BASIC SCIENCE ARTICLE

Effect of digestion on stability of palivizumab IgG1 in the infant gastrointestinal tract

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Author contributions

J.L., B.J.K., V.D.-M., B.N.P.S., Y.W., A.O., M.A., A.O., B.P.S. and D.C.D conceptualized the study. J.L. and B.J.K designed the experiments, performed data acquisition and analyzed data. J.L. drafted the article. D.C.D supervised the experiments. All authors revised, adapted, and approved final version of the manuscript.

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Impact

1. What is the key message of your article?

- Binding activity of palivizumab IgG1 decreased (26 to 99%) across each phase of *in vivo* infant digestion as measured by both anti-idiotype and RSV F protein-specific ELISAs.

- Palivizumab was likely degraded by proteases and changes in pH introduced in the gut.

- Antibodies generated for passive protection of the infant gastrointestinal tract from pathogens will need to be more resistant to digestion than the model antibody fed to infants in this study, or provided in higher doses to be most effective.

2. What does it add to the existing literature?

- The monoclonal antibody IgG1 tested was not stable across the infant gastrointestinal tract.

- The observation of palivizumab reduction was unlikely due to dilution in the gastrointestinal tract.

- The results of this work hint that provision of antibody could be effective in preventing enteric pathogen infection in infants.

3. What is the impact?

- Orally-delivered recombinant antibodies will need to either be dosed at high levels to compensate for digestive losses or be engineered to better resist digestion.

- Provision of enteric pathogen-specific recombinant antibodies to at-risk infants could provide a new and previously unexplored pathway to reducing the infection in infants.

- The strategy of enteric recombinant antibodies deserves more investigation throughout medicine as a novel means for treatment of enteric disease targets.

BACKGROUND: Potentially, orally-administered antibodies specific to enteric pathogens could be administered to infants to prevent diarrheal infections, particularly in developing countries where diarrhea is a major problem. However, to prevent infection, such antibodies would need to resist degradation within the gastrointestinal tract.

METHODS: Palivizumab, a recombinant antibody specific to respiratory syncytial virus (RSV), was used in this study as a model for examining the digestion of neutralizing antibodies to enteric pathogens in infants. The survival of this recombinant IgG1 across digestion in 11 infants was assayed via an anti-idiotype ELISA and RSV F protein-specific ELISA. Concentrations were controlled for any dilution or concentration that occurred in the digestive system using mass spectrometry-based quantification of co-administered, orally supplemented, indigestible polyethylene glycol (PEG-28).

RESULTS: Binding activity of Palivizumab IgG1 decreased (26 to 99%) across each phase of *in vivo* digestion as measured by both anti-idiotype and RSV F protein-specific ELISAs.

CONCLUSION: Antibodies generated for passive protection of the infant gastrointestinal tract from pathogens will need to be more resistant to digestion than the model antibody fed to infants in this study, or provided in higher doses to be most effective.

INTRODUCTION

Among children aged < 5 years in developing countries, diarrhea is a leading cause of illness and death (1). The proportion of deaths attributed to diarrhea in this age group is approximately 25.2% in Africa, 10.0% in the Americas, 30.4% in the Eastern Mediterranean, 10.6% in Europe, 11.0% in the Western Pacific and 31.3% in South-East Asia (2). In developing countries, the major contributors to diarrhea in children < 5 years include rotavirus, calicivirus, enteropathogenic and enterotoxigenic *Escherichia coli* (3), *Shigella* spp., *Vibrio cholerae* and *Campylobacter jejuni* (4). Breastfeeding protects infants against infectious diarrhea due to maternal antibodies and other bioactive proteins (5). We hypothesized that oral supplementation of enteric pathogen-specific recombinant antibodies could survive digestion and protect against neonatal enteric infection similarly as human milk antibodies. To prevent infection, such antibodies need to resist gastrointestinal digestion to remain biologically active. The extent to which recombinant antibodies might survive across the infant digestive tract, however, remains unknown. Palivizumab (Synagis®), a recombinant humanized monoclonal immunoglobulin G1 (IgG1) that binds to the RSV fusion protein and stops viral replication, is the only Food and Drug Administration (FDA)-approved monoclonal antibody for preventing infections in high-risk infants (albeit for intramuscular injection) (6). To examine recombinant antibody digestion across the infant gastrointestinal tract, palivizumab was selected as a model protein. Though palivizumab is respiratory pathogen-specific, our goal is to translate our findings to diarrheal pathogen-specific recombinant antibodies. Palivizumab added to human milk fed to infants was used in this study as a model for neutralizing antibodies against enteric pathogens. Infants who were already admitted to the Doernbecher Children's Hospital Neonatal Intensive Care Unit that already had an indwelling nasogastric or orogastric feeding tube were recruited to participate in this study. The development of new oral antibody therapy

to treat or prevent enteric infections in newborns is critical because infants are more at risk to infectious diseases than children due to their immature immune system.

