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Research paper

Long-term direct visualization of passively transferred fluorophore-conjugated antibodies

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ABSTRACT

The use of therapeutic antibodies, delivered by intravenous (IV) instillation, is a rapidly expanding area of biomedical treatment for a variety of conditions. However, little is known about how the antibodies are anatomically distributed following infusion and the underlying mechanism mediating therapeutic antibody distribution to specific anatomical sites remains to be elucidated. Current efforts utilize low resolution and sensitivity methods such as ELISA and indirect labeling imaging techniques, which often leads to high background and difficulty in assessing biodistribution. Here, using the *in vivo* non-human primate model, we demonstrate that it is possible to utilize the fluorophores Cy5 and Cy3 directly conjugated to antibodies for direct visualization and quantification of passively transferred antibodies in plasma, tissue, and in mucosal secretions. Antibodies were formulated with 1–2 fluorophores per antibody to minimally influence antibody function. Fluorophore conjugated Gamunex-C (pooled human IgG) were tested for binding to protein A, *via* surface plasmon resonance, and showed similar levels of binding when compared to unlabeled Gamunex-C. In order to assess the effect fluorophore labeling has on turnover and localization, rhesus macaques were IV infused with either labeled or unlabeled Gamunex-C. Plasma, vaginal Weck-Cel fluid, cervicovaginal mucus, and vaginal/rectal tissue biopsies were collected up to 8 weeks. Similar turnover and biodistribution was observed between labeled and unlabeled antibodies, showing that the labeling process did not have an obvious deleterious effect on localization or turnover. Cy5 and Cy3 labeled antibodies were readily detected in the same pattern regardless of fluorophore. Tissue distribution was measured in macaque vaginal and rectal biopsies. The labeled antibody in macaque biopsies was found to have similar biodistribution pattern to endogenous antibodies in macaque and human tissues. In the vaginal and rectal mucosa, endogenous and infused antibodies were found primarily within the lamina propria. In the mucosal squamous epithelium of the vaginal vault, significant antibody was also observed in a striated pattern in the superficial, nonviable, stratum corneum. Endogenous antibody distribution in both human and macaque squamous tissues exhibited a similar pattern as seen with the labeled and unlabeled antibodies. This proof-of-principle study reveals that the labeled antibody is stable and physiologically similar relative to endogenous antibody setting the stage for future work to better understand the mechanisms of how antibodies reach unique anatomical sites. Direct visualization of fluorophore-conjugated antibodies following passive infusion can be utilized to assess the kinetics of biodistribution of infused antibodies and may be a useful approach to monitor and predict efficacy of therapeutic antibodies.

1. Introduction

Monoclonal antibodies are the fastest growing sector of the therapeutic protein market (Ecker et al., 2015). Understanding how antibodies are metabolized and biodistributed following IV infusion is critical. Likewise, some therapeutic antibodies need to be delivered to specific sites, such as mucosal surfaces, or have unique characteristics such as glycoform, that facilitate optimal effector domain, and in turn

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therapeutic function (Johansson and Ekman, 1999; Hristodorov et al., 2013). Although ELISA and indirect immunofluorescence are used routinely to assess antibody localization and turnover, there are many drawbacks to using these methods. For example, false positive results from ELISA and high secondary background in tissue have made assessing IV infused IgG unreliable (Guven et al., 2014; Ivell et al., 2014; Terato et al., 2014). Thus, the development of alternative approaches to accurately measure antibody distribution and turnover is imperative.

Intravenous infusion of antibodies is actively administered in the clinic for a myriad of conditions ranging from states of immunodeficiency, cancer, infection, and autoimmunity. Multiple factors dictate how long antibodies persist in circulation and how effective they are following IV infusion. These include, but are not limited to subclass, Fc glycosylation, the neonatal Fc receptor (FcRn) and antigen binding affinities (Tabrizi et al., 2010; Zheng et al., 2011). The rate at which antibodies turn over, as well as their biodistribution, are critical components for the efficacy of protection that these antibodies possess.

Fluorophore conjugation to antibodies is not a new method; in fact Coon et al. first pioneered it back in the 1940s (Coons et al., 1941, 1942). The concept of using fluorophore-conjugated antibodies *in vivo* has been met with mixed reactions. Direct labeling of antibody is a highly sensitive method for imaging, as it does not rely on secondary techniques to visualize. However, other studies illustrate that conjugation of antibodies can alter the antibody-antigen interaction, leading to non-specific staining, with fluorophore to fluorophore variation depending on the degree of labeling (Pittman et al., 1967; Gruber et al., 2000; Vira et al., 2010).

The method described here utilizes conjugation of fluorophores to antibodies for direct fluorescent intensity measurements within plasma, mucosal secretions and tissue following IV infusion in rhesus macaques. We show that the conjugation of the fluorophore to the antibody does not noticeably affect its turnover, distribution kinetics, or localization upon IV infusion. This method has the potential for a multitude of downstream applications. For example, beyond the direct detection of infused antibodies, this method could potentially identify optimal bioavailability of therapeutic proteins by monitoring their behavior in competitive or multiplexed infusion experiments.

