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REVIEW ARTICLE

BASICS OF HUMAN LEUKOCYTE ANTIGENS (HLA) SYSTEM AND ITS APPLICATION IN CLINICAL SPECIALTIES

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ABSTRACT: Human leukocyte antigen (HLA) is complex immune system with many applications in transfusion and transplant medicine. A lot of clinical challenges such as febrile non-hemolytic transfusion reactions, transfusion-related acute lung injury, and graft-versus-host-disease are caused by HLA antibodies. Histocompatibility in transplant scenario is not the only function of HLA antigens but the main role is to present peptides to immune system and regulate cellular and humoral immunity for the fate of graft. HLA Class I (A, B, and C) and HLA Class II (DR, DQ, and DP) antigens are different in structure and function. Their typing methods have progressed from serology-based techniques to sequence-based typing to next-generation sequencing. Cross-matching techniques have also changed from complement-dependent cytotoxicity to microbead‑based assay to flow cytometry. Finally, HLA and its disease association has long been established, particularly so in cases of autoimmune disorders. The article will be helpful for beginners to get introduced about this unique system and principles in transplant immunology.

KEYWORDS: Human Leukocyte Antigen, Major Histocompatibility Complex, Transfusion, Transplantation

INTRODUCTION:

Human leukocyte antigen (HLA) is a specialized branch of immunology dealing with histo-compatibility. This article is intended to serve as an introduction to the basic concepts of HLA, its role in presentation of peptides to the immune system and coordinating cellular and humoral immunity and clinical applications viz. transfusion, transplantation, and Immunogenetics. It is important in transfusion-associated problems such as platelet refractoriness, febrile non-hemolytic transfusion reactions (FNHTRs), transfusion-related acute lung injury (TRALI), and graft versus host disease (GvHD).

In 1930, early work of Gorer on antigens responsible for allograft rejection in inbred mice led to the discovery of Major Histocompatibility Complex (MHC) [1]. Its human counterpart is termed as HLA.

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Jean Dausset (in 1954) observed that patients who had multiple blood transfusions showed allo-leukoagglutinins produced by infusion of cells bearing allo-antigens not present in recipient.[2] Also, seven patients with multiple transfusions agglutinated leukocytes from about 60% of the French population but not the leukocytes of those seven patients.[3] Payne et al observed patients with febrile nonhemo-lytic transfusion reactions (FNHTR) often contained leukoagglutinins.[4] Later microdropletlympho-cytotoxicity test was introduced by Terasaki et al in 1964 at the First International Histocompatibility Workshop (IHW) [5,6] Eventually HLA-typing was aided with computer programs by Rood and Leeuwen to study the serologic complexities. [7] In 1967, the term HLA was approved by WHO Committee.[8]

It was thought that HLA antigens were coded by two closely linked loci, each coding for multiple alleles[9,10,11] until a third locus was defined (codes 10 different Ags). Additional research disclosed that lymphocytes from two different individuals would undergo blast transformation and divide when mixed and cultured in vitro; known as the mixed lymphocyte reaction (MLR).[12, 13] It was discovered that MLR was negative when leukocytes from a pair of HLA-identical siblings were mixed together, indicating that HLA gene products were responsible for MLR activity with a technique called mixed lymphocyte culture (MLC).[14] In 7th IHW 'D' and 'DR' locus were defined serologically.[15] For the work on MHC and genetic control of immune response Snell, Dausset and Benacerraf were awarded Nobel Prize for Medicine in 1980. Since then the field of HLA is been getting flourished continuously with researches, newly added information and clinical applications.

STRUCTURAL, BIOCHEMICAL ASPECTS, NOMENCLATURE AND PROPERTIES OF HLA SYSTEM:

- ***General structure:***

HLA is a series of closely linked groups of genes (3Mbp stretch in chromosome 6p; 21.1-21.3) and its protein products expressed on leukocytes that determine major histocompatibility factor (surface Ags) responsible for recognition and elimination of foreign tissues (**Figure 1.**). HLA loci are multi-allelic (gene occupying the locus can be any one of several alternate forms). As each allele determines a distinct product/ antigen the system is highly polymorphic. The system bears many unique properties as well discussed subsequently. HLA is divided into three regions:

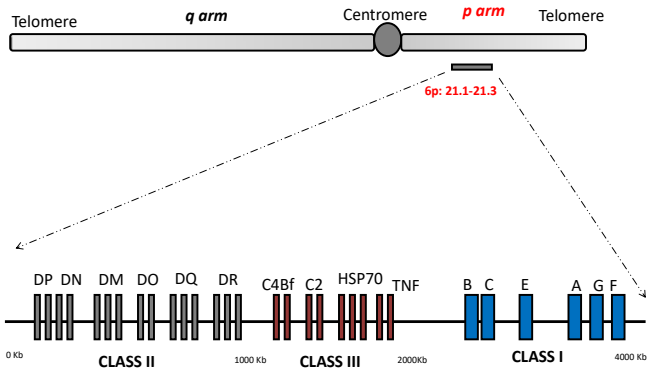

Figure 1. Structure of HLA gene products class I and II molecules

Class-I region: encodes genes for classic/ major histocompatibility molecules i.e. HLA-A, B (most polymorphic), and C. Non-classic genes are minor and their exact role is yet in research viz. E, F, and G (present on placental trophoblast). Class-I proteins are present on most of nucleated cells, in plasma and adsorbed on platelet surface. A vestigial amount remained on mature red cell are Bennett-Goodspeed Ags (Bg^a, Bg^b, Bg^c which are HLA-B7,-B17, -A28).