This study aimed to determine the binding activity of palivizumab IgG1 across infant *in vivo* digestion. As it is unclear the extent to which *in vivo* digestion can be replicated by *in vitro* and *ex vivo* digestion, we examined the survival of this recombinant antibody in parallel studies via *in vitro* (7) and *ex vivo* (8).

MATERIALS AND METHODS

Sample collection

This study was approved by the Institutional Review Board of Oregon Health & Sciences University (OHSU IRB #18274). The use of palivizumab in neonates was given a may-proceed clearance by the United States FDA (IND #140999). Infant inclusion criteria were the following: infants already admitted to Doernbecher Children's Hospital Neonatal Intensive Care Unit, greater than 34 weeks corrected gestational age, an indwelling nasogastric or orogastric feeding tube and tolerating full enteral feeding volumes. Infants were excluded from the study if they had anatomic or functional gastrointestinal disorders that would impact protein digestion, were medically unstable, were non-viable, or had disorders that would be expected to affect normal digestion. After obtaining informed consent from parents, a tube was placed into the distal duodenum or proximal jejunum through a nare, with the position of the tube port confirmed by abdominal x-ray. Only those infants with the side port of the post-pyloric tube at or beyond the third part of the duodenum to the proximal jejunum were eligible to have small intestinal fluid samples obtained. Gastric, intestinal and stool samples were collected from 11 infant pairs (infant demographics are shown in Table 1 and Supplementary Table S1). Feeds of either human milk or formula were prepared with palivizumab and PEG-28 (see concentrations in Supplementary Table S1). PEG is not absorbed nor digested within the gastrointestinal tract and is generally recognized as safe by the FDA (9,10). Feeds were delivered via naso- or orogastric tubes over 30 min or less. From the same feed, a volume (0.5–2 mL) of each infant's gastric contents was collected 1 h after the initiation of feeding in a 3-mL syringe from the feeding tube via suction, placed into sterile vials on ice, then frozen. Samples were collected from the nasojejunal/duodenal tube via gravity flow as the digesta passed the collection tube port into sterile vials on ice, and then frozen. Stool samples were collected from diapers into sterile containers. Gastric, intestinal and stool samples were stored

at –80 °C. All samples were transported to Oregon State University on dry ice and stored at – 80 °C upon arrival. Demographic information for each participant was extracted from the medical record and securely stored electronically.

Anti-idiotype palivizumab ELISA

Binding activity of palivizumab in all samples was determined via ELISA as described by Demers-Mathieu et al. (11) with some modifications. Samples were diluted with 10% of human AB serum (Corning, Manassas, VA, USA) in PBST at 200x and 400x for milk, gastric and intestinal samples, and 1x and 2x for stool samples. The concentration of palivizumab measured by ELISA from each feed was denoted as 100% stability, and the percentage stability from gastric, intestinal and stool was calculated based on the measured concentration in each sample compared with the concentration in the feed.

RSV F protein-specific IgG ELISA

The RSV F protein-specific IgG concentrations of samples were quantified by ELISA as described by Lueangsakulthai et al. (8) with modifications. Samples were diluted with 1% BSA (Thermo Scientific, Waltham, MA, USA) at 400x and 800x for milk, gastric and intestinal samples, and 1x and 2x for stool samples. The concentration of palivizumab measured by ELISA from each feed was denoted as 100% stability and percent stability within each sample was calculated as described previously.

Extraction of PEG-28

All milk, gastric, intestinal and stool samples were centrifuged at 4,000 × g, 4 °C for 30 min. The infranatant including proteins and PEG-28 were collected into new Eppendorf tubes. Centrifuging and collecting the infranatant were performed in duplicate with the same

