery fundamental to all subsequent techniques that have been developed. PCR had a revolutionary impact on molecular biology research in general and influenced multiple clinical applications. It swiftly became an accepted tool in many disciplines, including HLA. The simple idea of Mullis, which began as an exercise in trying to use oligonucleotides for detection of subtle differences between DNA sequences, has lived up to all its promises.

Emergence of nucleotide sequence data for the alleles of HLA genes permitted the rapid development of many PCR-based techniques and reagents. Conversely, the PCR technique greatly reduced the effort required in subsequent sequencing of new alleles.[40,41] PCR-based methods may be broadly classified into three categories: (i) those which generate a product containing internally located polymorphisms which can be identified by a second technique, (e.g. PCR-sequence specific oligonucleotide (SSO) probing, PCR-RFLP, PCR followed by sequencing); (ii) those in which the polymorphism is identified directly as part of the PCR process,

although there are post-amplification steps, [e.g. PCR-sequence specific primer (SSP)]; and (iii) confor-mational analysis in which different mutations generate specific conformational changes in PCR products. The latter are identified by electrophoretic analysis e.g. heteroduplex analysis. The two main methods most frequently adopted to clinical histocompatibility have been SSO and SSP, although at regular intervals a novel method or a novel variation of an existing method are reported.

Many factors were found to influence the specificity of the PCR amplification, including sequence of the primer (i.e. length, GC content), free Mg^{2+} concentration, ratio of primer to target, buffer and polymerase concentration.[42] Conditions must be optimised for each primer pair. It is extremely important to take precautions to prevent contamina-tion of samples with previously amplified DNA.[43] Contamination of reagents can be minimised by preparing solutions in facilities which have not been exposed to amplified products, aliquoting reagents for single use and using dedicated equipment and consumables.

The use of thermostable Taq DNA polymerase[44] meant it was possible to avoid the inactivation of the polymerase, which had necessitated the addition of enzyme after each denaturation step. This develop-ment led to the automation of PCR by a variety of simple temperature - cycling devices. Taq polymerase significantly increased the specificity and overall yield of the reaction. In addition to the above, the ability to discard radioactive methods and replace them with detection systems that could be more easily and safely applied, led to the introduction of PCR techniques in many clinical laboratories. The implementation of the salting-out method for DNA extraction[45] provided a further advantage to the clinical laboratory. This procedure avoided the toxicity of organic solvents and was much cheaper and less time consuming.

Sequence Specific Oligonucleotides (SSO)

The literature contains many alternative methods for SSO typing. The major differences between these are the length and sequence of oligonucleotide probes, and the reporter molecule and its detection. Initially ^{32}P-labelled allele-specific oligonucleotides were hybridised to an amplified conserved region of exon 2 of the HLA-DQα gene,[46] but soon after biotin

was used as a label.[47] The PCR-SSO method was quickly applied to other loci; DPβ,[48] DQβ[49,50] and DRβ[51,52] with various procedures using ^{32}P, biotin or horseradish peroxidase-labeled probes. Methods in the clinical laboratory have tended to use either a substrate in a coloration development system[53] or a substrate which generates a chemiluminescent signal.[54]

The SSO method can be customised for each application. For example, approximation of HLA-DR serological specificities requires detection of shared polymorphic sequences which encode the epitopes detected by antibodies. These shared sequences identify families of alleles that belong to the same serologically-defined specificity groups. This level of typing is often referred to as "low resolution" or "generic" SSO. Alternatively, "high resolution" SSO typing can distinguish all known alleles. High resolution SSO usually requires selective ampli-fication of a group of related alleles. For example, all HLA-DRB1*04 alleles are specifically amplified with selected PCR primers and then the DNA is hybridised with a panel of probes which distinguish each HLA-DRB1*04 allele.[55]

Today, most laboratories prepare one membrane for each probe in the assay. This procedure is facilitated using a 96 well manifold. The use of automation not only eliminates the reuse of membranes after dehybridization, but it also mini-mizes sample to sample variation in loading and provides a relatively large surface area to aid in evaluation of hybridization dots of varying intensity. Other laboratories have used a robotic work station for both this and the amplification aspect of the technique.[56] Previously, when dot blot manifolds were not available, DNA was directly spotted on to membranes,[57] transferred from gels using a Southern transfer technique,[58] or spotted using a vacuum dot blot apparatus. Many laboratories reduce the number of different wash temperatures by the addition of tetramethylammonium chloride (TMAC), which reduces the effect of GC content on the stability of the hybrids and, providing the probes are of the same length, enables membranes to be washed at the same temperature. Some laboratories avoid the use of TMAC due to its toxic properties.

The SSO technique has proved very reliable, robust and accurate. Good amplification always gives a clean and clear cut SSOP hybridisation while almost

all the problematic typing results encountered are due to poor amplification. In a quality control exercise the percentage of correct HLA oligotypes was 99.8% for HLA-DR and 99.8% for HLA-DQB1 based on 9,244 HLA-DRB1 and 7,244 HLA-DQB1 assignments.[59] A recent study using two different SSOP methods in different laboratories found an error rate of 0.1% in testing 2,604 samples for HLA-DP.[60]

DNA typing techniques were initially applied to class II genes rather than class I for several reasons. The requirement for replacing class II serology was thought to be more urgent as serological typing for class II antigens was difficult (HLA-DR and -DQ) or impossible (HLA-DP). [Although Parham made a compelling case that we were deluding ourselves into thinking that serology was good enough for typing HLA-A and -B specificities.[63]]. Class II was thought to be more important for transplantation and disease association. Furthermore DNA typing for class I was destined to be more complex because sequence polymorphisms in class I genes are located in two exons. However, once the benefits of developing and applying the methods to class II loci were apparent, attention turned to class I genes. Initially the methods were used to define alleles in selected specificity groups such as HLA-A2, -A68, -A69[64] or HLA-A2, -A3, -B44,[65] alleles of a single specificity e.g. HLA-B27,[66,67] or to identify specificities difficult to detect by serology e.g. HLA-Cw6.[68] Two methods for the determination of a complete locus system proved to be the foundation for development of SSO class I methods.[69,70] These methods were later improved by better resolution and methods of probe labelling.[71-76]

The biggest problem in class I typing has been the large number of probes required to give unambiguous results for certain heterozygote individuals, i.e. instances where two or more pairs of alleles give the same probe reaction pattern. The choice in SSO for class I has basically come down to whether a laboratory prefers performing one PCR amplification and use a very large number of probes, or use a two-stage typing system with an initial medium resolution followed by high resolution, depending on the results of the first typing. Regardless of which method is used, allele typing is much more difficult for HLA-B than HLA-A, due to the vast polymorphism at HLA-B.