Class-II region: encodes genes for HLA-DR, -DP, and -DQ composed of both α and β chains. DP molecules are the product of DPA1 and DPB1 alleles; DPB2 and DPA2 are pseudo genes (genes

with mutations that prevent gene activation or transcription). DQ molecules are the product of DQA1 and DQB1 alleles. DR molecules use DRA but can use alleles coded by DRB1 (classic DR specificities), DRB3 (DR52 molecules), DRB4 (DR53), and DRB5 (DR51). It also codes for 2 minor proteins i.e**.** DM and DO, used in the internal processing of antigens. They load the antigenic peptides from pathogens onto the HLA molecules of antigen-presenting cells (APCs). Class-II proteins occur only on APCs, macrophages, endothelial cells, activated T cells, and B cells.

Class-III region: encodes structurally and functionally diverse molecules, including C2, C4, Bf (the complement factors), 21-hydroxylase, and tumor necrosis factor (TNF). Also, glyoxalase-1 (GLO) and phosphoglucomutase-3 (PGM-3) are linked.[16]

- ***Biochemical nature of HLA molecules:***

a. HLA molecules: (Figure-2)

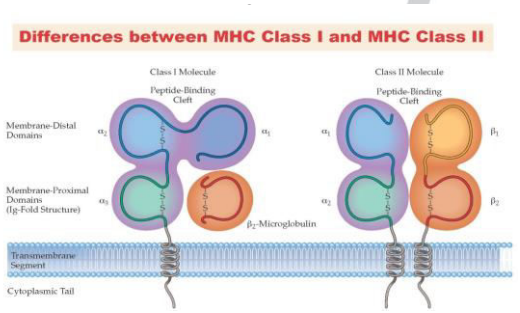

Figure 2. Location of the HLA system in human chromosome and its constituents. Print (Courtesy: Wikipedia)

They are globular glycoproteins each composed of two non-covalently linked chains. Class-I (A, B, C) molecules consist of a heavy chain (MW-45kD) coded by chromosome-6 associated non-covalently with β_2-microglobulin (coded by chromosome-15), a non-polymorphic protein (MW-12kD) found in serum and urine. Out of three α domains, α_1 and α_2 domains contain the polymorphic regions conferring antigen specificity. The heavy chain folds into three domains and is inserted through the cell membrane via a hydrophobic sequence.[17]

Class-II molecules consist of two similar-sized chains (MW-33kD α and 28-kD β) associated non-covalently throughout their extracellular portions. In these molecules, both chains are inserted through the membrane via hydrophobic regions. The extracellular portions of these chains fold into two domains.[18, 19] Class-I and II molecules are also alike in that most of the polymorphism is expressed in the portion of the molecule farthest from the cell membrane.[20]

Bjorkman elucidated 3D structure of HLA-A_2 molecule via X-ray crystallography and showed α_1 and α_2 domains forms peptide-binding site. It holds processed peptides for presentation to T-cells. In class-II both chains contribute to form this groove. [21]

The surface topography (by 3D-configuration) is large, irregular, containing multiple, non-repeating sites i.e. antigenic determinants or Epitopes that are potentially immunogenic. An epitope is estimated to involve a minimum of 5-6 amino acid residues, but larger sequences may also often present. Serologically defined epitopes are located primarily in and around the peptide groove and are finite. The epitopes recognized by T-lymphocytes are less precisely mapped, very numerous, and distinct from the serologically defined ones.[22]

b. Natural Killer (NK) Cell Receptors:

Different receptors on NK-cells provide activating or inhibitory signals in response to target cells. NK-cells utilize killer cell immunoglobulin-like receptors (KIRs) to distinguish the unhealthy targets from a healthy self.[23, 24]

c. MHC-Class I Chain-related Protein A and B (MICA and MICB):

They are highly polymorphic genes located in Class-III region having structural homology with classical

HLA-molecules except there are no binding to β2-microglobulin and no functional peptide-binding groove. They are expressed on epithelial (gastrointestinal tract), and endothelial cells, fibroblasts, monocytes, dendritic cells, and function as stress-induced antigens.[25]

- ***Nomenclature:***

Before 1991 IHW, provisionally identified specificities carried the initial letter w (for "workshop") inserted between locus letter designation and temporary antigen specificity number.[26] Now it is retained to distinguish between HLA-C locus and complements. With the introduction of molecular techniques numbers of alleles are increasing. There are currently 27,273 HLA and related alleles included in IPD-IMGT/HLA Database (last update: March 2020).[27] The complex nomenclature includes:

1. HLA- designates MHC
2. A capital letter indicates a specific locus (A, B, C, D, etc.) or region. All genes in the D-region are prefixed by letter D and followed by a second capital letter indicating the subregion (DR, DQ, DP, DO, DN, etc.)
3. Loci coding for the specific Class-II α and β peptide chains are identified next (A1, A2, B1, B2)
4. Specific alleles are designated by * followed by a two-digit numeral defining the unique allele. Following two, a two-digit numeral defines the variant of the specific allele. The * signifies that specificity is typed by molecular technique only. E.g. serologically defined HLA-B27 specificity is actually made up of seven distinct allelic variations; now defined as HLA-B *2701 through *2707.
5. A fifth digit, such as HLA-Cw *02021 and *02022 indicates that two variants differ by a silent nucleotide substitution but not in amino acid sequence. Digits five through six denote any synonymous mutations within the coding frame of the gene. The seventh and eighth digits distinguish mutations outside the coding region (exon).
6. Letters such as L (low expression), N (null), Q (questionable role), or S (secreted) may follow an allele's designation to specify an expression level or other non-genomic data known about it.
7. Thus, a completely described allele may be up to 9 digits long, not including HLA-prefix and locus notation. (**Figure-3**)

HLA-A*02:101:01:02:N

Signs	Meaning
HLA	HLA as prefix for the system
-	Hyphen to separate gene from prefix
A	Gene
*****	Separator
02	Allele group
:	Field separators
101	Specific HLA protein
01	Synonymous DNA substitution in Exon (coding region)
02	Differences in Intron (non-coding region)
N	Expression level of gene, additional information

Figure 3. HLA nomenclature (molecular, current in use)

- ***Some important properties of the HLA system***

a. Inheritance:

The entire set of A, B, C, DR, DQ, and DP antigens on one chromosome is called a *haplotype* which is inherited by children as a unit/ *en block*. Genetic crossovers and recombination in the HLA region are uncommon (<1%).[28] Alleles are expressed co-dominantly i.e there is an equal expression of both alleles. Each person has two alleles for each locus. An individual can be heterozygous (two different alleles on one locus) or homozygous (both alleles on that locus are the same).