procedure. Thirty microliters of commercial PEG-28 (100 ng/µL, Sigma Aldrich, St. Louis, MO, USA) were prepared as a stock solution of the PEG standard. All samples and the standard underwent identical experimental procedures prior to mass spectroscopy (MS) analysis. One hundred and fifty microliters of chilled ethanol were mixed with 30 µL samples and the standard (stored at –20 °C for 1 h). Samples were placed in a –20 °C freezer for 1 h and centrifuged at 12,000 × g, 4 °C for 20 min. Supernatants containing PEG-28 from samples and standards were collected into new tubes and dried using a SpeedVac (Genevac, Gardiner, NY, USA). To avoid clogging the nano-LC column with intact proteins remaining in samples, trypsin digestion for both samples and the standard was performed. Pellets containing proteins were mixed with 100 µL of 50 mM NH_4HCO_3 (Thermo Scientific), denatured by reduction with 2 µL of 550 mM dithiothreitol (incubated at 50 °C for 50 min, Promega, Madison, WI, USA) and alkylation with 4 µL of 450 mM iodoacetamide (incubated at RT for 1 h in the dark, Sigma Aldrich). Denatured proteins in the pellets were enzymatically digested using trypsin (Promega) with shaking at 300 rpm, 37 °C for overnight and combined with each supernatant. Samples and the standard were purified using C18-based solid-phase extraction. The cartridges were preconditioned with 5 mL of ultrapure water, 5 mL of 80% acetonitrile (ACN) with 0.1% trifluoroacetic acid and 5 mL of ultrapure water. Samples were loaded on the cartridges, washed with 5 mL of ultrapure water and eluted with 5 mL of 80% ACN with 0.1% trifluoroacetic acid. All samples and standards were dried using a SpeedVac. All dried samples (30 µL) and the standard (100 µL) were reconstituted with 3% ACN with 0.1% formic acid. To make a standard curve, the standard was diluted with the reconstitution solvent at 20,000×, 10,000×, 4,000×, 2,000×, 1,000×, 400×, 200×, 100×, 40× and 20×.

Quantitation of PEG-28 using parallel reaction monitoring (PRM)-based LC/MS

PEG-28 concentration in standards and samples was determined as described by Kim et al. (12) with some modifications. For PRM, PEG-28 at m/z 417.933 (z=3) was selected as a precursor compound in MS/MS analysis. Collision-induced dissociation was used with 35% of normalized collision energy to fragment PEG-28. MS/MS spectra were acquired in the positive-ionization mode over a m/z range of 300 to 1,500 by the Orbitrap at a resolution of 60,000.

MS data processing

Skyline (MacCoss Lab, University of Washington, USA) was used to make a standard curve and calculate the concentrations of PEG-28 in the standards and samples. MS/MS raw data sets generated by PRM were imported into the Skyline and peak areas were extracted using the transition list including the single charge state of total 10 fragment ions: m/z 327.2013 (DP7), 371.2276 (DP8), 459.2800 (DP10), 503.3062 (DP11), 547.3324 (DP12), 591.3586 (DP13), 635.3848 (DP14), 679.4111 (DP15), 723.4373 (DP16) and 767.4635 (DP17). These fragment ions were extracted from raw files using the following parameters in the Skyline: m/z 0.01 in Method match tolerance; DIA in Acquisition method; Orbitrap in Product mass analyzer; and All ions in Isolation scheme 60,000 at m/z 200 in Resolving power.

Statistical analyses

One-way ANOVA followed by Tukey's multiple comparison test (GraphPad Prism software, version 8.2.1, La Jolla, CA, USA) was applied to compare the samples from *in vivo* gastrointestinal digestion. Differences were designated significant at $p < 0.05$. Pearson's Correlation Coefficient test was applied to compare the percentage stability correlation of each sample. The correlation was designated as strong correlation if the coefficient value (r) was

between 0.50 and 1, medium correlation if the coefficient value (r) was between 0.30 and 0.49 and low correlation if the coefficient value (r) was below 0.29.

RESULTS

Survival of palivizumab IgG1 across *in vivo* gastrointestinal digestion

The average percentage stability of palivizumab IgG1 was detected by the anti-idiotype and RSV F protein-specific ELISA in milk, gastric, intestinal and stool samples of 11 infants. The average percentage stability of palivizumab detected by anti-idiotype ELISA across *in vivo* gastric, intestinal and stool samples decreased 34.2%, 72.2% and 100%, respectively (Fig. 1a). The average percentage stability of palivizumab detected by RSV F protein-specific ELISA across *in vivo* gastric, intestinal and stool samples decreased 25.8%, 70.9% and 100%, respectively (Fig. 1b). The percentage stability of palivizumab across *in vivo* infant gastrointestinal digestion as measured by the anti-idiotype and RSV F protein-specific ELISA were strongly correlated (gastric contents $r = 0.65$, intestinal contents $r = 0.91$ and stool $r = 0.97$). The results demonstrated that palivizumab was degraded across each phase of *in vivo* gastrointestinal digestion ($p < 0.05$).