Although the SSO technique is perfectly suited for analysing large numbers of samples, it is not suitable for analysing individual or small numbers. The alternative technique of reverse dot blotting uses the same amplification procedure and SSO probes, but the panel of probes is bound to a solid support and the amplified sample is labelled and hybridised to that support. A single hybridization and stringency wash allows the detection of sequence polymorphisms present in the chosen sample. There are several variations of the reverse dot blot. One method uses poly-T tails to attach the oligonucleotide to the membrane, thus increasing the binding efficiency of the oligonucleotide and sparing the specific binding sequence from forming the attachment bonds. The immobilised array of probes hybridises to the PCR product which has incorporated biotinylated primers during the amplification process.[61] Streptavidin horseradish peroxidase conjugate is added and positive reactions are detected using a coloured soluble substrate. Alternatively visualisation of positive signals is performed by chemiluminescence.[62]

PCR-RFLP

Initially this method used the availability of sites in the nucleotide sequences to employ restriction endonucleases which recognized allelic variations, to digest PCR amplified HLA genes (HLA-DR, -DQ, -DP).[79-81] However, small bands located close to each other on the polyacrylamide gels sometimes obscure precise analysis and some heterozygotes can not be discriminated.[82]

These problems have been overcome for HLA-DR by a modified PCR-RFLP method using informative restriction enzymes, which have a single recognition site present in some alleles but not in others and using group specific primers to avoid cross hybridisation with other genes.[83,84] This method was also applied to HLA-DQB1, -DQA1 and -DPB1 genes [85,86] and, in some instances, simultaneous digestion of amplified DNA with two or more enzymes has been applied. [87] One of the first indications that HLA-DP matching may be important in bone marrow transplantation was reported using the PCR-RFLP method.[88] A recent innovation was the use of consecutive rounds of PCR-RFLP.

After the first digestion of the PCR product, the cleaved fragment was extracted from the gel and used as template for a second PCR-RFLP.[89] For the "allele

walking" (the term used by authors to describe this method) to proceed, a previous "cutting" was required.

Sequence Specific Primers (SSP)

In the early 1990's publications appeared on a method called PCR-SSP,[92,93] the design of which was based on the amplification refractory mutation system (ARMS). [94] The principal of this method is that a completely matched primer will be more efficiently used in the PCR reaction than a primer with one or several mismatches. Specificity is determined by the use of sequence specific primers in which a 3' single-base mismatch inhibits the priming of non-specific reactions.[94] Because Taq polymerase lacks 3' to 5' exonuclease activity, even if primer pairs do anneal non-specifically, they will not amplify efficiently. Thus only the desired allele or alleles will be amplified and the amplified product can then be detected by agarose gel electrophoresis.

Other investigators have used multiplex PCR i.e. having several primer pairs in the same reaction.[95,96] Sizing of the PCR product is necessary for interpretation, necessitating that the gel be run longer to separate the PCR fragments. The SSP method is ideal for typing individual samples, but is costly and requires high capacity thermal cyclers for larger numbers of samples. One laboratory reduced this problem by instigating a two-stage technique - low resolution followed by high resolution according to the first result.[98] As the method takes less than five hours, it can be applied to cadaveric transplantation.

Much of the earlier work in class I was performed by Browning and colleagues who developed a low-resolution typing system for HLA-A,[99] and quickly followed with a more extensive system to cover all HLA-A alleles.[100] The same group designed a low resolution primer panel for HLA-B.[101] Other laboratories used the SSP system as a supplement to serology by only typing for alleles of certain serological specificities,[102] or only determining HLA-B*27.[103] Bunce et al. developed a SSP system for HLA-C[104] and a high resolution system for HLA-B.[105]

The products of many alleles of the HLA-C locus are difficult to detect by serological methods due to the low expression of HLA-C molecules at the cell surface and to the corresponding lack of suitable antisera. Thus having a DNA typing system for HLA-C proved very useful. In addition, systems were developed for other loci which complemented each other, so that complete HLA-A, -B. -C, -DRB1, -DRB3, -DRB4, -DRB5 and -DQB1 typing could be performed simultaneously.[106] This method, termed "phototyping", has a resolution equivalent to high quality serology and could be completed within three hours.

The SSP method suffers from the disadvantage that the end-step of gel electrophoresis is not suitable for large numbers of samples or for automation. A novel method has been reported which removes the electrophoresis and combines high throughput with speed and high resolution. The method uses fluorogenic probes, each of which has a reporter and a quencher dye. When the probe is intact the proximity of the two dyes results in suppression of the reporter fluorescence. During PCR-SSP, if the target of interest is present, the probe specifically anneals between the forward and reverse primer site. The nucleolytic activity of the Taq polymerase cleaves the probe resulting in an increase in fluorescence. Taq polymerase does not cleave free probe - the enzyme requires sequence complementarity between the probe and template for cleavage to occur. After cleavage, the shortened probe dissociates from the target and polymerisation of the strand continues. This process occurs in every cycle and allows direct detection of PCR product. Using the 14 sequence specific primer pairs selected originally by Olerup[107] and three probes, Trucco and his colleagues applied the method to HLA-DQB1 allelic typing.[108] Other investigators have applied this method to class I typing using two different fluorogenic probes and 24, 48 and 16 primer mixes for HLA-A, -B and -C respectively.[109]

Heteroduplexes

During the primer-annealing stage of each cycle of the PCR, a proportion of coding strands of each DRB locus allele may hybridise to the noncoding strands of a different DRB locus allele and vice versa. This double stranded DNA will thus be mismatched in some regions (heteroduplexes) leading to alteration in the conformation of the DNA molecule. This conformation varies for each DR haplotype and can be detected by the modified migration in nondenaturing polyacrylamide gels, as the heteroduplex will move more slowly than the homoduplexes (complementary strands). A single mismatch of nucleotide can cause a marked

electrophoretic retardation and thus even subtypes involving a single substitution can be detected. PCR-heteroduplex analysis was first applied to HLA matching in 1990, in an analysis of HLA-DR allotypes encoded by the second exons of HLA-DRB genes.[110]

Additional bands are formed in heterozygotes which are not present in either of the patterns of the individual alleles and are caused by heteroduplex formation in trans i.e. between PCR products from two different haplotypes. This phenomenon was used in the DNA crossmatch test whereby DNA from two different individuals are co-amplified in the PCR. If the individuals are identical for HLA-DR then the banding pattern of the mixture will be the same as the banding pattern of each of the individuals, but if the individuals are not identical the mixture will contain extra bands. This method proved very useful in analysing HLA class I identical individuals for HLA-DR disparity in the selection of unrelated donors to be used for bone marrow transplantation.[111] A modification of heteroduplex analysis is the inclusion of third party DNA to generate new heteroduplexes (spiking) which enhance discrimination between otherwise similar patterns.[112] A further refinement was the use of temperature gradient gel electrophoresis which analyzes DNA not only on the basis of differential heteroduplex formation between different single strands, but also according to the thermal stability of the homoduplex, which is directly dependent on its nucleotide sequence.[113]

In reference strand conformation analysis (RSCA), DNA from a homozygous reference sample is amplified using primers, one of which is fluorescent-labelled at its 5' end.[141] The sample under test is amplified and the PCR product mixed with the reference PCR product to form heteroduplexes. These are resolved in an automated DNA sequencer with only the fluorescent-labelled duplexes being observed and identified according to the distance they have migrated.

