Breakthrough gastrointestinal COVID and intra-host evolution consequent to combination monoclonal antibody prophylaxis

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Runnning Title: genetic evolution of SARS-CoV-2 in primate gut

Summary: Breakthrough COVID was observed after experimental SARS-CoV-2 infection in a rhesus macaque undergoing low-dose monoclonal antibody prophylaxis that resulted in unique intrahost evolution of the virus within the gut of the infected animal.

The authors do not have commercial or other associations that might pose a conflict of interest.

Abstract

Breakthrough gastrointestinal COVID was observed after experimental SARS-CoV-2 upper mucosal infection in a rhesus macaque undergoing low-dose monoclonal antibody prophylaxis. High levels of viral RNA were detected in intestinal sites contrasting with minimal viral replication in upper respiratory mucosa. Sequencing of virus recovered from tissue in three gastrointestinal sites and rectal swab revealed loss of furin cleavage site deletions present in the inoculating virus stock and two amino acid changes in spike that were detected in two colon sites but not elsewhere, suggesting compartmentalized replication and intestinal viral evolution. This suggests suboptimal antiviral therapies promote viral sequestration in these anatomies.

Key words: SARS-CoV-2, nonhuman primates, genetic evolution, suboptimal therapy

Introduction

The COVID-19 pandemic, caused by SARS-CoV-2, has been the subject of much recent research regarding therapeutics and viral evolution[1]. A monoclonal antibody (mAb) therapy, produced by Regeneron, currently has emergency use authorization to be administered to COVID patients relatively early in the course of disease with successful results[2]. Other mAb-based therapies are currently under consideration as well, with one combination undergoing clinical trials (NCT04700163) targeting two separate locations within the receptor binding domain (RBD) of spike protein[3]. This mAb combination has been shown to protect prophylactically[4] and therapeutically[5] in a Rhesus macaque (*Macaca mulatta*; RM) model of SARS-CoV-2 infection, with viral loads in the respiratory and gastrointestinal tract significantly blunted.

Infection by SARS-CoV-2 has increasingly been seen as a gastrointestinal disease in addition to the typical respiratory disease it has been associated with since the start of the pandemic[6], with symptoms including nausea, vomiting, diarrhea and abdominal pain present in 40% of infected individuals[7]. Intestinal damage has been noted during autopsy of individuals with fatal cases of disease[8], including viral protein detection in multiple locations[6]. Approximately 50% of patients admitted to hospitals for active disease exhibit digestive symptoms, with 5% of patients displaying digestive symptoms in the absence of respiratory complications[9].

Recently, the Delta variant of SARS-CoV-2 (B.1.617.2) has outpaced other variants of concern to become dominant[10]. This was preceded by other variants including B.1.1.7 (alpha), B.1.351 (beta) and P.1 (gamma)[11]. Some of the sequence changes seen within these variants include those that aid in immune escape, such as those in E484 of spike RBD, that can lower neutralization capacity by over an order of magnitude[12]. Mutations arising that display

differential antibody binding properties are of significant concern for antibody therapeutic and prophylaxis approaches to combating the ongoing pandemic.

In this report, we focus on one RM, LT54, from a study that used a combination antibody therapy as a prophylaxis against SARS-CoV-2 infection[4]. We identified high levels of viral genome and subgenomic RNA within intestinal compartments after respiratory clearance. Intra-host evolution of virus was seen, with sequences changing in accordance with enhanced replication capacity, as well as site-specific differences potentially indicating preferential replication.

Methods

Virus and Cells

Virus used for animal inoculation was strain SARS-CoV-2; 2019-nCoV/USA-WA1/2020 (BEI# NR-52281) prepared on subconfluent VeroE6 cells (ATCC# CRL-1586) and confirmed via sequencing. VeroE6 cells were used for live virus titration of biological samples and were maintained in DMEM (#11965092, Thermo Scientific, USA) with 10% FBS.

Animals and Procedures

A total of 16 Rhesus macaques (*Macaca mulatta*), between 3 and 11 years old, were utilized for this study. All Rhesus macaques (RMs) were bred in captivity at TNPRC. The RMs were infused with 20, 6, or 2 mg/kg mAb cocktail three days before challenge. They were then exposed via intratracheal/intranasal (IT/IN) installation of viral inoculum (1mL intratracheal, 500 μL per nare, total delivery 2e+6 TCID50).

