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Direct quantitative measurement of the kinetics of HLA-specific antibody interactions with isolated HLA proteins

Sunil Daga[a,b,i,*], Harry Moyse[c], David Briggs[d], David Lowe[e], Neil Evans[c], James Jones[e], Rico Buchli[g], Curtis McMurtrey[f], Arend Mulder[h], William Hildebrand[f], Frans Claas[h], Robert Higgins[a,i], Daniel A. Mitchell[f,1], Daniel Zehnder[a,i,1]

[a] Renal Department, UHCW NHS Trust, Coventry, UK
[b] Renal Department, St. James's University Hospital, Leeds, UK
[c] School of Engineering, University of Warwick, Coventry, UK
[d] H & I department, NHSBT, Birmingham, UK
[e] H & I department, Royal Liverpool University Hospital, Liverpool, UK
[f] University of Oklahoma Health Science Center, Oklahoma City, OK, United States
[g] Pure Protein LLC, Oklahoma City, OK, United States
[h] Immunohaematology and Blood Transfusion, Leiden University Medical Center, 2333 ZA Leiden, The Netherlands
[i] Warwick Medical School, University of Warwick, Coventry, UK

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ABSTRACT

HLA specific antibodies vary in their pathogenicity and this is likely to be the net effect of constant chain usage, quantity, specificity, and affinity. Here we have measured the affinity of human monoclonal antibodies for a range of HLA proteins. Purified antibodies and ligands allowed dynamic interactions to be measured directly by surface plasmon resonance. Physiochemical differences between pairs of ligands were quantified using electrostatic mismatch and hydrophobic mismatch scores.

All antibodies were characterized by fast on-rates and slow off rates but with a wide range of association rates ($k_{on,}$ 3.63–24.25 × 10^5 per mol per second) and dissociation rates (k_{off}, 0.99–10.93 × 10^{-3} per second). Dissociation constants (K_D) ranged from 5.9 × 10^{-10} M to 3.0 × 10^{-8} M. SN320G6 has approximately a twenty-fold greater affinity for HLA A2 compared with SN607D8, but has a similar affinity for HLA-A2 and B57. In contrast, SN607D8 has greater than a twofold greater affinity for HLA-A2 compared with A68. Similarly, WK1D12 has about a threefold greater affinity for HLA-B27 compared with B7. The higher affinity interactions correlate with the specificity of stimulating antigen. This is the first study to directly measure the binding kinetics and affinity constants for human alloantibodies against HLA.

1. Introduction

HLA specific antibodies are a relative barrier to solid organ transplantation. Higher levels, which typically precipitate a cytotoxic crossmatch, can cause hyperacute rejection [1]. Lower levels may be tolerated or be associated with acute rejection and/or poor outcome [2–6]. Post-transplantation, the appearance of donor HLA-specific antibodies (DSA) is often, but not always, associated with acute or chronic rejection and transplant glomerulopathy [3,7–10]. The ability of an HLA-specific antibody or antibody mixture (e.g. serum) to activate the classical pathway of the complement system appears to be related antibody pathogenicity, although this functionality is not necessarily proportional to measured antibody level [11–14]. Antigen specificity (or the degree of expression of the target antigen) is a further important criterion related to pathogenicity as, for example, transplantation can be successful in the presence of cytotoxic positive crossmatches due to HLA Class II antibodies [15].

The specificity of HLA antibodies is defined by reaction patterns against different alleles sharing specific amino acid residues [16]. These are generally considered to be the nominal epitopes allowing binding of the antibody and tend to be reduced to the minimal number of required residues, sometimes to a single amino acid. It has been suggested that the critical interactions for binding involve those residues within a 3 Å diameter patch, termed an eplet [17,18]. However, the exact

* Corresponding author at: Renal Department, St. James's University Hospital, LTH NHS Trust, Leeds, UK.
E-mail address: sunildaga@nhs.net (S. Daga).
[1] Equal contributions.

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conformation and orientation of the interaction has yet to be shown for any HLA protein-anti-HLA antibody complex [19]. The footprint of the antibody complementarity determining regions (CDRs) can be up to 900 Å^2 in surface area [20,21], and therefore a larger number of amino acid residues within the HLA protein, perhaps up to 25, will likely be involved in determining the nature of the interaction, imparting both specificity and affinity. Importantly, the reactivity of HLA proteins with antibody can vary even when sharing the critical epitope/eplet. It is speculated that the difference in reactivity is due to a difference in release of binding energy which is proportional to the total surface area of interaction [22,23]. The immunogenicity of the HLA antigen, defined as its ability to evoke/stimulate a *de novo* antibody response can also vary and is believed to be related to electrostatic and hydrophobic properties of amino acid residues within polymorphic regions of the HLA protein [24–26].

Current solid phase single antigen bead assays measure mean fluorescence intensity (MFI) and this readout is often used to estimate amounts of antibodies, although this has not been validated. High value MFIs will have components relating to both higher absolute antibody concentrations and to stronger antibody binding (affinity). Resolving the distinction between concentration and affinity is important because affinity measurements are likely to provide a greater insight into antibody function. During the immune response, the affinity of antibodies typically increases by affinity maturation. This is generated via somatic hypermutation of immunoglobulin gene segments, leading to clonally selected variations in amino acid sequences that favor increased binding to target antigens [27]. Only one study has previously determined the affinity of HLA antibodies, and that was of mouse monoclonal antibodies against human HLA-A2 protein expressed on human B-cell lines. That study shows marked differences in affinity between F_{ab} fragments from two separate monoclonal antibodies (determined to be in the micromolar and nano-molar range, respectively), which were due to difference in the dissociation rates [28]. The authors used a saturation binding technique with radio-labeled antibodies. Our study is the first to directly measure the affinities of human allo-antibodies against HLA.