Sequence Based Typing (SBT)

One of the drawbacks of SSO or SSP is that, although they are capable of detecting a single base difference in DNA sequence between two alleles, they are not likely to detect a new undefined allele, unless the variation happens to be at the specific site detected by the probe or the primer. Methods based

on sequencing have come to the fore. The sequencing technology advanced with the introduction of dye-labelled primers and fluorescent automated sequencing.[90] Group-specific amplification is perform-ed in order to limit the number of allele sequences in any sequencing template, otherwise DNA from both haplotypes would be present. This, in turn, simplifies the software-based allele assignments-suitable computer software was one of the greatest problems with this technique. Initially in typing for HLA-DR alleles PCR amplification used one set of primers with common sequence to all HLA-DRB1 alleles. However this meant the simultaneous amplification of alleles of other HLA-DRB loci (e.g. DRB3, DRB4) and led to difficulties in assignment of the DRB1 alleles. However by using a series of primers, the sequence of each being specific for some DRB1 alleles, but which do not share sequences with other DRB genes this problem has been nullified. This strategy has also been applied to SSOP typing.

In a recent comparative study between two centres, it was concluded that neither the manufacturer of the DNA sequencer nor the method of sequencing influenced the result. The most critical step was the amplification reaction.[91] On some occasions preferential amplification was observed, whereby one allele was more likely to be amplified than another when they were present in the heterozygote state.

The great advantage of SBT is its accuracy. It is the only technique, which directly detects the nucleotide sequences of an allele, thus allowing an exact assignment. It requires very expensive equipment. Nevertheless, it should be only a matter of time until generally accepted, easy to perform protocols will be available, thus leading to wider use of SBT. The advent of capillary based sequencers has been a tremendous boost in reducing the sophis-tication of the approaches required in the laboratory; laboratories with no previous experience in sequencing have quickly adapted to this method. It is now feasible to lease the equipment required thus reducing the initial heavy financial outlay. Although it is not always necessary to have resolution to the allele level, the automation and reduction in time required for sequencing has mean that this is easy to obtain.

Choice of Method

The use of a specific technique will depend on the laboratory's requirements. The choice will be influenced by clinical urgency and requirement, sample numbers, availability of equipment, staff skills and budget. Some laboratories, depending on their needs, may use a combination of methods. Because of the ease of storage and transport of DNA samples, or reference cell lines, and the fact that reagents can be made and not continually searched for, as in serology, some laboratories have been able to assist laboratories to set-up the techniques.

According to the clinical application, high or low resolution typing may be required. Kidney transplant candidates, for instance, do not necessarily have to be typed at a high resolution level, because only the 'broad' serological specificities (eg. DR1-DR10) are usually taken into consideration for organ allocation. For unrelated bone marrow transplantation purposes, high resolution typing is required.

Many of the methods referenced in this article only cover polymorphisms for HLA class I occurring in exons 2 and 3 and for class II in exon 2. It should be remembered that some alleles only differ in sequence outside these regions. A list of these alleles can be found on the IMGT/HLA database at http://www.ebi.ac.uk/imgt/hla. At present there are 34 such pairs of alleles (including ten in which the pair of alleles only differ in silent substitutions) in class I, but only two at HLA-DR.

New Alleles

The advent of DNA techniques has led to a massive increase in the number of HLA alleles, either through the detection of alleles not recognised by serological methods or because alleles which differ from one another by one or a few nucleotides can be differentiated, a process not possible by serological methods. In 1992, the number of antigens detected by serology was HLA-A, (25), -B (32), -DR (34), whereas by January 2003 the number of alleles was HLA-A (263), -B. (503), -DR (327). A World Health Organisation committee for HLA nomenclature is responsible for the naming of new alleles and reports of new alleles are furnished monthly in H&I journals. Data is now available on the IMGT/HLA website.

On many occasions laboratories attempt, at great cost, to incorporate the detection of all alleles, even if many are absent in their population. Many alleles are only present in one individual or his/her family, or

may be the product of an erroneous sequencing – several alleles have subsequently been withdrawn after being present on the IMGT/HLA database. In the N.Ireland population, from our results of allele typing 1,000 individuals, we only found 103 of 422 HLA Class I alleles published at the time of testing; 30/90 (33.3%) for HLA-A; 50/254 (19.7%) for HLA-B; 23/78 (29.5%) for HLA-C.[114-116] Bearing in mind the number of new alleles determined since that study, it is likely that less than 15% of HLA Class I alleles identified at present are in this Caucasian population from Northern Europe. One way to alleviate this problem, would be for the laboratory to know the frequency of the alleles in the population under test. This would enable a prediction of the most probable alleles. Recently a website www.allelefrequencies.net has been instigated to collect the frequencies of alleles in various populations. This enables an investigator to examine the frequency of all alleles, or a specific allele, in one or many populations. Data will be available, not only for HLA alleles, but for alleles of other polymorphic loci connected to the Histocompa-tibility and Immunogenetics field e.g. cytokine, TAP, MIC, KIR receptors.

Occasional difficulties arise in attempting to link the allele name based on sequence to the serology of the associated antigen. For example most laboratories have assigned the HLA-B7 serological type to the allele HLA-B*2708 which was given that name based on close sequence homology to HLA-B*2701. On other occasions, there is no serological information. Some alleles are hybrids of two "broad" alleles. For example HLA-DR*1107 which at the second exon is composed of two parts, (one from HLA-DRB1*11 and the other from HLA-DRB1*03) has been named because the initial half of the sequence is identical to HLA-DRB1*11 alleles.[117] Knowledge of serological functional behaviour of the allele would be important in such instances. Several alleles have been reported whose products are not expressed. The mutations causing these non-expressions are sometimes located outside the exons normally analysed by DNA methods. Until all these alleles are identified and DNA tests extended to include their polymorphisms, serology will have a role to play.[77,78,118] Although it must be pointed out that the frequency of these null alleles is very low.

HLA Associated Diseases

DNA techniques have provided a new impetus to the study of HLA and disease. They have removed the imprecise nature of serology allowing associations to be more accurately defined. HLA susceptibility to insulin-dependent diabetes mellitus has now been shown to be related to the presence of non-aspartic acid residues at position 57 on both DQβ chains[119] and to the presence of arginine at position 52 on the DQα chain.[120] More recent evidence has suggested a combination of DR and DQ molecules is involved.[121] It has been shown that whilst some alleles of HLA-DR4 are associated with susceptibility to rheumatoid arthritis (DRB1*0401, DRB1*0404) others are not (DRB1*0402, DRB1*0403).[122] It is now possible to denote common sequences in the HLA alleles associated with a disease. In rheumatoid arthritis the sequence (QKRRA/QRRAA) within the third hypervariable region of the HLA-DRB1 molecule is present in the alleles associated with this disease.[123] DNA techniques have shown that in nearly every case of coeliac disease the same DQαβ dimer is present in patients, either encoded in cis (on the same haplotype) or in trans (one chain encoded on the maternal haplotype and one chain encoded on the paternal haplotype).[124] The increased knowledge derived from all of the above studies will help to find the mechanisms responsible for the disease.

HLA in Anthropology

The extent of allelic diversity within the HLA complex makes it an especially valuable system for the study of population admixture. The more the HLA system has been studied, the greater the diversity discovered.

Among a group of subtypes, the common pattern is for one subtype to be widely distributed throughout the world's population and the others to be more localized. The sequence of each local subtype is usually identical to the common subtype, except within a short segment. For example, 65 alleles of the HLA-A2 serologically defined antigen have been discovered.