The animals were monitored twice daily for the duration of the study, with collections of mucosal swabs (nasal, pharyngeal, rectal), as well as bronchioalveolar lavage, taken pre-exposure as well as post-exposure days 1, 3, and at necropsy. Blood was collected pre-exposure,

as well as 1, 2, 3, 5 and at necropsy. Physical examinations were performed daily after exposure, and necropsy occurred between 7 and 9 days post-exposure. During physical examination, rectal temperature and weight of each animal was performed. No animals met humane euthanasia endpoints during this study. During necropsy, tissues were collected in media, fresh frozen, or in fixative for later analysis.

Sample Collection and RNA Isolation

Swabs were collected in RNA/DNA Shield (Zymo Research, Irvine, CA). RNA was isolated using the Zymo Quick-RNA Viral kit, with the addition of the swab into the collection column to ensure complete removal of fluid. BAL cells and tissues were collected in Trizol, tissues were homogenized, and RNA was isolated using a RNeasy Mini Kit (#74106, Qiagen, Germany) after phase separation with chloroform.

Quantification of Viral RNA using Quantitative Real-Time PCR

Isolated RNA was analyzed in a QuantStudio 6 (Thermo Scientific, USA) using TaqPath master mix (Thermo Scientific, USA) and appropriate primers/probes [4] with the following program: 25°C for 2 minutes, 50°C for 15 minutes, 95°C for 2 minutes followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Signals were compared to a standard curve generated using *in vitro* transcribed RNA of each sequence diluted from 10^8 down to 10 copies. Positive controls consisted of SARS-CoV-2 infected VeroE6 cell lysate. Viral copies per swab or BAL were calculated by multiplying mean copies per well by volume in the total swab extract or BAL, while viral copies in tissue were calculated per microgram of RNA extracted from each tissue.

cDNA conversion

cDNA was generated using Protoscript II (New England Biolabs, Ipswich, MA) as follows: 10 µl Template RNA, 1µl 10 µM random hexamers, and 1µl 10mM dNTPs were incubated at 65°C for

5 minutes and then place directly on ice for 1 minute. The following was then added: 4 µl PSII buffer, 2 µl 100mM DTT, 1 µl RNase inhibitor, and 1µl PSII reverse transcriptase and incubated at 42°C for 50 minutes, then 70°C for 10 minutes, followed by a hold at 4°C.

Sequencing

DNA libraries were made using the standard SWIFT Normalase Amplicon Panels protocol (SWIFT Biosciences, Ann Arbor, MI) utilizing the SNAP UD indexing primers. The libraries were normalized to 4nM and pooled. Paired-end sequencing (2 x 150) was performed on the Illumina (San Diego, CA) MiSeq platform.

Data analysis

Primer sequences were trimmed, and sequence reads were aligned to the SARS-CoV-2 genome (WA1/2020 isolate, accession MN985325) using the built-in mapping function in Geneious Prime software. Variants were called that were present at greater than 10% of reads at that site.

Results

The animal which is the subject of this report (LT54) was a male Indian origin TNPRC purpose-bred and reared male approximately four (4) years in age and a weight of 6.50 kg when assigned to the treatment study, both measures of which were comparable to other animals in the study cohort[4]. The only clinically remarkable aspect of this animal prior to assignment was a history of intermittent soft stool. Antibody combination (C-135-LS and C-144-LS, 2 mg/kg IV) was administered prophylactically three days to mucosal SARS-CoV-2 challenge. Clinical development of experimentally induced COVID-19 in this animal was generally mild. Daily veterinary physical examination yielded saturated blood oxygen measurements of >98% and auscultation within normal limits throughout disease course up until study termination and necropsy at day +7 post infection. Transient sinus arrhythmia was noted day +2 post infection.

Mild anorexia early in COVID-19 infection resulted in negligible weight loss (~6% of total body weight at termination of study). Viral loads via genomic (N gene) and subgenomic (E gene) RNA were followed for one week. Swab samples were acquired from pharyngeal, nasal, and rectum, as well as cells isolated from bronchoalveolar lavage (BAL) fluid. In both RT-qPCR assays (genomic N and subgenomic E) viral loads in LT54 increased 1 to 2 days post infection at respiratory sites, before resolving by necropsy at +7 days post infection (Figure 1). Swab analysis showed near complete protection against replicating virus throughout the study, with subgenomic E analysis unable to detect virus in any respiratory site except pharyngeal (Figure 1). This indicates robust respiratory protection from challenge post prophylactic administration of antibodies.