PEG-28 concentration quantified via MS

The concentration of PEG-28 in milk, gastric, intestinal and stool samples of 11 infants was quantified via MS. The average concentration of PEG-28 detected by MS across *in vivo* feed, gastric, intestinal and stool samples was 0.16, 0.18, 0.18 and 0.29 ng/µL, respectively (Fig. 2). There were no significant differences in PEG-28 concentration across sample type, indicating no consistent sample type dilution or concentration effect. This finding suggests that the observation of palivizumab reduction was unlikely due to dilution in the gastrointestinal tract.

Survival of palivizumab normalized with PEG-28 across *in vivo* gastrointestinal digestion

The average percentage stability of palivizumab IgG1 by the anti-idiotype and RSV F protein-specific ELISA normalized with indigestible PEG-28 in milk, gastric, intestinal and stool

samples of 11 infants. The average percentage stability of palivizumab detected by anti-idiotype ELISA across *in vivo* gastric, intestinal and stool samples when normalized to PEG-28 concentration decreased 40.5%, 62.2% and 100%, respectively (Fig. 3a). The average percentage stability of palivizumab detected by RSV F protein-specific ELISA across *in vivo* gastric, intestinal and stool samples when normalized to PEG-28 decreased 26.5%, 61.6% and 100%, respectively (Fig. 3b). The percentage stability of palivizumab across *in vivo* infant gastrointestinal digestion as measured by the anti-idiotype and RSV F protein-specific ELISA normalized with PEG-28 were strongly correlated (gastric contents $r = 0.80$, intestinal contents $r = 0.93$ and stool $r = 0.97$). The results of the average percentage stability of palivizumab IgG1 detected by both anti-idiotype and RSV F protein-specific ELISA normalized for dilution using the concentration of co-fed PEG-28 demonstrate that palivizumab was degraded after *in vivo* gastrointestinal digestion.

DISCUSSION

Enteric infection, whether acute or chronic, is a predominant cause of death in infants in developing countries (13). Additionally, chronic diarrhea is a major cause of growth stunting (14,15), which is strongly correlated with intellectual and psychosocial impairment in survivors (16,17). In the newborn, maternal transplacental-transferred IgG and human milk-derived secretory IgA and IgG enhance infant host defense to prevent infectious disease (13). It has long been recognized that breastfeeding reduces the risk of infectious diseases in infants and young children (18-21), including diarrheal disease (22-24). Given that not all infants receive human milk to enhance their protection against diarrhea, provision of recombinant antibodies as a supplement could help prevent enteric infections. To reduce the incidence, or prevent enteric infection, exogenous antibodies would need to survive across the gastrointestinal tract with remaining biological activity to target and inactivate pathogens.

To date, monoclonal antibodies have not been tested for use in the gastrointestinal tract, nor has there been any report of the fate of any monoclonal in the gastrointestinal tract, including retention of structural integrity or activity. We report the first study of the fate of a recombinant IgG1 in across *in vivo* digestion as a first step towards investigation of the possibility of enterally-dosed monoclonals targeted at enteric pathogens as one method to reduce infant and childhood diarrheal disease.

Palivizumab is a recombinant monoclonal IgG1 that binds to the A antigenic site of RSV F protein, and is FDA approved for use in neonates by intramuscular route to confer passive immunity to RSV in high risk infants and children (25). We selected palivizumab to examine the extent to which recombinant IgG1 can survive within the infant gastrointestinal tract because of its approved use in infants, its standard monoclonal structure (humanized IgG1), and an assayable *in vitro* activity. Infants were fed with human milk or formula containing palivizumab and indigestible PEG-28 (used as a marker of dilution/concentration to normalize

palivizumab concentration). The use of an *in vivo* study design is advantageous over an *in vitro* digestion design, as it better reflects the reality of the extent to which monoclonal antibodies survive within the infant gut.

The percentage stability of palivizumab decreased across each phase of gastrointestinal digestion (feed to gastric to intestinal to stool samples) based on both the anti-idiotype and anti-RSV F protein ELISAs. This finding was consistent whether or not palivizumab concentrations were normalized for dilution/concentration based on PEG-28. Palivizumab was likely degraded by proteases and changes in pH introduced in the gut. Protease-catalyzed primary structure cleavages and pH induced secondary and tertiary structural changes would be expected to destroy antibody activity if extensive. The observed digestion of palivizumab conformed to that reported in previous studies showing that various monoclonal IgG1 are cleaved in the hinge region after *in vitro* incubation with pepsin at 37 °C, pH 4.0 (26-31) and trypsin at pH 8.0 (29), releasing bivalent fragments F(ab′)2. As detection of palivizumab via the ELISA methods used herein requires the antibody to possess both an intact Fc and Fab segment, cleavage at the hinge region by pepsin and trypsin could cause the observed palivizumab reduction across the infant gastrointestinal tract.