Figure-4a illustrates the segregation of HLA-haplotypes in a family. The two haplotypes of the father as a and b, and of the mother as c and d. Each offspring inherits two haplotypes, one from each parent. Thus, only four possible haplotypes: ac, ad, bc, and bd can be found in the offspring. It can be calculated that 25% of the offspring will have identical HLA-haplotypes, 50% will share one HLA-haplotypes, and 25% will share no HLA-haplotypes.

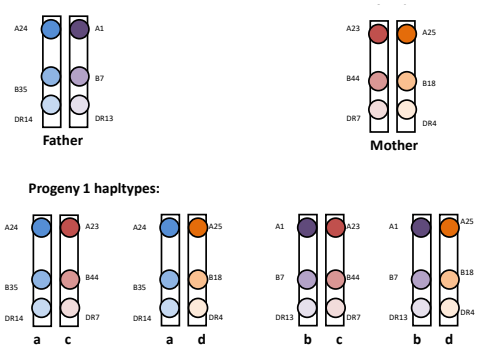

Figure 4a. HLA-haplotypes inheritance

A parent and child can share only one haplotype, making an identical match between the two unlikely. It should also be apparent that uncles, grandparents, and cousins are very unlikely to have identical haplotypes. These are important factors in searching for a well-matched organ/ blood donor. It is necessary to evaluate HLA-antigen composition in prospective donor-recipient pairs before transplantation since pre-sensitization to HLA-antigens may cause rapid rejection.[29]

b. Crossing Over:

An event that infrequently complicates HLA-typing interpretation and haplotype determination is crossing over (or recombination). **Figure-4b** explains that exchange of genetic material occuring during meiosis between the paired chromosomes. The farther apart two loci are on a given chromosome, the more likely it is that genetic exchanges will occur e.g. recombination between HLA-A and HLA-DP occurs commonly, whereas between HLA-DQ and HLA-DR is a rare event. Crossing over has the effect of rearranging the genes on the chromosome to produce new haplotypes unique in a defined population.

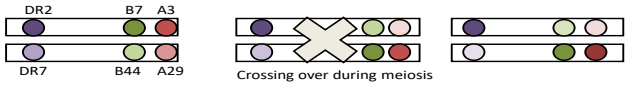

Figure 4b. Recombination event between HLA-B and HLA-DR

c. Linkage Disequilibrium:

It is the occurrence of HLA-genes more frequently in the same haplotype than would be expected by chance alone. In a randomly mating population at Hardy-Weinberg equilibrium, the occurrence of two alleles from closely linked genes will be the product of their individual gene frequencies. If the observed value of the joint frequency is significantly different from the expected frequency (the product of the individual allele frequencies), the alleles are said to be in linkage disequilibrium.[30]

d. Cross Reactivity:

It is a phenomenon in which an antiserum directed against one HLA-antigenic determinant reacts with other HLA-antigenic determinants as well. Cross-reactive antigens share important structural elements with one another but retain unique, specific elements.[31-33] Dausset et al suggested that antibodies might detect public specificities shared by multiple HLA-gene products.[34] The broadly reactive antibodies used in van Rood and Leeuwen's original computer-derived HLA clusters also defined many of the currently defined major cross-reactive groups (CREGs). Based on these reactions, most specificities can be grouped into major CREGs (**Table-1**.).[35, 36] e.g. the HLA-A locus antigens A2, A23, A24, and A28 share a common determinant and therefore make up the A2 CREG. The majority of HLA-alloantibodies are IgG. Antibodies to HLA molecules can be divided into two groups:

1. Those that detect a single HLA-gene product ("private" antibodies binding to an epitope unique to one HLA-gene product)
2. Those that detect more than one HLA-gene product. These may be "public" (binding to epitopes shared by more than one HLA-gene product) or cross-reactive (binding to structurally similar HLA-epitopes).[37]
3. The monoclonal HLA antibody (MoAb) is produced by fusing HLA antibody-producing B cells with plasmacytoma lines.[38]

Table 1. The broadly reactive antibodies used in van Rood and von Leeuwen's original computer-derived HLA clusters

Major CREGs	Associated alleles
A1	A1, 3, 36, 9(23, 34), 10(25,26,34,66), 11, 19(29, 30, 31, 32, 33), 28(68,69), 43, 74, 80
A2	A2, 9(23,24), 28(68,69), B17
B5	B5(51,52), 15(62,63,75,76,77), 17(57,58), 18, 35, 12(49,50), 53, 70(71,72),78, 37
B7	B7, 42, 22(54,55,56), 27, 40(60,61), 13, 41,46,47,48,81,37,73
B8	B8, 14(64,65), 16(38,39), 18, 59, 67
B12	B12(44,45), 221(49,50), 13, 40(60,61), 41, 48, 82
4c	A9(23,24), A25, A32, Bw4
6c	Bw6, Cw1, Cw3, Cw7

- ***Antigenic Splits:***

Improvement in serologic methods allowed antigens believe to represent a single specificity to be "split" into specificities that were serologically distinct.[39] e.g. HLA-B5, -B51, -B52. The parent is written in parentheses like "HLA-B51(5)."

