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Kinetic epitope mapping of monoclonal antibodies raised against the *Yersinia pestis* virulence factor LcrV

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ABSTRACT

Five monoclonal antibodies, mAb7.3, mAb29.3, mAb46.3, mAb12.3 and mAb36.3, raised to the LcrV virulence factor from *Yersinia pestis* were characterised for their Fab affinity against the purified protein and their Fc affinity to Protein A/G as a proxy for the FcγR receptor. Kinetic measurements were performed label-free in a localised particle plasmon array reader. The Fc-ProteinA/G complex first-order half-life was determined for each antibody and fell in the range of 0.8–3.8 h. The Fab first-order half-lives had ranged from 3.4 to 9.2 h although two antibodies, mAb12.3 and mAb36.3, showed low affinity interactions. Competitive binding studies of mixtures of the Fab-active antibodies were performed to measure the relative binding efficiency of one antibody in the presence of the other. A geometric relative positioning of the epitopes of mAb7.3, mAb29.3 and mAb46.3 was determined based on the footprint locus of the antibody and the percentage of competitive binding. The two known protective antibodies mAb7.3 and mAb29.3 showed greater interference, indicating epitopes close to one another compared to the non-protective mAb46.3 antibody. The Fab-Fc complex half-life screen and epitope mapping are potentially useful tools in the screening of therapeutic antibodies or vaccine candidates.

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1. Introduction

Epitope mapping is an essential step in the characterisation of antibodies designed for therapeutic use (Gershoni et al., 2007). The location of the epitope on a protein is difficult to determine absolutely, with many methods identifying linear epitopes rather than conformational epitopes. However, folding proteins may not presever access to the linear epitope with three-dimensional peptide conformations controlling the charge interaction with the Fab region of the antibody. (Van Regenmortel, 1996; Zhang et al., 2011). The most common methods of epitope mapping include array-based oligopeptide scanning (Linnebacher et al., 2012), X-ray crystallography (Hill et al., 2009), phage display (Rowley et al., 2004), site-directed mutagenesis (Mata-Fink et al., 2013) and proteolysis monitored by mass spectrometry (Suckau et al., 1990). Conformational epitopes that are in close proximity to one another on the antigen surface will compete kinetically for the binding site, interfering with the simple binding kinetics model when the footprints of the antibodies overlap geometrically. Careful monitoring of the binding kinetics using label-free surface plasmon resonance (SPR) allows the kinetic interference to be determined and the relative location of the epitopes estimated.

The relative location of an epitope can in part explain why one antibody is protective whereas another antibody is not. A number of antibodies have been raised to the organism *Yersina pestis*, specifically against the low calcium response virulence protein (LcrV) encoded by the 70 kb plasmid (pYV/pCD1); proteins encoded by this plasmid effect type III secretion by the bacterium (Price et al., 1989; Williamson et al., 1995). LcrV is present on the surface of the bacterium and is critically required for the pathogenesis of plague, explaining its potency as a vaccine candidate (Broz et al., 2007; Williamson et al., 2005).

Characterisation of the Fab and Fc affinity and the relative location of the epitopes is a new technique for assessing the performance of potential immunotherapeutics, such as monoclonal antibodies specific for LcrV. The Fab affinity, specificity and cross-reactivity can be screened using an array of antigens using label-free localised plasmon technology, monitoring the kinetics of the binding events from which the association and dissociation rate constants can be derived (Olkhov et al., 2009; Olkhov and Shaw, 2008). The relative location of the epitopes can be determined from competitive binding studies of varied mAb cocktails by comparing the observed signal with the expected signal (defined as the cumulative response of the individual mAbs). Epitope close to one another will prevent the binding of antibodies

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to their close-by epitopes, excluded in space by the volume of antibody. The protein structure of an IgG antibody (Ahmed et al., 2014) indicates recognition, Fab, and effector responses, Fc, characterisation of the Fab affecting affinity and the Fc region (Ahmed et al., 2014). The Fc affinity for the IgG Fc receptor (FcγR) can be screened similarly using the bacterium evasion protein, protein A/G, as a proxy (Eliasson et al., 1989; Eliasson et al., 1988; Sikkema., 1989).

Others have demonstrated correlations between measured binding affinities and functionality. Whilst Tang et al., described a positive relationship between the Fab-affinity of IgG antibody and antibody-dependant cellular cytotoxicity (ADCC) (Tang et al., 2007), lower affinity antibodies directed at the human epidermal growth factor receptor 2 (HER2) were less susceptible to catabolism by the target cells. Here, increasing the affinity of the antibody for the target had a negative effect on target-mediated clearance by phagocytosis, but, like Tang et al., a positive effect on ADCC activity.

Label-free SPR studies has previously been demonstrated as a tool for epitope mapping (Safsten, 2009) based on the signal derived from sequential mAb injections and can be used as a platform to perform micro-array based mapping methods without the need for secondary labels (Alfthan, 1998).

This study aims to identify the location of antibody epitopes on the LcrV protein surface and to determine the affinity of the interaction between the Fab region of the mAbs raised in mice against LcrV antigen and the Fc region of the same mAbs for protein AG (PAG). A further aim was to determine if this approach to identify high affinity antibody–antigen interactions could be used more widely to aid the selection of antibodies able to elicit passive immunity.