This contrasts with viral RNA loads measured in the rectal site, seen as a delayed increase in relation to respiratory viral RNA trends that did not resolve by necropsy. Controls exhibit an increase at this site as well, though not to the extent of LT54 (Figure 1). Tissues collected at necropsy indicate complete protection in respiratory sites of LT54, with controls still exhibiting high amounts of viral RNA in almost all sites (Figure S1A). The intestinal sites of LT54 show the opposite pattern, with high amounts of viral RNA in all sites except the duodenum. The highest levels were seen in the jejunum and descending colon (Figure S1B), potentially indicating preferential replication in those sites, though this could be related to sampling error rather than biological preference. The ileum of LT54 shows minimal changes upon infection (Figure S1C), despite positive viral staining via immunofluorescence (Figure S1D).

Intra-host viral evolution is of interest due to the continued appearance of variants of concern throughout the pandemic. We sequenced virus at each site it was present to determine evolutionary patterns present in LT54 during this study. Present in our challenge inoculum at

low levels were deletions in the furin cleavage site that disappeared during challenge, as has been seen before[13-15], presumably due to lack of replication favorability. The H655Y and N149K changes were seen in the jejunum and transverse colon (Figure 2). H655Y has been reported before in a primate model of SARS-CoV-2 infection, with the change being near the furin cleavage site likely favoring increased replication[15]. N149K is found in the N-terminal domain of spike protein, in an area less well characterized than RBD. This site is not targeted by the antibodies administered, so is unlikely to have arisen due to this prophylactic approach. Rather, this is more likely reflective of the low dose of antibody administered, as well as a more rapidly decreasing antibody level in LT54 than in others of the same low-dose cohort. Interestingly, the sequences seen in the descending colon and rectum were identical to the WA1/2020 isolate (Figure 2).

Discussion

Here, we present data from one animal that was challenged with SARS-CoV-2 post prophylactic administration of combination anti-spike antibody therapy. Despite robust respiratory protection afforded using this antibody combination, high viral loads were observed in the intestinal mucosa. In addition, sequence changes were present indicating increased replication, as well as site specific changes that may indicate increased replication at those sites allowing for those evolutionary patterns to emerge. Finally, we consider the disappearance of the furin cleavage site deletion present in the challenge inoculum to be further evidence of the importance of an intact furin cleavage site in the *in vivo* infection and replication of SARS-CoV-2. Further work may include adapting these sequence changes into current circulating strains to determine the effect, if any, on replication and pathogenesis *in vivo*.

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Figure 1. RT-qPCR assessed viral RNA loads post SARS-CoV-2. Challenge Viral RNA was quantified for SARS-CoV-2 genomic N and subgenomic E content in pharyngeal, nasal and rectal swabs (A, B, C, respectively) and BAL cells (D).

Figure 2. Sequence changes found in gastrointestinal sites of LT54 post SARS-CoV-2 challenge. Viral RNA was sequenced at anatomical sites indicated, with frequency and amino acid substitutions relative to the WA1/2020 patient isolated noted with their relative frequency within the sample.