- ***Polymorphism:***

Polymorphism at the HLA loci is extreme, evolved to counter the different peptides. Each molecule differs from the other in its amino-acid sequence. As of March 2020, the total allele number has reached 26889. HLA-A, HLA-B, and HLA-C have 6082, 7256, and 5842 alleles, respectively. Class-II contains 7302 alleles.[40] However, polymorphism is also population specific. In the Indian population, the common haplotypes are A26-B8-DR3, A1-B57-DR15, A2-B44-DR15, A2-B60-DR15. Two loci haplotypes, i.e., A11-B35/A24-B40/DR3-DQ2/DR15-DQ6/DR4-DQ3 are observed with appreciable frequency.[41] From the transplantation perspective, it is quite difficult to match HLA-types between two populations.

PATHOPHYSIOLOGICAL ROLE OF CLASS-I AND II MOLECULE:

MHC allows T-cells to bind, recognize, and tolerate itself (autorecognition). It is also the chaperone for intracellular peptides that are complexed with MHCs and presented to T-cell receptors (TCRs) as potential foreign antigens. MHC interacts with TCR and its co-receptors to optimize binding conditions for the TCR-antigen interaction, in terms of antigen-binding affinity, specificity, and signal transduction effectiveness. The MHC-peptide complex is a complex of auto/alloantigen. After binding, T-cells should in principle tolerate auto-antigen but activate when exposed to alloantigen. Disease states occur when this principle is disrupted.(**Figure-5A**)

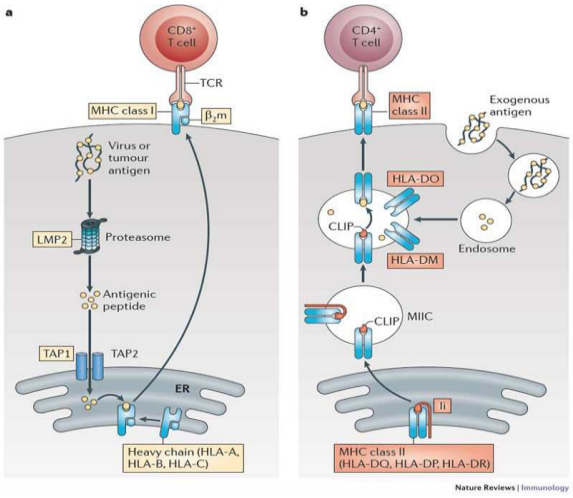

Figure 5A. Patho-physiological role of the HLA system. print Courtesy: Nature reviews

- ***Antigen presentation:***

MHC molecules bind to both TCR and CD4/CD8 co-receptors on T-lymphocytes, and antigen epitope held in the peptide-binding groove interact with variable Ig-like domain of the TCR to trigger T-cell activation.[42]

- ***Tissue allorecognition*:**

MHC molecules in complex with peptide epitopes are essential ligands for TCRs. T-cells become activated by binding to the peptide-binding grooves of any MHC molecule that they were not trained to recognize during positive selection in the thymus.

Peptides are processed and presented by two pathways (**Figure -5B**)

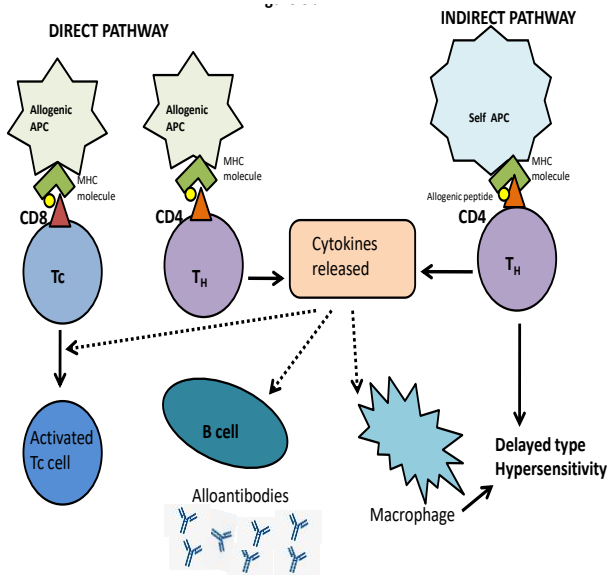

Figure 5B. Allo-recognition pathways

a. In Class-II, phagocytes take up entities by phagocytosis into phagosomes through B-cells exhibit the more general endo-cytosis into endosomes to fuse with lysosomes whose acidic enzymes cleave the uptaken protein into many different peptides. With physicochemical dynamics in molecular interaction with the particular MHC Class-II variants borne by the host, encoded in the host's genome, a particular peptide exhibits immuno-dominance and loads onto MHC Class-II molecules. These are trafficked to and externalized on the cell surface.[43]

b. In Class-I, any nucleated cell normally presents cytosolic peptides, mostly self-peptides derived from protein turnover and defective ribosomal products. During viral infection, intracellular microorganism infection, or cancerous transformation, such proteins degraded in the proteosome are as well loaded onto Class-I molecules and displayed on the cell surface. T-lymphocytes can detect a peptide displayed at 0.1%-1% of the MHC molecules

- ***From transplantation perspective:***

a. Direct alloantigen recognition occurs when T-cells bind directly to an intact allogeneic MHC molecule on graft (donor) APC

b. Indirect allogenic recognition occurs when allogenic MHC from the graft is taken up and processed by recipient APCs and peptide fragments of allo-MHC containing polymorphic amino-acids residue are bound and presented by self(recipient) molecule

c. In short,Class-I molecules process endogenous peptides via CD8+ T-cells whereas Class-II molecules process exogenous peptides via CD4+ T-cells