Through the use of real time biosensor techniques (by surface plasmon resonance), we have been able to measure binding kinetics and the affinity constants of antibodies against different HLA proteins that share epitopes/eplets but differ in adjacent residues. This approach was used to determine quantitatively the importance of epitope configurations in relation to affinity. Here, we used this technique to measure the affinity of clinically relevant anti-HLA antibodies.

2. Material and methods

2.1. Biotinylated soluble recombinant HLA proteins

Biotinylated soluble recombinant HLA proteins (sHLA) were provided by Pure Protein LLC (Oklahoma City, OK, USA) [29]. Products of the following genes were studied; HLA-A*01:01, A*02:01, A*68:01, B*57:01, B*07:02, and B*27:05. We will refer to these proteins as HLA-A1, A2, A68, B57, B7, and B27, respectively, for simplicity. The concentration of the purified biotinylated molecules was determined using the Micro BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL) using bovine gamma globulin (Sigma; Poole, UK) as a protein standard.

2.2. Human monoclonal HLA-specific antibodies

Human monoclonal HLA-specific antibodies (mAbs) were secreted by human hybridomas produced by Epstein-Barr virus (EBV) transformation of B-lymphocytes from HLA antibody positive multi-parous women (sensitized during pregnancy), followed by fusion and subcloning of antibody producing EBV lines (Table 1). SN607D8 and SN203G6 were derived from the same multiparous donor (HLA type A*24 A*29, B*07, B*44, C*07, C*16) sensitized during pregnancy (child's HLA type A*02, A*29, B*44, B*57). WK1D12 was derived from a female donor (HLA type A*01, B*08, C*07) sensitized during pregnancy (child's HLA type A*01, A*11, B*08, B*27, C*01, C*07).

Table 1
Characteristics of IgG human monoclonal HLA-specific antibodies. Epitope designations use the nomenclature from the HLA epitope registry (www.epregistry.br) and are based on reaction pattern rather than a known binding specificity. *Corresponding to the alleles from which the proteins were derived.

mAb	Immunizing HLA	Bead assay-defined HLA specificities*	Epitope designation	Isotype
SN607D8	A2	A*02:01/03/06, A*68:01/02, A*69:01	142TKH	IgG1, κ
SN203G6	A2 and/or B57	A*02:01/03/06, B*57:01/03, B*58:01	62GE	IgG1, λ
WK1D12	B27	B*27:05/08, B*07:02, B*13:02, B*40:01/02/06, B*81:01	163EW	IgG1, κ

Antibody-containing supernatants were dialyzed (8000 MWCOSpectra/Por® Dialysis) against PBS overnight. The supernatants were further processed by affinity chromatography using HLA protein immobilized on Sepharose beads [29] and ion-exchange chromatography using Q-Sepharose. HLA-A2 (from A*02:01) and B7 (from B*07:02) were separately coupled to cyanogen bromide activated Sepharose and the resulting columns were used to enrich mAbs SN203G6 and SN607D8, and WK1D12 respectively. Overall this approach yielded highly purified monoclonal IgG and avoided contamination with residual bovine IgG and other serum components from the hybridoma culture medium. The homogeneity of the purified IgG was confirmed via SDS-PAGE using 4–12% gradient polyacrylamide gels. The concentration of purified antibodies was determined using Lowry's assay [30]. CDC defined antibody specificities were confirmed in a single antigen bead assay (One Lambda, Canoga Park, CA) as per manufacturer's instructions. Dose response curves from antibody doubling dilution series were used to calculate effective concentrations that gave 50% of maximal MFI signal (EC-50). Hill-type EC-50 values were calculated using curve fitting performed in MATLAB.

2.3. Surface plasmon resonance

Sensorgrams were obtained using the Bio-Rad Proteon XPR36 biosensor platform [13,14]. 2.5 μg/ml of biotinylated HLA protein was immobilized on neutravidin-coated sensor chips (ProteOn™ NLC Sensor Chip #1765021) with a flow rate of 25 μl/min over 300 s. Purified monoclonal HLA-specific antibodies were flowed over the chip at a rate of 25 μl/min (slowest rate for the equipment to allow maximum binding) for 960 s to obtain equilibrium phase (where the sensorgram of binding phase plateaus) and at 37 °C in the Association phase to represent physiological binding temperatures. Following this, the running buffer (PBS containing 0.05% Tween 20) was passed over for 960 s at a flow rate of 25 μl/min for the dissociation phase. Interactions of all three HLA-specific antibodies were studied in duplicate over a range of six different concentrations (3.125–100 nM) against the same surface concentration of immobilized HLA protein.

The data obtained from the sensorgrams were modelled using an implementation of differential evolution [31] on MATLAB software (Mathworks®) with the simulation tool FACSIMILE (MCPA Software) [32]. Together these allow for curve fitting and simultaneous determination of kinetic Association (k_{on}) rates, dissociation (k_{off}) rates and the Dissociation Constant K_D (as the ratio of the dissociation, k_{off}, and Association, k_{on}, rate constants).

2.4. Molecular differences between the allele pairs

The PyMOL Molecular Graphics System, Version 1.1 Schrödinger, LLC was used to model the crystallographic structures of HLA-A2 (PDB