Only two of these are found in a Northern Ireland Caucasian population, with one allele (HLA-A*0201) representing 96% of HLA-A2. This allele also represents more than 90% of HLA-A2 alleles in a Mexican population. However, in a Singapore Chinese population, only 20% of HLA-A2 alleles are HLA-A*0201, and HLA-A*0207, at 25%, is the most frequent HLA-A2 allele.[125] Thus DNA techniques have been able to show differences in allele frequencies amongst populations which could not be found by serology.

Kidney Transplantation

Mention has previously been made of the beneficial effect on graft survival if HLA-DR typing can be performed more accurately.[27] Using serological techniques, it had been impossible to reach any conclusion on the effects of matching for HLA-DQ and -DP in kidney transplantation. With the advent of DNA technology, there has been some indication that matching for HLA-DQ may have some benefit in graft survival,[126,127] but this question remains difficult to answer because of the strong linkage disequilibrium between HLA-DR and -DQ. Initially no effect of matching for HLA-DP alleles was found in transplants from a single centre[127] or in transplants selected from multiple centres with no mismatching at the HLA-A, -B and -DR loci.[128] However an effect of HLA-DP matching in transplants with varying degrees of HLA-A, -B and -DR matching has been reported in second but not first transplants.[129] Although no allowance has been made for linkage disequilibrium, it is known that linkage between HLA-DP and -DR is limited compared to that between HLA-DQ and -DR.

Using serological techniques it was previously shown in kidney cadaveric transplantation that matching for HLA-A and -B splits was more influential than matching for the broad antigens.[130] Additionally, a study examined HLA-A and -B specificities using SSO and SSP in kidney cadaveric transplants, that had zero mismatches for HLA-A and -B (by serology) and HLA-DR (by RFLP).[97] This study did not take the resolution of typing to the allele level, but still found an increase in graft survival of 15% between those transplants with 0mm for HLA-A, and -B after testing with medium resolution SSO and SSP, compared to transplants which were found to have mismatches at either HLA-A or -B by SSO or SSP.

Retrospective studies such as the above are required to determine at what resolution matching should be applied and whether polymorphic sequences at certain parts of the HLA molecule are

more important than others. At present in transplantation, a difference of one HLA specificity is called a mismatch. One has only to look at the sequence of alleles to see that DRB1*0101 (HLA-DR1) and DRB1*0103 (HLA-DRBR) have significantly fewer amino acid differences than DRB1*0101 and DRB1*0401 (HLA-DR4). In addition, analysis of matching on transplantation success has mainly been performed using serological typing at the broad level whereby specificities such as HLA-B44 and -B45 have been considered as matched. However they are quite different in sequence and, in fact, HLA-B45 is closely related to HLA-B50.

Naturally any structural difference must be shown to be functionally important and the peptide-binding specificity of the allele will need to be considered.[131] The more relevant parameter in the choice of a donor may not be the number of mismatches but the kind of mismatch.

Hematopoietic Cell Transplantation

The DNA methods have meant that most laboratories typing for bone marrow transplantation no longer rely on the mixed lymphocyte culture test. In addition, the methods have enabled HLA testing before birth, in order to ascertain if the cord blood would be suitable to use for a sibling requiring a stem cell transplantation. The application of high resolution DNA typing for HLA alleles in bone marrow transplantation has shown that nearly 20% of transplants that were initially typed as identical for HLA-DR by serology were not matched at the allele level, and that there was a significant correlation between DRB1 mismatches and decreased survival.[132] It has already been shown that the one nucleotide difference between HLA-B*0707 and HLA-B*0702 can be recognised by CTLs.[133] This difference is functionally relevant and similar differences have also been shown in other recipient/donor pairs serologically matched for B14,[134] B35,[135,136] B41[137] and B44.[138] With the ability to type to the allele level it will be very difficult for many recipients to receive a transplant from an allelic-matched donor. Hopefully new studies will be able to find the answer on what must be matched for and which mismatches can be ignored.

In the compilation of stem cell donor registries one of the problems has been the recording of donor types obtained by DNA methods when these methods have given alternate types which cross the existing serological boundaries.

A suitable method needs to be found which alleviates this problem and permits existing serological and DNA data to "communicate" with each other. In addition, an SSO probe pattern is interpreted according to the sequence information available at that time. However, as new HLA alleles are found they may have the same probe pattern. Therefore the type assigned to a probe pattern will differ with time. The same problem would also arise with SSP.

As suggested by Hurley, the recording on registries of sequence polymorphisms may go some way toward solving these problems.[139]

Future

The chemistry of probe synthesis has evolved to the stage where probes can be synthesised on surfaces such as glass or silicon. The use of large arrays of oligonucleotides on a solid support (DNA chips) which can then be hybridised with a labelled target sequence[140] should be feasible for HLA typing. The next few years will see further expansion. This will be the result of the needs in other fields for technological improvements in direct sequencing technology and automation.

The tests that become available should have flexibility in their resolution enabling laboratories to economically purchase what they require. For example a medium resolution system would at present be adequate for renal transplantation whereas high resolution to the allele level will probably be required for marrow transplantation.

REFERENCES

1. Wake CT, Long EO, Mach B. Allelic polymorphism and complexity of the genes for HLA-DR β chains-direct analysis by DNA-DNA hybridization. Nature 1982; 300(5890):372-4.
2. Coppin HL, Weissman SM, McDevitt HO. A study of HLA-B-locus polymorphism with a specific hybridization probe. In: Albert ED, Baur MP, Mayr

WR, editors. Histocompatibility Testing 1984. New York, Springer-Verlag, 1984: 554.

3. Tilanus MGJ, Bosch ML, Gerrets R. Giphart MJ. Molecular analysis of HLA: haplotype-specific DNA hybridization patterns using class II cDNA probes. In: Albert ED, Baur MP, Mayr WR, editors. Histocompatibility Testing 1984. New York: Springer-Verlag, 1984: 577.

4. Angelini G. de Preval C, Ferrara GB, Mach B. Analysis and segregation of HLA-DR by DNA typing: correlation with supertypic specificities. In: Albert ED, Baur MP, Mayr WR, editors: Histocompatibility Testing 1984. New York: Springer-Verlag, 1984: 582.

5. de Preval C, Gorski J. Mach B. Molecular complexity of the HLA-DR, DQ, and DP genes and genotypic split of HLA-DR serological speci-ficities by "DNA typing". In: Albert ED, Baur MP, Mayr WR. Editors. Histocompatibility Testing 1984. New York: Springer-Verlag, 1984: 569.

6. Sood AK, Pereira D, Weissman SM. Isolation and partial nucleotide sequence of a cDNA clone for human histocompatibility antigen HLA-B7 by use of an oligodeoxynucleotide primer. Proc Natl Acad Sci USA 1981; 78(1):616-20.

7. Grumet FC, Fish L, Moossazadeh J. Ness D, Duceman BW. An HLA-B locus probe clarifies endonuclease polymorphism of major histocompa-tibility complex class I genes. Mol Biol Med 1983; 1(5): 501-9.

8. Koller BH, Sidwell B. De Mars R. Orr HT. Isolation of HLA locus-specific DNA probes from the 3' - untranslated region. Proc Natl Acad Sci USA 1984; 81(16):5175-8.