In this paper, five mAbs (mAbs 7.3, 12.3, 29.3, 36.3, and 46.3) were studied to derive the Fab and Fc binding affinities of the antibodies and identify the epitope location *via* a novel epitope mapping technique.

2. Experimental procedures

2.1. Chemicals

Self-assembling monolayer (SAM) components: HS-$(CH_2)_{17}$-$(OC_2H_4)_3$-OH (used as a 'spacer') and HS-$(CH_2)_{17}$-$(OC_2H_4)_6$-OCH_2COOH (used as a 'linker'), were obtained from ProChimia Surfaces (Poland). N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 16-mercaptohexadecanoic acid (MHDA), bovine serum albumin (BSA) (98%), human fibrinogen (FBR) (60%, with 40% buffer salts; the protein content is $>80\%$ clottable FBR) and human serum albumin (HSA) ($>96\%$) were obtained from Sigma-Aldrich. Recombinant Protein A/G (PAG) was purchased from Pierce. Sheep polyclonal antibodies to BSA (aBSA) (23 mg/mL, IgG fraction) were supplied by AbD Serotec. Human Serum (HS) (male AB) was obtained from Biosera. For the secondary antibodies, all were polyclonal and produced in goat with anti-IgG (H+L) purchased from Bethyl labs at a concentration of 1 mg/mL.

2.2. Proteins and monoclonal antibodies

Recombinant LcrV protein (200 mg/mL) of *Y. pestis* was produced as previously described (Carr et al., 1999). All mAbs used were raised in Balb/c mice against LcrV and details of the antibody production are available (Hill et al., 1997). All of the mAbs were characterised as IgG1 isotype. mAb7.3 was produced from the splenocytes of mice immunised with SDS-treated LcrV.

2.3. Array analysis

All experiments were performed on the Array Reader platform as described in detail elsewhere (Olkhov et al., 2009; Olkhov and Shaw, 2008, 2010) but described briefly here. Changes in the localised plasmon properties of biofunctionalized nanoparticles, in a 96-spot array format, were illuminated in a total internal reflection configuration, and the scattered light was monitored in real time. Arrays are printed from an inkjet printer using a solution of 4 nm seed nanoparticles. The arrays are then removed from the printer into a developing solution where the seed nanoparticles grow to form truncated polyhedral with an overall diameter of ~120 nm. Arrays can be stored in this form until required. To create the biosensor assay surface, unfunctionalized gold nanoparticle arrays were taken from storage and cleaned with water and iso-1-propanol (IPA). They were then functionalised with 1:10 (linker:spacer) mixture of the SAM and activated with EDC:NHS for 1 h. The activated arrays were then functionalized with proteins by printing BSA, CRP, fibrinogen (1 mg/mL), PAG (2 mg/mL), F1 (1.4 mg/mL), LcrV (1 mg/mL) and the F1V fusion protein (625 μg/mL) from solutions in PBS directly over the nanoparticle containing array spots. Each assay contained 16 spots which were then averaged, reducing the observed noise and confidence in the observed signal. Recombinant proteins LcrV and PAG were used in specific assays designed to capture target antibodies from buffered solutions. Fibrinogen and BSA were used as negative control and a reference surface respectively throughout the course of experiments.

After installation, the functionalised arrays underwent washing with regeneration buffer and blocking with a 500 μL human serum albumin solution. There were five mAbs studied in total, referenced as mAb7.3, mAb12.3, mAb29.3, mAb36.3 and mAb46.3. Each mAb was tested individually and prepared following a 2-fold dilution protocol starting at 20 nM. A 4 min baseline was first collected, this was followed by a 10 min association phase and a 15 min dissociation phase before the surface was regenerated; this process was repeated for all concentrations and all mAbs. Each mAb assay was also calibrated at the start and end of the data set with a mAb7.3 10 nM injection, to account for any loss in response over the time course of the experiment.

The data are fitted using a global fit procedure based on the Langmuirian adsorption isotherm (Langmuir, 1917) allowing a surface with a finite number of binding sites to be filled over time in a 1:1 ratio. The rate equation is given by (Olkhov and Shaw, 2010)

$$\theta(t) = \frac{\vartheta(t)}{\vartheta_m} = \frac{k_a[P]}{k_a[P] + k_d}(1 - \exp(-\ k_a[P] + k_d\ t)) \quad (1)$$

where θ is the surface coverage, a fraction of the occupied binding sites; $[P]$ is the concentration of protein (antibody) in solution; and k_a and k_d are the adsorption and desorption rate constants, respectively. $\vartheta(t)$ refers to the experimental observable, namely change in scattered brightness and ϑ_m refers to maximum coverage reached for a given analyte. In the case of very strong interactions when the dissociation is negligible, $k_d \ll k_a[P]$, Eq. (1) can be reduced to the following single exponential form:

$$\vartheta \approx \vartheta_m\,(1 - \exp(-\ k_a[P]t)) \quad (2)$$

The parameter ϑ_m is used for the epitope mapping procedure to define the number of surface binding sites filled based on the analyte injection.

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