2. Materials and methods

2.1. Ethics statement

All macaques (*Macaca mulatta*) were housed at the Tulane National Primate Research Center (TNPRC) in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International standards. All primate studies were reviewed and approved by the Tulane University Institutional Animal Care and Use Committee under protocol number P0240. Rhesus macaques were provided *ad libitum* with Monkey chow (Lab Fiber Plus Primate diet-DT, PMI Nutrition International, St. Louis, MO) and supplemented with fruits, vitamins and Noyes' treats (Research Diets, New Brunswick, NJ). All clinical procedures were carried out under the direction of a laboratory animal veterinarian and were performed under anesthesia using ketamine, often in combination with telazol, with all efforts made to minimize stress, improve housing conditions, and to provide enrichment opportunities (*e.g.*, objects to manipulate in cage, varied food supplements, foraging and task-oriented feeding methods, interaction with caregivers and research staff).

2.2. Macaques

Two sets of macaques were intravenously injected with labeled and unlabeled Gamunex-C at either 50 mg/kg or 100 mg/kg. In the first set of macaques, macaque IL24 received 50 mg/kg of unlabeled Gamunex-C and macaque BV98 received 50 mg/kg of Cy3 labeled Gamunex-C IgG. In the second set of macaques, macaque HL03 received 100 mg/kg of unlabeled Gamunex-C and macaque JM01 received a mixture of 50 mg/kg of Cy3 labeled Gamunex-C IgG and 50 mg/kg of Cy5 labeled Gamunex-C IgG for a total of 100 mg/kg. JM01 was 4.7 kg, HL03 was 7.3 kg, BV98 was 10.6 kg and IL24 was 7.6 kg at time of injection. Vaginal and rectal biopsies were collected weekly for 8 weeks from each animal. Tissue samples were preserved in optimal cutting temperature (OCT) compound and stored at −80 °C. Plasma, vaginal Weck-Cel® fluid, and cervicovaginal mucus were collected at 0 h, 2 h, 4 h, 24 h, 48 h, 72 h, 1 week and every week thereafter out to 8 weeks.

2.3. Tissue source and culture

Cervical tissue samples were obtained from consenting donors following surgery at Northwestern Memorial Hospital. All samples were collected in accordance with IRB guidelines and with ethics approval. Tissue was collected and processed within 2 h of surgery, dissected into 1 cm^3 ectocervical explants. All donor tissue was then snap-frozen at −80 °C, in OCT. Tissue was sectioned (12μm), immunofluorescently stained for endogenous antibodies and imaged by deconvolution microscopy (GE DeltaVision).

2.4. Immunofluorescence

Sectioned tissues were fixed in 3.7% formaldehyde in PIPES buffer and blocked with normal donkey serum prior to staining. For adherens junction identification in macaque tissues, anti-E-cadherin (BD Pharmingen) was used. To identify endogenous antibodies in human samples, tissues were stained with anti-IgG (BD Biosciences). Macaque endogenous antibodies were also detected with macaque specific anti-IgG (BD Biosciences). Likewise, 8E11 (KeraFAST) was used to identify passively transferred unlabeled Gamunex-C antibodies in rhesus macaque tissue. Secondary antibodies labeled with Rhodamine RedX (Jackson ImmunoResearch) or Cy5 (Jackson ImmunoResearch), were also utilized. Antibody specificity was confirmed by negative results with respective isotype and secondary control antibodies. Hoechst DAPI (Invitrogen) was used for staining of nuclear material. After staining, mounting medium (DakoCytomation) and coverslips were applied and sealed with clear nail polish.

2.5. Imaging and image analysis

Images were obtained by deconvolution microscopy on a DeltaVision RT system collected on a digital camera (CoolSNAP HQ; Photometrics) using a 40 × or 100 × oil objective. In order to fully visualize antibody localization within tissues, 40 × panel images were acquired to include the epithelium and lamina propria. To assess mean fluorescent intensities in tissue, we took ten 100 × images of each tissue type per macaque. We measured the mean fluorescent intensity of each image using in-house algorithms written in IDL 7.1 (Harris Geospatial Solutions) and the Bio-Formats library (Linkert et al., 2010).

2.6. Fluorophore labeling of antibodies

Gamunex-C (pooled human IgG) was purchased from the Northwestern pharmacy. Prior to labeling, Gamunex-C was diluted to 50 mg/mL in 10 mL PBS and buffer exchanged into PBS using two 10 mL Zeba columns (Thermo Fisher), 5 mL for each column. 500 mg of Gamunex-C was labeled using 0.5 mg of Sulfo-NHS-ester Cy5 or Cy3 (Lumiprobe) in 10 mL PBS with 100 mM Sodium Bicarbonate. The reaction was covered from light and allowed to rock gently for 1 h at room temperature. Following the labeling reaction, the 10 mL reaction was passed through two 10 mL Zeba columns (5 mL per column) buffer exchanged with PBS to remove free dye. This step was repeated again to ensure free dye removal. Finally the labeled antibody was passed through a 0.45 μm filter and stored at 4 °C, protected from the light, until infusion.

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