9. Driesel AJ, Romer K, Schunter F, Laryea MD, Schneider EM, Wernet P, et al. DNA polymorphism of the major histocompatibility class I genes and their association with serologically defined haplotypes. Immunogenetics 1985; 21(6):529-38.

10. Cann HM, Ascanio L, Paul P. Marcadet A, Dausset J. Polymorphic restriction endonuclease fragment segregates and correlates with the gene for HLA-B8. Proc Natl Acad Sci USA 1983; 80(6):1665-8.

11. Cohen D, Paul P. Font MP, Cohen O, Sayagh B, Marcadet A, et al. Analysis of HLA class I genes with restriction endonuclease fragments. Implications for polymorphism of the human major histocompatibility complex. Proc Natl Acad Sci USA 1983; 80(20):6289-92.

12. Smeaton I, Summers CW, Harris R. Strachan T. Restriction fragment length polymorphism at the HLA-C locus. Immunogenetics 1987; 25(3):179-83.

13. Trapani JA, Michelson CA, McKenzie IFC. A 3.5 Kilobase Taq I restriction fragment of genomic DNA segregates with HLA-B27. Immunogenetics 1985; 21(2):189-92.

14. Erlich HA, Stetler D, Sheng-Dong R, Ness DG, Grumet C. Segregation and mapping analysis of polymorphic class I restriction fragments: detection of a novel fragment. Science 1983; 222(4619):72-4.

15. Barker D, Schafer M, White R. Restriction sites containing CpG show a higher frequency of polymorphism in human DNA. Cell 1984; 36(1): 131-8.

16. Bidwell JL Bidwell EA, Savage DA, Middleton D, Klouda PT, Bradley BA. A DNA-RFLP typing system that positively identifies serologically well-defined and ill-defined HLA-DR and DQ alleles, including DRw10. Transplantation 1988; 45(3): 640-6.

17. Carlsson B, Wallin J, Bohme J, Moller E. HLA-DR-DQ haplotypes defined by restriction fragment analysis. Correlation to serology. Human Immunol 1987; 20(2): 95-113.

18. Angelini G, Preval CD, Gorski J. Mach B. High-resolution analysis of the human HLA-DR polymorphism by hybridization with sequence-specific oligonucleotide probes. Proc Natl Acad Sci USA 1986; 83(17): 4489-93.

19. Gorski J, Niven MJ, Sachs JA, Mach B, Cassell PG, Festenstein H, et al. HLA-DRα, -DXα and DRβlll gene association studies in DR3 individuals. Human Immunol 1987; 20(4):273-8.

20. Amar A, Holbeck SL, Nepom GT. Specific allelic variation among linked HLA class II genes. Transplantation 1987; 44(6):831-5.

21. Le Gall I, Millaseau P, Dausset J, Cohen D. Two DRβ allelic series defined by exon II specific synthetic oligonucleotide genomic hybridization: A method of HLA typing? Proc Natl Acad Sci USA 1986; 83(20):7836-40.

22. Tiercy JM, Gorski J, Betuel H, Freidel AC, Gebuhrer L, Jeannet M, et al. DNA typing of DRw6 subtypes: Correlation with DRB1 and DRB3 alleleic sequences by hybridization with oligonucleotide probes. Hum Immunol 1989; 24(1):1-14.

23. Ucla C, van Rood JJ, Gorski J, Mach B. Analysis of HLA-D micropolymorphism by a simple procedure: RNA oligonucleotide hybridization. J Clin Invest 1987; 80(4):1155-9.

24. Freeman SM, Noreen HJ, Bach FH. Oligonucleotide probing. Applications to HLA typing. Arch Pathol Lab Med 1988; 112(1):22-7.

25. Middleton D, Savage DA, Cullen C, Martin J. Discrepancies in serological tissue typing revealed by DNA techniques. Transplant Int 1988; 1(3): 161-4.

26. Mytilineos J, Scherer S, Opelz G. Comparison of RFLP-DRβ and serological HLA-DR typing in 1500 individuals. Transplantation 1990; 50(5): 870-3.

27. Opelz G, Mytilineos J, Scherer S, Dunckley H, Trejaut J, Chapman J, et al. Survival of DNA HLA-DR typed and matched cadaver kidney transplants. Lancet 1991; 338(8765):461-3.

28. Bidwell JL, Bidwell EA, Wood NAP. HLA-DR and -DQ typing by DNA-RFLP analysis. In: Hui KM, Bidwell JL, Eds: Handbook of HLA typing techniques. London, CRC Press, 1993:71.

29. Ploegh HL, Orr HT, Strominger JL. Molecular cloning of a human histocompatibility antigen cDNA fragment. Proc Natl Acad Sci USA 1980; 77(10):6081-5.

30. Lee JS, Trowsdale J, Bodmer WF. cDNA clones coding for the heavy chain of human HLA-DR antigen. Proc Natl Acad Sci USA 1982; 79(2):545-9.

31. Long EO, Wake CT, Gorski J, Mach B. Complete sequence of an HLA-DR β chain deduced from a cDNA clone and identification of multiple non-allelic DR β chain genes. EMBO J 1983; 2(3):389-94.

32. Wiman K, Larhammaer D, Claesson L, Gustafsson K, Schenning L, Bill P, et al. Isolation and identification of a cDNA clone corresponding to an HLA-DR antigen beta chain. Proc Natl Acad Sci USA 1982; 79(6):1703-7.

33. Strachan T, Sodoyer R, Damotte M, Jordan BR. Complete nucleotide sequence of a functional class I HLA gene. HLA-A3: implications for the evolution of HLA genes. EMBO J 1984; 3(4):887-94.

34. Sodoyer R, Damotte M, Delovitch TL, Trucy J, Jordan BR, Strachan T. Complete nucleotide sequence of a gene encoding a functional human class I histocompatibility antigen (HLA-CW3). EMBO J 1984; 3(4): 879-85.

35. Arnot D, Lillie JW, Auffray C, Kappes D, Strominger JL. Inter-locus and intra-alleleic polymorphisms of HLA class I antigen gene mRNA. Immunogenetics 1984; 20(3):237-52.

36. Figueroa F, Klein J. The evolution of MHC Class II genes. Immunol Today 1986; 7: 3, 78.

37. Gregersen PK, Todd JA, Erlich HA, Long E, Servenius Bo, Choi E, et al. First domain sequence diversity of DR and DQ subregion alleles. In: Dupont B, editors. Immunobiology of HLA. New York, Springer-Verlag, 1989: 1027.

38. Saiki R, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, et al. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 1985; 230(4732):1350-4.

39. Mullis KB, Faloona F. Specific synthesis of DNA in vitro via a polymerase catalysed chain reaction. Meth Enzymol 1987; 155:335-50.

40. Gyllensten UB, Erlich HA. Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. Proc Natl Acad Sci USA 1988; 85(20):7652-6.

41. Ennis PD, Zemmour J, Salter RD, Parham P. Rapid cloning of HLA-A, B cDNA by using the polymerase chain reaction: frequency and nature of errors produced in amplification. Proc Natl Acad Sci USA 1990; 87:2833.

42. Innis MA, Gelfand DH. Optimization of PCRs. In: Innis MA, Gelfand DH, Sninski JJ, White TJ, Eds: PCR Protocols. London: Academic Press, 1990: 3.