In addition, these findings agree with our previous work indicating that *ex vivo* gastric and intestinal digestion in infant gastric and intestinal fluids resulted in the degradation of palivizumab IgG1, as well as recombinant palivizumab reformatted as IgA and secretory IgA (sIgA). However, results from the gastric and intestinal *ex vivo* digestion experiment (8) indicated that the naturally-occurring RSV F protein-specific IgG and sIgA/IgA were stable. Likewise, a gastric and intestinal *in vitro* digestion experiment (7) demonstrated that the naturally-occurring RSV F protein-specific IgG was stable across digestion. These observations provide evidence that the isotype structure or the post-translational modification could influence the survival of a monoclonal antibody across gastrointestinal digestion. Protein

engineering to make the structure of recombinant antibodies more similar to naturally-occurring antibodies could enhance their resistance to digestion and increase their functionality within the gut. For example, available production techniques that create human-like glycosylation could enhance antibody stability (32,33). This may inform design of future enteral recombinant antibodies.

This study is exploratory and limited by small numbers, as well as the use of a model monoclonal antibody, rather than a recombinant antibody targeted against a diarrheal pathogen. However, a study that does not have these restrictions is not currently feasible. In addition, a comparison of how different factors such as feeds (human milk vs. infant formula) and gestational age affect the extent of palivizumab digestion could not be conducted due to a limited sample size.

CONCLUSION

Provision of enteric pathogen-specific recombinant antibodies to at-risk infants could provide a new and previously unexplored pathway to reducing diarrhea in the regions where the burden of acute and chronic diarrhea remains significant. Such a strategy, if effective, would be in large part dependent on the production of antibodies at a much lower cost than current methodologies, and would need to fit within larger, multipronged approaches to acute and chronic childhood diarrhea prevention. In addition, the results of this work suggest that to be effective in preventing enteric pathogen infection in infants, orally-delivered recombinant antibodies will need to either be dosed at high levels to compensate for digestive losses or be engineered to better resist digestion. Indeed, Sah et al. (34) revealed that palivizumab at higher concentration (1,000 µg/mL) was more stable in terms of RSV neutralization capacity in gastric and intestinal digestion than that dosed at a lower concentration (60 µg/mL). As infants are exposed to a variety of enteric pathogens, an effective strategy to reduce pathogenic diarrhea would likely require multiple recombinant monoclonal antibodies (one monoclonal antibody per pathogen). Purified human milk antibodies could also be used to protect against enteric pathogens as they are more resistant to digestion and their polyreactive properties allow them to neutralize various pathogenic microbes. The strategy of enteric recombinant antibodies deserves more investigation throughout medicine as a novel means for treatment of enteric disease targets or microbiome "sculpting"; the example of *C. difficile* disease is a prime example, offering the advantages of specificity and avoidance of non-specific and increasingly ineffective antibiotic-based strategies.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/) contains supplementary material.

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FIGURE LEGENDS

Fig. 1 Average percentage stability of palivizumab IgG1 across infant gastrointestinal digestion from (a) anti-idiotype and (b) RSV F protein-specific ELISA. Letters *a*, *b*, *c* and *d* show statistically significant differences between groups ($p < 0.05$) using one-way ANOVA followed by Tukey's multiple comparison tests to compare the percentage stability of palivizumab in each sample type compared with the palivizumab concentration in the feed sample for each infant.

Fig. 2 Concentration of PEG-28 across infant gastrointestinal digestion quantified via MS. One-way ANOVA followed by Tukey's multiple comparison tests was used to compare the concentration of PEG-28 in each sample type compared with the PEG-28 concentration in the feed sample for each infant.

Fig. 3 Average percentage stability of palivizumab IgG1 across infant gastrointestinal digestion from (a) anti-idiotype and (b) RSV F protein-specific ELISA normalized with PEG-28 quantified via MS. Letters *a*, *b*, *c* and *d* show statistically significant differences between groups ($p < 0.05$) using one-way ANOVA followed by Tukey's multiple comparison tests to compare the percentage stability of palivizumab in each sample type with the palivizumab concentration in the feed sample for each infant.