TECHNIQUES OF HISTOCOMPATIBILITY TESTING:

HLA laboratories perform various tests to support transplant programs including HLA-typing of the recipient and prospective donor, cross-matching, antibody screening, and identification. Serologic cross-matching is performed by cytotoxicity and flow cytometric techniques.[44]

- ***HLA Antigen Detection:***

Agglutination methods initially used to define the HLA-complex have been succeeded by a precise microlymphocytotoxicity test.[45] It requires 1-2 µL of serum from ACD/ phenol-free heparinized blood. A purified lymphocyte suspension is prepared by layering whole blood on a Ficoll-Hypaque gradient and centrifugation. Lymphocytes collected at the gradient's interface are harvested, washed, and adjusted to appropriate test concentrations. HLA-A, B, C typing is performed on this lymphocyte suspension. Class-II typing requires purified B-lymphocyte suspension generally prepared by Nylon wool separation [46, 47] and Fluorescent labeling.[48]

In the lymphocytotoxicity test (LCT), isolated lymphocytes are added to specific HLA typing sera. If the lymphocytes contain antigen against specific antisera (Class I/II), Ag-Ab binding will take place and added complement (rabbit derived) causes membrane damage. The damaged cells allow uptake of vital stains such as eosin. Under the inverted phase-contrast microscope identification of the stained cells is counted and the presence of specific antigen is assigned after grading and scoring. Inherent drawbacks of tests are cross-reactivity, unavailability of monospecific antisera, difficult identification due to weak expression of Ag, and the possibility of false-positive (FP) and false-negatives (FN). MoAbs can circumvent the problem of cross-reactivity.[49]

Nowadays, immunomagnetic beads are used to positively select (target cells rosette on beads) lymphocyte subpopulations for typing, for both classes antigens.[50] It provides rapid isolation, a high degree of purity, and the use of immunofluorescence. Nowadays, in the molecular era, HLA-typing is performed by DNA hybridization techniques.[51]

In Sequence Specific Oligonucleotides (SSO) typing PCR-amplification of a chosen sequence is carried using primers flanking that sequence. The amplified DNA is immobilized on a membrane and hybridized with selected, labeled oligonucleotide probes (dot-blot). The reverse of this technique is utilized in where probes are immobilized on membranes and hybridized with amplified DNA (reverse dot-blot). Although SSO is powerfully reliable and accurate, it has been superseded by the more rapid technique of PCR-SSP.[52-54]

In Sequence-Specific primer (SSP), oligonucleotide primers are designed to obtain amplification of specific alleles or groups of alleles. A completely matched primer will be more efficiently used in the PCR-reaction than a primer with one/ more mismatches. The assignment of alleles is based on the presence or absence of amplified products normally detected by agarose gel electrophoresis and transillumination.[55-56]

In Sequence-Based Typing (SBT) direct nucleotide sequencing of HLA genes is utilized for high-resolution typing and in the definition of a new allele. Detection is not based on the use of sequence specific oligonucleotide probe and prior knowledge of the nucleotide sequences.[51,52] Sequencing methods can be differentiated by the template, cloned or genomic DNA, and can be automated or manual. Software, which aligns the derived sequences against established libraries, facilitates the analysis and assignment of alleles. [57-60]

Currently more advanced high-throughput HLA-typing methods using Next Generation Sequencing (NGS) have been developed. NGS facilitates sequencing of the entire genomic DNA, provides complete information of all relevant HLA genes, minimum ambiguities, ability to identify new alleles, null alleles, and finally a shorter turnaround time. Null alleles are those for which no HLA products are expressed at the cell surface. Over 529 null alleles have been identified across HLA Class-I and II, e.g. A*01:01:01:02N.[61-62]

- ***HLA Antibody Detection Methods:***

The standard CDC is the most common. While for screening recipient serum samples, a more sensitive technique Amos-modified, extended incubation, or AHG should be employed. Standard CDC tests rarely detect 100% of the antigen-binding specificities of cross-reactive antibodies.[63, 64] The assay has several disadvantages viz viability of cells leading to FP-results, a necessity to maintain a reliable and consistent antigen panel that reflects the ethnic composition of the patient population, inability to detect non-complement-fixing antibodies. Also, differentiation between HLA-specific and non–HLA-specific antibodies and between IgG/ IgM antibodies is not possible.

In transplant; unknown serum (recipient) is tested against a panel of cells or soluble antigen of known HLA phenotype (say, donors). Targets from a large panel of donors must be selected if antibodies to all HLA specificities are to be detected. A panel of at least 30 carefully selected targets is required for initial screening in the determination of panel reactive antibody (PRA), and a panel of at least 60 cells is required for accurate antibody identification.[39]

Enzyme-Linked Immunosorbent Assay (ELISA) uses purified HLA antigens, instead of lymphocytes/ cells, as targets. The increased specificity of the assay offers the advantage of recognizing FP non-HLA reactions as well as distinguishing class-I and II specificities. Also, it differentiates between IgG/ IgM antibody isotypes by using an appropriate secondary antibody. ELISA is a screening assay.[39]

Flow Cytometry detects antibody binding directly. Complement activation is not necessary. Similar to ELISA, flow screening can distinguish IgG/IgM antibodies using secondary antibodies. It can detect non-complement-fixing antibodies because the binding of the antibodies rather than complement fixation is measured. It utilizes T and B-lymphocytes as targets or, in a newer technique, purified HLA antigens coated onto microparticles of 2-4µm in diameter.[39] e.g. In the Luminex platform. Microparticles/beads coated with purified class-I or II antigens attaches with antibodies present in patient sera specifically. After incubation of serum with beads, followed by staining with a fluorescently tagged anti-human IgG, the anti-HLA IgG-positive serum shows a fluorescent channel shift as compared with the negative serum termed as MFI (Median Fluorescent intensity). %PRA is represented by the % of pooled beads that react positively with the serum. In the current situation, the Luminex platform is highly popularized and is been used by most laboratories across the world.