43. Higuchi R, Kwok S. Avoiding false positives with PCR. Nature 1989; 339:237.

44. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, et al. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988; 239:487.

45. Millar SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988; 15:859.

46. Saiki RK, Bugawan TL, Horn GT, Mullis KB, Erlich HA. Analysis of enzymatically amplified β-globin and HLA-DQα DNA with allele-specific oligonucleotide probes. Nature 1986; 324:163.

47. Saiki R, Chang CA, Levenson CH, Boehm CD, Kazazian HH, Erlich HA. Rapid genetic analysis of enzymatically-amplified DNA with non-radioactive allele-specific oligo-nucleotide probes. Am J Human Genetics 1987; 41:A237.

48. Bugawan TL, Horn GT, Long CM, Mickelson E, Hansen JA, Ferrara GB, et al. Analysis of HLA-DP alleleic sequence polymorphism using the in vitro enzymatic DNA amplification of DP-α and DP-β loci. J Immunol 1988; 141:4024.

49. Horn GT, Bugawan TL, Long CM, Erlich HA. HLA class II antigens and susceptibility to insulin-dependent diabetes mellitus. Proc Natl Acad Sci USA 1988; 85:6012.

50. Morel C, Zwahlen F, Jeannet M, Mach B, Tiercy JM. Complete analysis of HLA-DQB1 polymorphism and DR-DQ linkage disequilibrium by oligonucleotide typing. Hum Immunol 1990; 29:64.

51. Erlich HA, Scharf SJ, Long CM, Horn GT. Analysis of isotypic and allotypic sequence variation in the HLA-DRb region using the in vitro enzymatic amplification of specific DNA segments, In: Dupont B. Ed: Immunobiology of HLA. New York: Springer-Verlag, 1989: 181.

52. Baxter-Lowe LA, Hunter JB, Casper JT, Gorski J. HLA gene amplification and hybridization analysis of polymorphism. J Clin Invest 1989; 84:613.

53. Nevinny-Stickel C, Bettinotti MdIP, Andreas A, Hinzpeter M, Muhlegger K, Schmitz G, et al. Non-radioactive HLA class II typing using polymerase chain reaction and digoxigenen-11-2'-3' dideoxyuridine-triphosphate-labelled oligonucleotide probes. Human Immunol 1991; 31:7.

54. Shaffer AL, Falk-Wade JA, Tortorelli V, Cigan A, Carter C, Hassan K, et al. HLA-DRw52- associated DRB1 alleles: Identification using polymerase chain reaction-amplified DNA, sequence-specific oligo-nucleotide probes, and a chemiluminescent detection system. Tissue Antigens 1992; 39:84.

55. Gao X, Fernandez-Vina M, Shumway W, Stastny P. DNA typing for class II HLA antigens with allele-specific or group-specific amplification. I. Typing for subsets of HLA-DR4. Human Immunol 1990; 27: 40.

56. Caillat-Zucman S, Garchon HJ, Bach JF. Automation of large-scale HLA class II oligotyping using a robotic work station. In: Tsuki K, Aizawa M, Sasasuki T, editors. HLA 1991. Oxford: Oxford University Press, 1992:346.

57. Kafatos FC, Jones WC, Efstratiadis A. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridisation procedure. Nucleic Acids Res 1979; 7:1541.

58. Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 1975; 98:503.

59. Ng J, Hurley CK, Carter C, Baxter-Lowe LA, Bing D, Chopek M, et al. Large-scale DRB and DQB1 oligonucleotide typing for the NMDP registry: progress report from year 2. Tissue Antigens 1996; 47:21.

60. Noreen H, Steiner L, Davidson M, Johnson S, Segall M, Begovich AB. Six new DPB1 alleles identified in a study of 1,302 unrelated bone marrow donor-recipient pairs. Tissue Antigens 1997; 49:512.

61. Bugawan TL, Begovich AB, Erlich HA. Rapid HLA-DPB typing using enzymatically amplified DNA and non-radioactive sequence-specific oligo-nucleotide probes. Immunogenetics 1990; 32: 231.

62. Buyse I, Decorte R, Baens M, Cuppens H, Semana G, Emonds MP, et al. Rapid DNA typing of class II HLA antigens using the polymerase chain reaction and reverse dot blot hybridization. Tissue Antigens 1993; 41: 1.

63. Parham P. Typing for class I HLA polymorphism: past, present and future. Eur J Immunogenet 1992; 19: 347.

64. Fernandez-Vina MA, Falco M, Sun Y, Stastny P. DNA typing for HLA Class I alleles: I. Subsets of HLA-A2 and of A28. Human Immunol 1992; 33: 163.

65. Tiercy JM, Djavad N, Rufer N, Speiser DE, Jeannet M, Roosnek E. Oligotyping of HLA-A2, -A3, and B44 subtypes: Detection of subtype incompatibilities between patients and their serological matched unrelated bone marrow donors. Human Immunol 1994; 41: 207.

66. Hill AVS, Allsop CEM, Kwiatkowski D, Anstey NM, Greenwood BM, McMichael AJ. HLA class I typing by PCR: HLA-B27 and an African B27 subtype. Lancet 1991; 337: 640.

67. Dominguez O, Coto E, Martinez-Naves E, Choo SY, Lopez-Larrea C. Molecular typing of HLA-B27 alleles. Immunogenetics 1992; 36: 277.

68. Sakkas LI, Vaughan RW, Panayi GS, Welsh KL. Application of polymerase chain reaction (PCR) to HLA-C locus typing. Eur J Immunogenet 1991; 18: 185.

69. Oh SH, Fleischhauer K, Yang SY. Isoelectric focusing subtypes of HLA-A can be defined by oligonucleotide typing. Tissue Antigens 1993; 41:135-142.

70. Yoshida AJ, Kimura A, Numano F, Sasazuki T. Polymerase chain reaction based analysis of polymorphism in the HLA-B gene. Human Immunol 1992; 34:257-266.

71. Allen M, Liu L, Gyllensten U. A comprehensive polymerase chain reaction-oligonucleotide typing system for the HLA class I A locus. Human Immunol 1994; 40:25.

72. Gao X, Jakobsen IB, Serjeantson SW. Characterization of the HLA-A polymorphism by locus-specific polymerase chain reaction amplification and oligonucleotide hybridization. Human Immunol 1994; 41:267.

73. Cereb N, Maye P, Lee S, Kong Y, Yang SY. Locus-specific amplification of HLA class I genes from genomic DNA: locus-specific sequences in the first and third introns of HLA-A, -B and -C alleles. Tissue Antigens 1995; 45: 1.

74. Middleton D, Williams F, Cullen C, Mallon E. Modification of an HLA-B PCR-SSOP typing system leading to improved allele determination. Tissue Antigens 1995; 45:232.

75. Fleischhauer K, Zino E, Bordignon C, Benazzi E. Complete generic and extensive fine specificity typing of the HLA-B locus by the PCR-SSOP method. Tissue Antigens 1995; 46:281.

76. Williams F, Mallon E, Middleton D. Development of PCR-SSOP for HLA-A typing of bone marrow registry donors. Tissue Antigens 1997; 49: 61.

77. Ishikawa Y, Tokunaga K, Tanaka H, Nishimura M, Muraoka M, Fujii Y, et al. HLA-A null allele with a stop codon, HLA-A*0215N, identified in a homozygous state in a healthy adult. Immunogenetics 1995; 43: 1.