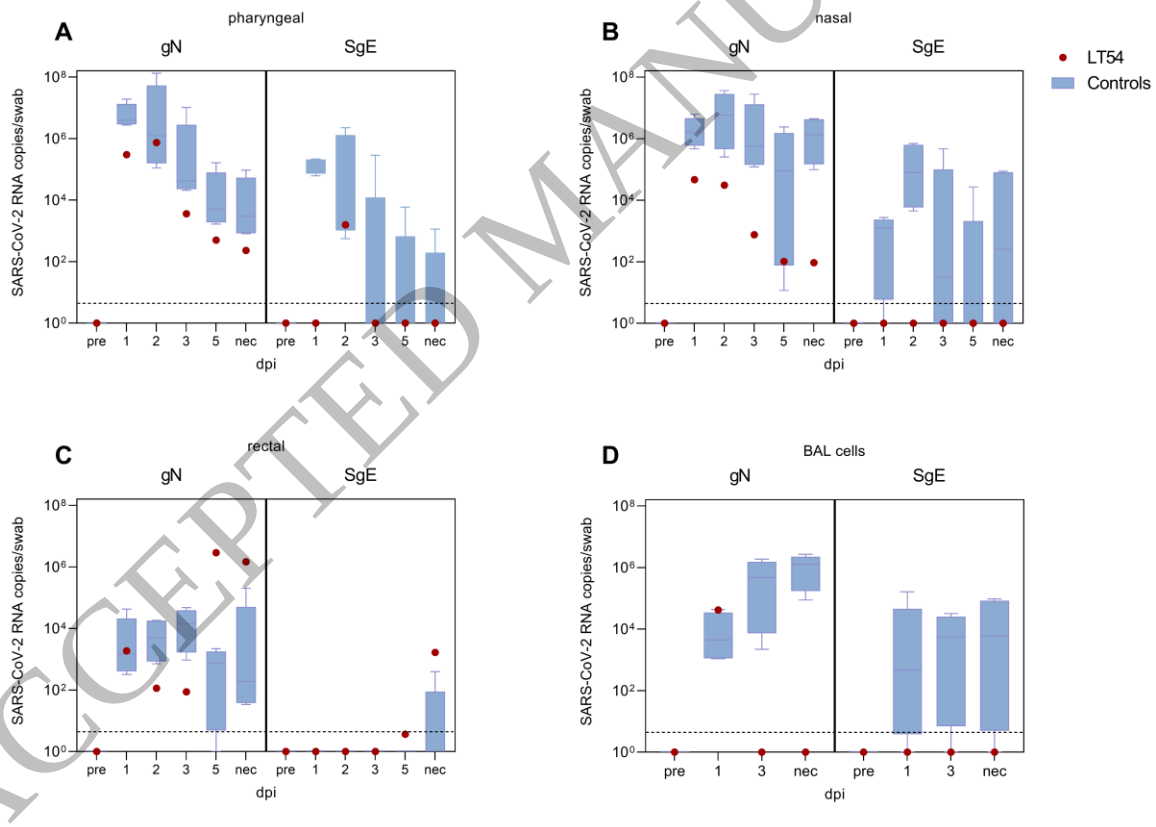

Figure 1
159x113 mm (x DPI)

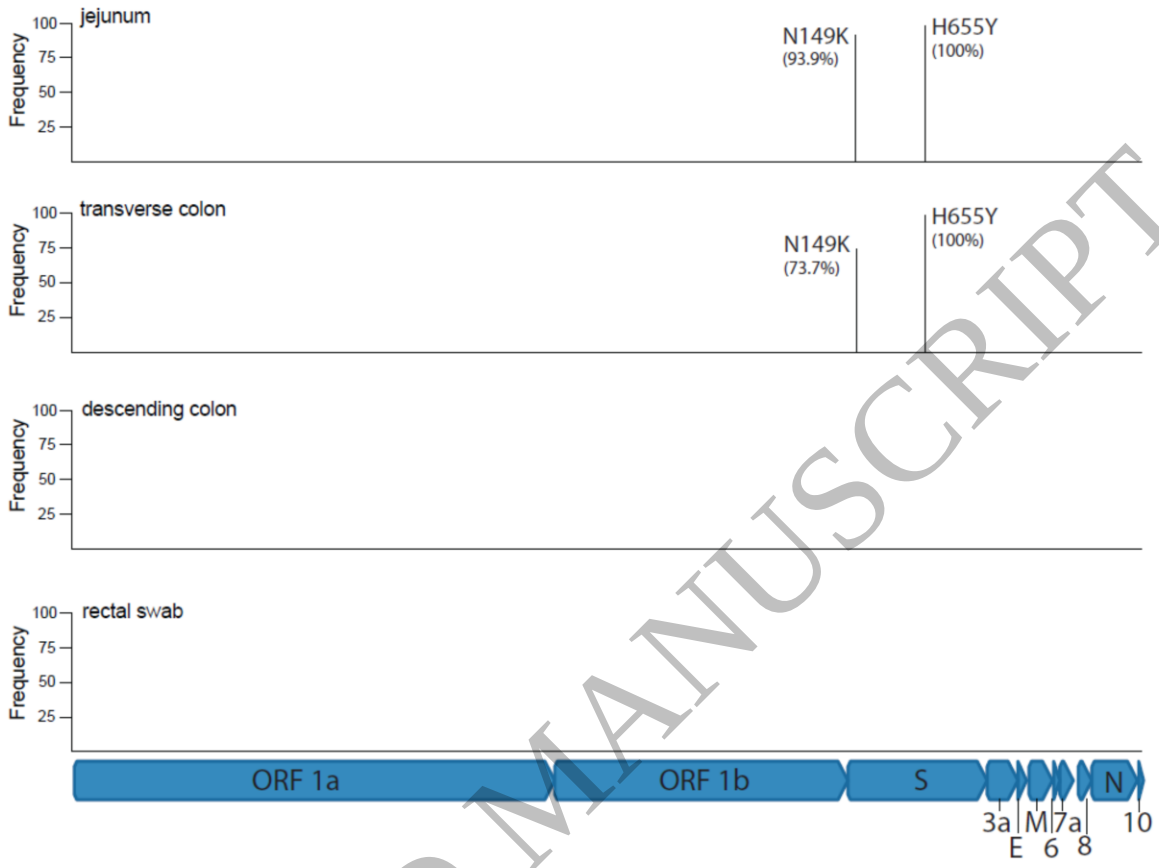

Figure 2
159x123 mm (x DPI)