- ***HLA-Crossmatch Techniques:***

a. **CDC-XM:** It is most widely used technique because of rapid and reproducible results, small volumes of antisera and cells. The primary purpose of cross-matching before transplantation/ transfusion is to identify antibodies in the recipient to antigens present on the prospective donor to avoid graft rejection.
b. **Flow-cytometry XM (FCXM):** This sensitive method facilitates the detection of low levels of antibodies in potential recipients. Certain cases of irreversible rejection during the first few days after transplantation may be a result of low levels of antibody undetected by less sensitive techniques.[65, 66] Bray et al observed that FCXM is highly sensitive in detecting donor HLA-antibodies.[67]
c. **Microbeads based Immunoassay (Luminex-XM):** This solid-phase immunoassay, detects only antibodies directed against HLA-antigen. It can detect donor-specific antibodies (DSA) without the requirement for donor HLA-typing. The introduction of solid-phase assays led to a second revolution in histocompatibility

testing with the use of purified antigens bound to artificial surfaces. It has augmented sensitivity and specificity to detect even low titer antibodies important in chronic rejection of the graft.[68]

d. **Virtual-XM**: Determination of DSA "virtually" by comparison of HLA-antibody specificities of the recipient with the HLA-typing of the donor, softwares are available. e.g. HLA-Matchmaker.[69]

CLINICAL SIGNIFICANCE OF HLA SYSTEM:

As mentioned by Erik Thorsby, histocompatibility is not the only function of HLA-antigens, this complex may better be called major immune response complex.[70] The HLA system is still of primary clinical importance in transfusion and transplantation with wide application in human genetics and disease associations.

- ***Role in Paternity testing:***

It involves the analysis of genetic markers from the mother, child, and alleged father to determine whether the tested man could be the biological father of a child. Combinations of various genetic markers are used, including RBC markers and enzymes, serum proteins, HLA typing, and DNA testing.[71]

- ***Role in Disease Association: (Table-2)***

Table 2. Disease association with HLA genes

Diseases	HLA association	RR
Celiac disease	DQ2	>250
Ankylosing spondylities	B27	>150
Narcolepsy	DQ6	>38
Subacute thyroiditis	B35	14
Type 1 diabetes	DQ8	14
Multiple sclerosis	DR15, DQ6	12
Rheumatoid arthritis	DR4	9
Juvenile rheumatoid arthritis	DR8	8
Grave's disease	DR17	4

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Although many diseases associated with HLA have a relative risk value > 2, none exceed the strength of the association of HLA-B27 with ankylosing spondylitis. To date, 530 diseases have been studied.[72] The exact cause for the association of HLA to disease is unclear. This susceptibility may somehow be related to altered immunologic responsiveness, multiple gene interactions, and environmental factors. Although the study of HLA and disease associations is very important in understanding disease susceptibility and manifestation, HLA alone is not clinically useful as a diagnostic tool. It has been determined that HLA-antigens are associated with disease susceptibility to a greater extent than any other known genetic marker.[72] Data from these studies show weak association of disease and relative risks of less than 2.[73]

In Indain population HLA-A1, HLA-B8, and HLA-DR17 haplotypes are frequently associated with autoimmune diseases. The reported prevalence of HLA-B27 in AS and other spondyloarthropathies varies from 18%–94% as compared to 1.4%–8%. HLA-B*27:05 was the most common subtype associated with AS in India.[74, 75]

- ***Role in Platelet Transfusion:***

The incidence of HLA-alloimmunization and platelet refractoriness in repeated transfusion recipients is as high as 20-71%.[76] Immune mechanisms are established after the exclusion of nonimmune causes such as sepsis, DIC, drug, and hypersplenism. Immune-mediated platelet refractoriness is usually caused by antibodies against HLA-antigens, platelet-specific (PSA) or ABH antigens.[77] Leukocyte reduction to $<5 \times 10^6$ in blood products can reduce or prevent HLA-alloimmunization which is achieved by third-generation leukocyte filters.

Alloimmunization to the HLA results in refractoriness to platelet transfusions since HLA-I antigens are expressed variably on platelets.[78-82]

Refractoriness is failure to achieve a rise in the circulating platelet count 1hour after the infusion of adequate numbers of platelets. Considering the highly polymorphic nature of the HLA system, it is impossible to obtain sufficient numbers of HLA-typed donors to provide HLA-matched platelets for alloimmunized patients.

Duquesnoy et al demonstrated that platelet transfusions from donors mismatched only for cross-reactive antigens can effectively provide hemostasis for refractory patients termed as permissible mismatch platelets (**Table-3**) e.g. an HLA-A1, B7; -A11, B22 recipient might benefit from the platelets of an A1, B7; A3, B27 donor because A3 and A11 and B27 and B22 are crossreactive. As a result of these observations, the donor pool necessary to sustain an HLA-matched platelet program can be reduced from 8000-10,000 to manageable numbers of 2000-5000.[83]

Table 3. Match grades for HLA matched platelets (Ref: aabb,17th edition)

Grade	Antigen match level	Probable outcome
A	4 antigen match	Ideal match
B	2 or 3 antigen match Unmatched antigen is unknown/ cross-reactive	Adequate response with variable increments considering associated clinical factors
B1U	1 antigen unknown/ blank	
B1X	1 cross-reacting group	
B2UX	1 antigen blank and 1 antigen cross-reactive	
B2X	2 antigen cross-reactive	
C	1 mismatched antigen	Poor to no response similar to unmatched
D	2 or more mismatched antigens	

Despite a perfect HLA match a poor recovery may be a result of sensitization to non-HLA antigens e.g. PSA. In contrast, excellent transfusion results are at other times obtained in the presence of a complete HLA mismatch. Good recovery may be a function of:

1. A restricted pattern of alloimmunization, private versus public Abs

2. Variable expression of HLA-antigens on the platelet surface

3. Leukocytes are more immunogenic than platelets, and refractoriness is probably initiated by HLA-antigens on the contaminating leukocytes. Evidence for this is based on a study by Brand et al in which they demonstrated a decreased rate of alloimmunization to RDPs when contaminating leukocytes were removed before transfusion.[84] Herzig and colleagues were also able to improve the transfusion response to HLA-matched platelets by removing the leukocytes.[85] Most institutes follow a basic algorithm for a prospective suitable donor in the refractory recipient is shown in the **Figure 6**.