78. Magor KE, Taylor EJ, Shen SY, Martinez-Naves E, Valiante NM, Wells RS, et al. Natural inactivation of a common HLA allele (A*2402) has occurred on at least three separate occasions. J Immunol 1997; 158: 5242.

79. Uryu N, Maeda M, Ota M, Tsuji K, Inoko H. A simple and rapid method for HLA-DRB and -DQB typing by digestion of PCR-amplified DNA with allele specific restriction endonucleases. Tissue Antigens 1990; 35: 20.

80. Maeda M, Murayama N, Ishii H, Uryu N, Ota M, Tsuji K, et al. A simple and rapid method for HLA-DQA1 genotyping by digestion of PCR-amplified DNA with allele specific restriction endonucleases. Tissue Antigens 1989; 34: 290.

81. Maeda M, Uryu N, Murayama N, Ishii H, Ota M, Tsuji K, et al. A simple and rapid method for HLA-DP genotyping by digestion of PCR-amplified DNA with allele specific restriction endonucleases. Human Immunol 1990; 27: 111.

82. Olerup O. HLA class II typing by digestion of PCR-amplified DNA with allele-restriction endonucleases will fail to unequivocally identify the genotypes of many homozygous and heterozygous individuals. Tissue Antigens 1990; 36: 83.

83. Yunis I, Salazar M, Yunis EJ. HLA-DR generic typing by RFLP. Tissue Antigens 1991; 38:78.

84. Ota M, Seki T, Fukushima H, Tsuji K, Inoko H. HLA-DRB1 genotyping by modified PCR-RFLP method combined with group-specific primers. Tissue Antigens 1992; 39:187.

85. Nomura N, Ota M, Tsujik, Inoko H. HLA-DQB1 genotyping by a modified RFLP method combined with group-specific primers. Tissue Antigens 1991; 38:53.

86. Ota M, Seki T, Nomura N, Sigimura K, Mizuki N, Fukushima H, et al. Modified PCR-RFLP method for HLA-DPB1 and -DQA1 genotyping. Tissue Antigens 1991; 38: 60.

87. Sawitzke AD, Sawitzke AL, Ward RH. HLA-DPB typing using co-digestion of amplified fragments allows efficient identification of heterozygous genotypes. Tissue Antigens 1992; 40: 175.

88. Nomura N, Ota M, Kato S, Inoko H, Tsuji K. Severe acute graft-versus-host disease by HLA-DPB1 disparity in recombinant family of bone marrow transplantation between serologically HLA-identical siblings: an application of the polymerase chain reaction-restriction fragment length polymorphism method. Human Immunol 1991; 32:261.

89. Nieto A, Tobes R, Martin J, Pareja E. Allele walking: a new and highly accurate approach to HLA-DRB1 typing. Application to DRB1*04 alleles. Tissue Antigens 1997; 49: 141.

90. McGinnis MD, Conrad MP, Bouwens AGM, Tilanus MGJ, Kronick MN. Automated, solid-phase sequencing of DRB region genes using T7 sequencing chemistry and dye-labeled primers. Tissue Antigens 1995; 46: 173.

91. Vooter CEM, Rozemuller EH, de Bruyn-Geraets D, van der Zwan AW, Tilanus MGJ, van der Berg-Loonen EM. Comparison of DRB sequence-based typing using different strategies. Tissue Antigens 1997; 49:471.

92. Olerup O, Zetterquist H. HLA-DR typing by PCR amplification with sequence-specific primers (PCR SSP) in 2 hours: An alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. Tissue Antigens 1992; 39: 225.

93. Olerup O, Zetterquist H. DR "low-resolution" PCR-SSP typing - a correction and an up-date. Tissue Antigens 1993; 41: 55.

94. Newton CR, Graham A, Heptinstall LE. Analysis of any point mutation in DNA. The amplification refractory system (ARMS). Nucleic Acids Res 1989; 17: 2503.

95. Park MS, Tonai R. Phenotype frequencies of the class II (DR, DQ) DNA alleles by the patterns of sequence-specific primer mixtures (SSPM) in four different populations and the probable haplotypes between DRB1 allele and DQB1 allele. In: Terasaki PI, Cecka M, Eds: Clinical Transplants 1992. Los Angeles, UCLA Tissue Typing Laboratory, 1992 p. 475.

96. Lo YMD, Mehal WZ, Wordsworth BP, Chapman RW, Fleming KA, Bell JI, et al. HLA typing by double ARMS. Lancet 1991; 338: 65.

97. Mytilineos J, Lempert M, Middleton D, Williams F, Scherer S, Opelz G. HLA class I DNA typing of 215 "HLA-A, -B, -DR zero mismatched" kidney transplants. Tissue Antigens 1997; 50: 355.

98. Savel-koul PHM, de Bruyn-Geraets DP, van den Berg-Loonen EM. High resolution HLA-DRB1 SSP typing for cadaveric donor transplantation. Tissue Antigens 1995; 45:41.

99. Browning MJ, Krausa P, Rowan A, Bicknell DC, Bodmer JG, Bodmer WF. Tissue typing the HLA-A locus from genomic DNA by sequence-specific PCR: comparison of HLA genotypes and surface expression on colorectal tumor cell lines. Proc Natl Acad Sci USA 1993; 90:2842.

100. Krausa P, Browning MJ. A comprehensive PCR-SSP typing system for identification of HLA-A locus alleles. Tissue Antigens 1996; 47: 237.

101. Sadler AM, Petronzelli F, Krausa P, Marsh SG, Gutteridge MG, Browning MJ, et al. Low resolution DNA typing for HLA-B using sequence-specific primers in allele- or group-specific ARMS/PCR. Tissue Antigens 1994; 44: 148.

102. Guttridge MG, Burr C, Klouda PT. Identification of HLA-B35, B53, B18, B5, B78 and B17 alleles by the polymerase chain reaction using sequence-specific primers (PCR-SSP). Tissue Antigens 1994; 44:43.

103. Olerup O. HLA-B27 typing by group-specific PCR amplification. Tissue Antigens 1994; 43: 253.

104. Bunce M, Welsh KI. Rapid DNA typing for HLA-C using sequence-specific primers (PCR-SSP): identification of serological and non-serologically defined HLA-C alleles including several new alleles. Tissue Antigens 1994; 43: 7.

105. Bunce M, Fanning GC, Welsh KI. Comprehensive, serologically equivalent DNA typing for HLA-B by PCR using sequence-specific primers (PCR-SSP). Tissue Antigens 1995; 45: 81.

106. Bunce M, O'Neill CM, Barnardo MCNM, Krausa P, Browning MJ, Morris PJ, et al. Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers. (PCR-SSP). Tissue Antigens 1995; 46: 355.

107. Olerup O, Aldener A, Fogdell A. HLA-DQB1 and -DQA1 typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours. Tissue Antigens 1993; 41: 119.

108. Fass SJ, Menon R, Braun ER, Rudert WA, Trucco M. Sequence-specific priming and exonuclease-released fluorescence detection of HLA-DQB1 alleles. Tissue Antigens 1996; 48: 97.