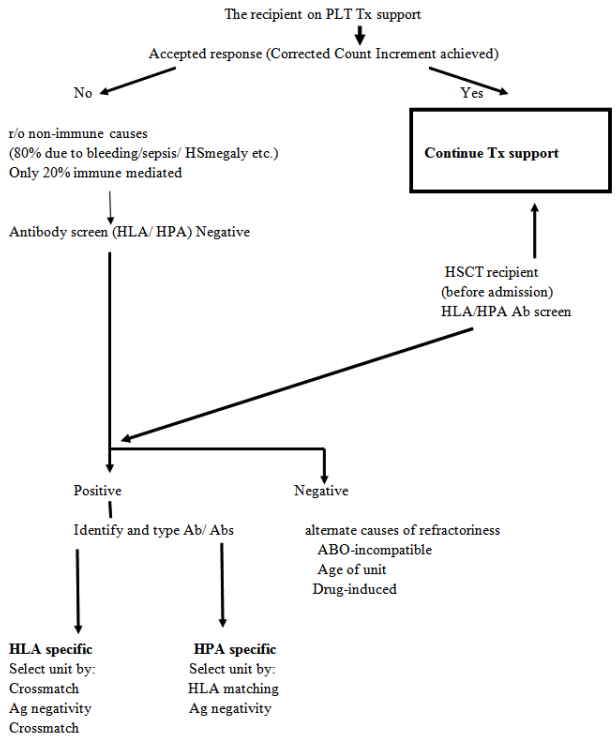

Figure 6. Approach to a patient of thrombocytopenia (with suspected platelet transfusion refractory patient)

- ***Role in Febrile Non-hemolytic Transfusion Reactions (FNHTRs):***

It is a temperature rise of >1°C above the baseline during or strongly after transfusion. Antibodies against HLA, HPA, HNA have been implicated in the pathogenesis. Recipient antibodies react with transfused Ags leading to release of cytokines (IL-1) causing fever.[16]

- ***Role in Transfusion Related Acute Lung Injury (TRALI)**:*

TRALI an acute non-cardiogenic pulmonary edema (NCPE) developed in response to transfusion is caused by passively transferred antibodies against HLA Class-I and II or HNA (mostly 3a).[86] Antibodies are usually in plasma of transfused components, less commonly in recipients.[87] The reaction probably activates complement cascade leading to neutrophil aggregation and sequestration in the lungs with the release of neutrophil granules and vascular damage. HLA laboratories can play a role in such situations by typing donor-recipient and interpreting corresponding passively transferred antibodies that have caused TRALI events in the recipient. Also, it will guide whether to keep the donor in donor-pool or not. This application has stimulated many centers to follow the 'Male donor/ nulliparous women plasma policy only'.

- ***Role in Transplantation*:**

When an immunosuppressed individual receives competent allogeneic T-lymphocytes from transfused blood products, these lymphocytes mount an immune attack against the recipient's cells causing TA-GvHD.[88] It has also been observed in patients with no apparent immunosuppression. This is due to a one-way HLA match (rejection direction) and a one-way HLA mismatch in GvHD direction. In this case, the donor is homozygous for HLA loci while the recipient is heterozygous for the same antigen as shown in Figure. For GvHD to occur Bilingham's criteria holds best. There is no effective treatment for GvHD and the only way is prevention.[89] Gamma irradiation (Cobalt/ Indium or x-rays) of the cellular blood products at a dose of 15-25 Gray (maximum 50) is the effective way of inactivating donor lymphocytes.

a. Role in Hematopoietic Stem Cell Transplantation (HSCT):

HSCT is used to treat patients with severe aplastic anemia, immunodeficiency disease, and various forms of leukemia. Since 1980, there has been a change in the proportion of patients treated with bone marrow transplantation for malignant versus nonmalignant diseases.[90]

Data from the Fred Hutchinson Cancer Research center indicate that the risk of graft rejection, GVHD, and death increase with one serologically defined antigen-mismatch, multiple allele mismatches, lower marrow cell doses, and positive serum cross match.[91] Data on unrelated donor transplantation from the National Marrow Donor Program (NMDP) and the Japanese Marrow Donor Program are similar.[92, 93]

In India most HLA-labs search a suitable donor based on a negative DSA-XM before transplant to minimize chances of antibody mediated graft rejection. In our laboratory, out of 18 haplo-transplants 12.5% recipient had HLA antibodies with positive DSA-XM with one patient having both class antibodies and had graft failure. Desensitization of these antibodies before transplant had better survival.