109. Albis Camps M, Ludeck H, Slateva K, Blasczyk R. The taqman fluorotyping technique - a new way for differential automated detection of amplified products in the HLA-A, B and C PCR-SSP. Eur J Immunogenet 1997; 24: 126. (Abstract).

110. Bidwell JL, Hui KM. Human HLA-DR/Dw allotype matching by analysis of HLA-DRB gene PCR product polymorphism (PCR "Fingerpints"). Technique 1990; 2: 93.

111. Clay TM, Bidwell JL, Howard MR, Bradley BA. PCR-fingerprinting for selection of HLA matched unrelated marrow donors. Lancet 1991; 337: 1049.

112. Bidwell JL, Clay TM, Wood NAP. Rapid HLA-DR-Dw and DP matching by PCR fingerprinting and related DNA heteroduplex technologies. In: Hui KM, Bidwell JL, Eds: Handbook of HLA typing techniques. London, CRC Press, 1993 99.

113. Uhrberg M, Hinney A, Enczmann J, Wernet P. Analysis of the HLA-DR gene locus by temperature gradient gel electrophoresis and its application for the rapid selection of unrelated bone marrow donors. Electrophoresis 1994; 15: 1044.

114. Williams F, Meenagh A, Maxwell AP, Middleton D. Allele resolution of HLA-A using oligonucleotide

probes in a two-stage typing strategy. Tissue Antigens 1999;54: 59-68.

115. Middleton D, Williams F, Hamill MA, Meenagh A. Frequency of HLA-B alleles in a Caucasoid population determined by a two-stage PCR-SSOP typing strategy. Human Immunology 2000; 61: 1285-1297.

116. Williams F, Meenagh A, Patterson C, Middleton D. Molecular diversity of the HLA-C gene identified in a Caucasian population. Human Immunology 2002; 63: 602-613.

117. Middleton D, Hughes DJ, Williams F, Graham CA, Martin J, Savage DA. A new DRB1 allele DRB1*1107 - a combination of DRB1*11 and DRB1*03. Tissue Antigens 1993; 42: 160.

118. Curran MD, Williams F, Little AM, Rima BK, Madrigal JA, Middleton D. Aberrant splicing of intron 1 creates a novel null HLA-B*1501 allele. Tissue Antigens 1999; 53: 244.

119. Todd JA, Bell JI, McDevitt HO. HLA-DQ gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. Nature 1988; 329:599.

120. Khalil I, d'Auriol L, Gobet M, Morin L, Lepage V, Deschamps I, et al. A combination of HLA-DQ beta Asp57 negative and HLA-DQ alpha Arg52 confers susceptibility to insulin-dependent diabetes mellitus. J Clin Invest 1990; 85: 1315.

121. Thorsby E. Invited anniversary review: HLA associated diseases. Human Immunol 1997; 53: 1.

122. Nelson JL, Mickelson E, Masewicz S, Barrington R, Dugowson C, Koepsell T, et al. Dw14 (DRB1*0404) is a Dw4-dependent risk factor for rheumatoid arthritis. Tissue Antigens 1991; 38: 145.

123. Gregersen PK, Silver J, Winchester RJ. The shared epitope hypothesis: an approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum 1987; 30: 1205.

124. Thorsby E, Lundin KEA, Ronningen K, Sollid LM, Vartdal F. Molecular basis and functional importance of some disease-associated HLA polymorphisms. Tissue Antigens 1989; 34:39.

125. Williams F, Middleton D, Savage D, Gorodezky C, Wilson DW, Fitzgerald JM, et al. Development of PCR-SSOP for the identification of HLA-A*02 subtypes and determination of HLA-A*02 frequencies within different ethnic populations. Tissue Antigens 1997; 49: 129.

126. Sengar DPS, Couture RA, Raman S, Jindal SL, Lazarovits AI. Role of class I and Class II HLA antigens in cadaveric renal transplantation. Transplant Proc 1987; 19: 3422.

127. Middleton D, Savage D, Trainor F, Taylor A. Matching for various HLA class II loci in cadaveric renal transplantation using DNA techniques. Transplantation 1992; 53: 1138.

128. Middleton D, Mytilineos J, Savage D, Ferrara GB, Angelini G, Amoroso A, et al. Matching for HLA-DPB1 alleles in zero mismatched HLA-A, -B, -DR renal transplants. Transplant Proc 1992; 24: 2439.

129. Middleton D, Mytilineos J, Deufel A, Williams F, Opelz G. Impact of HLA-DPB "epitope" matching in kidney graft survival. Eur J Immunogenet 1997; 24: 54. (Abstract).

130. Opelz G. Importance of HLA antigen splits for kidney transplant matching. Lancet 1988; 2:61.

131. Sidney J, Grey HM, Kibo RT, Sette A. Practical, biochemical and evolutionary implications of the discovery of HLA class I supermotifs. Immunol Today 1996; 17: 261.

132. Petersdorf EW, Longton GM, Anasetti C, Martin PJ, Mickelson EM, Smith AG, et al. The significance of HLA-DRB1 matching on clinical outcome after HLA-A, B, DR identical unrelated donor marrow transplantation. Blood 1995; 86: 1606.

133. Grundschober C, Rufer N, Adami N, Breur-Vriesendorp B, Jeannet M, Roosnek E, et al. Sequence of a new HLA-B7 variant, B*0707 that differs from the common B*0702 allele by one single residue in the peptide binding groove. Tissue Antigens 1997; 49: 508.

134. Gilman AL, Auger JJ, Terry L, Fomenberg N, Baxter-Lowe LA. HLA-B14 subtypes and their influence on allorecognition. Tissue Antigens 1996; 47: 253.

135. Rufer N, Breur-Vriesendorp B, Tiercy JM, Gauchat-Feiss D, Shi X, Slavcev A, et al. HLA-B35 subtype mismatches in ABDR serologically matched unrelated donor-recipient pairs. Hum Immunol 1994; 41: 96.

136. Gauchat-Feiss D, Rufer N, Speiser D, Jeannet D, Roosnek E, Tiercy JM. Heterogeneity of HLA-B35: oligotyping and direct sequencing for B35 subtypes reveals a high mismatching rate in B35 serologically compatible kidney and bone marrow donor/recipient pairs. Transplantation 1995; 60: 869.

137. Rufer N, Roosnek E, Kressig R, Jeannet M, Tiercy JM. Sequencing of a new HLA-B41 subtype (B*4102) that occurs with a high frequency in the Caucasoid population. Immunogenetics 1995; 41: 333.

138. Fleischhauer K, Keman NA, O' Reilly RJ, Dupont B, Yang SY. Bone marrow allograft rejection by host-derived allocytotoxic T lymphocytes recognizing a single amino acid at position 156 of the HLA-B44 class I antigen. N Engl J Med 1990; 323: 1818.

139. Hurley CK. Acquisition and use of DNA-based HLA typing data in bone marrow registries. Tissue Antigens 1997; 49: 323.

140. Southern M. DNA chips: analysing sequence by hybridization to oligonucleotides on a large scale. Trends in Genetics 1996; 12: 110.

141. Arguello JR, Little A-M, Pay AL, Gallardo D, Rojas I, Marsh SG, et al. Mutation detection and typing of polymorphic loci through double-strand conformation analysis. Nat Genet 1998; 18(2): 192-4.