Because of the continuous difficulty of finding well-matched related (1/3) and unrelated (50%) donors for HSCT, umbilical cord blood transplantation (UCBT) has increased over the past two decades[28] because of the functional and phenotypic immaturity of UCB lymphocytes and/or the reduced T-cell dose for reduced alloreactivity. The limiting factor of UCBT is cell dosage in one UCB unit. UCB does is of paramount importance in engraftment and survival after unrelated UCBT.[28]

NMDP registry was associated with AABB, ARC, CCBC, and has more than 50lakhs registered donors. In India, registries are limited e.g. MDRI (Marrow Donor registry of India) and DATRI.

b. Role in Kidney Transplant:

Transplantation is preferred over dialysis in treating patients with chronic renal failure (CRF) and end-stage renal disease (ESRD) because it is more cost-effective, and it usually returns patients to a state of relatively normal health. The best graft survival rates are obtained from HLA-identical, ABO-compatible siblings, but such donors are available for relatively few patients.[94, 95] Three general strategies are used to minimize graft rejection: Immunosuppressive agents, reduction of graft foreignness and, induction of tolerance.

Immunosuppressive agents such as Azathioprine, Prednisone, Thymoglobulin, Cyclosporine, and Tacrolimus are used to diminish the destructive immunologic responses to the graft. These agents are nonselective and carry risks of serious side effects, especially life-threatening infection.[96]

Antigen disparities that most influence graft rejection include the ABO-blood group antigens and HLA antigens. Although it is still not clear what combinations of HLA gene products promote optimal graft survival rates, it is evident that 0 and 1 antigen mismatches result in increased graft survival.[97]

In highly sensitized recipients, it is necessary to match for HLA-A and HLA-B because of the presence of class-I HLA antibodies. Sanfilippo and colleagues found that matching based on public cross-reactive antigens can provide the same association with graft outcome as private antigens. For highly sensitized recipients identification of antibodies is important.[98]

Two modalities, plasmapheresis and intravenous immune globulin (IVIG) are used in the treatment of rejection post and pre-transplant desensitization of the patient.[99] Plasmapheresis has been demonstrated to remove HLA-specific antibodies in many different clinical settings.[100, 101] IVIG has been used to modulate immune responses and suppress alloantibody. Several groups have had success using IVIG to decrease levels of anti-HLA antibody and to lower PRA among highly sensitized patients awaiting transplantation.[102, 103]

c. Role in Heart transplantation:

It is used to treat cardiomyopathies and end-stage ischemic heart disease. Because of the organ's extremely short total ischemic time (3 hours for hearts, compared with 72 hours for kidneys), HLA matching is not feasible. Total ischemic time is the amount of time during which there is no blood flow through the organ. The single most important HLA pretransplant test is the HLA-antibody screen. Recipients with no preformed HLA antibodies receive transplants without crossmatching. Those with preformed HLA antibodies require pretransplant cross matches to determine recipient-donor compatibility.

In a retrospective study by Yacoub et al, an additive effect of class I and II matching on graft survival was observed. Matching for class II antigens had a marked influence on increased graft survival, whereas matching for class I antigens alone had no influence on the outcome. [104]

d. Role in Orthotopic Liver transplantation:

It has become an established and successful therapeutic modality for patients with end-stage liver disease. Immunologic factors in recipient/donor matching for liver transplantation and recipient pre-sensitization have largely been ignored in the past. The consequences of HLA pre-sensitization and ABO incompatibility were recently underlined in two reports.

In the first, a retrospective analysis of preformed HLA-antibodies demonstrated 1-year graft survival of 40% in the pre-sensitized individuals as compared with 83% in the non-sensitized individuals. In the second, the survival of patients with emergency ABO-incompatible transplants was 30% compared with 76% in patients with emergency ABO-compatible grafts and 80% in patients with elective ABO-compatible grafts. [105-107]

e. Role in Lung Transplant:

Emphysema and cystic fibrosis account for the majority of double-lung transplants. The major indications for single-lung transplantation include pulmonary fibrosis (33%) and emphysema (41%). Also, single-lung transplants for primary hypertension are being performed instead of heart-lung transplantation. Short cold ischemic times for lungs, as with hearts, preclude prospective histocompatibility testing. However, the HLA matching between donor and recipient may play an important role in live-donor lung transplantation (2 -3 %) in an attempt to improve post-transplant conditions and graft survival rates.[108]

f. Role in Pancreas and Islet Cell transplant:

The primary indication for pancreas transplantation is diabetes. The majority of pancreas transplants performed are SPK (simultaneous pancreas/kidney transplants) 81%, with PAK (pancreas after kidney) 12% and PAT (pancreas alone transplant) 5%. HLA matching, as reported by one of the largest pancreas transplant centers, has a major effect on graft survival, particularly in PAK and PATs.[109] Because of increased risks of myocardial complications with pancreas transplantation, islet cell transplantation (ICT) has been actively pursued.

Although ICT is technically simple, difficulty has been encountered in the achievement of sustained engraftment due to insufficient cell numbers. To address this issue, a protocol was initiated in Alberta, Edmonton, to transplant islets from multiple donors to attain sufficient islet mass. Sufficient islet mass

was attained by transplanting islets from two donor pancreases in 6/7 patients.[110] To date, the effect of HLA matching has not been studied, but data are being stored for future analyses.

- ***Future perspective:***

Many reviews and protocols from different institutes summarizes the clinical evidence for HLA matching and donor selection in unrelated hematopoietic stem cell transplantation according to the resolution and loci of different HLA mismatches and also discusses special situations such as unidirectional mismatches, permissible HLA mismatches, and HLA matching in cord blood transplantation. Predicted Indirectly ReCognizable HLA Epitopes (PIRCHE) is a bioinformatic approach to predict potentially immunogenic peptides deriving from mismatched HLA allotypes in patients and their donors in transplantation medicine, which are presented in shared HLA molecules. In theory, low numbers of PIRCHE may correlate with reduced alloreactivity as compared to high numbers of PIRCHE.[111] In the way HLA still is that complicated system to be dealt cautiously to get better outcomes in transfusion and transplantations with a fine balance between donor and recipient pair.

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