Brief Report

SARS-CoV-2 Breakthrough Infections after Third Doses Boost IgG Specific Salivary and Blood Antibodies

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Abstract: SARS-CoV-2 breakthrough infections, associated with waning immunity, increase systemic antibody levels. In this study, we analyzed the impact of the infection timing on the magnitude of the systemic humoral response and whether breakthrough infections also boost antibody levels in the salivary compartment. We observed that the combination of infection plus vaccination, regardless of infection timing, produced a sharp increase of systemic antibodies, being higher in subjects infected after third doses. Moreover, despite high systemic antibody levels, breakthrough infections after dose 3 occurred and boosted antibody levels in the salivary compartment. These results lead to rethink the current vaccination strategies against COVID-19 and the use of salivary anti-SARS-CoV-2 antibodies for disease surveillance and vaccination follow-up.

Keywords: SARS-CoV-2 breakthrough infections; vaccines; anti-spike antibody levels; humoral response; IgG specific salivary antibodies

1. Introduction

SARS-CoV-2 breakthrough infections in fully vaccinated individuals were associated with low systemic antibody levels, suggesting third dose administration might reduce the risk of infection [1]. It was shown that one vaccine dose in previously infected individuals, as well as breakthrough infections in fully vaccinated subjects, increase the systemic immunity [2-5]. However, information regarding systemic antibody levels in individuals infected between doses or after third doses is scarce.

The systemic humoral response against SARS-CoV-2 has been extensively studied, while less attention has been paid to the specific salivary antibodies. Some studies in SARS-CoV-2-infected subjects have shown a correlation between the systemic and salivary anti-SARS-CoV-2 IgG antibody levels [6, 7], suggesting that salivary IgG could reflect the systemic antibody response. Studies have also reported specific salivary antibodies in individuals fully vaccinated mainly with COVID-19 mRNA vaccines and showed that specific salivary antibodies indicate seroconversion and correlate with serum neutralization [8, 9]. Salivary antibodies represent mucosal responses that could be relevant in vaccine prevention of oral and nasal SARS-Co-V-2 transmission.

In this work we analyzed the impact of SARS-CoV-2 infection on the magnitude of the systemic humoral response in subjects infected before vaccination or after the first, second or third vaccine doses. Salivary antibody levels before and following SARS-CoV-2 breakthrough infections were also investigated.

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2. Materials and Methods

Study design, participants and sample collection

Since the beginning of pandemic our laboratory monitored healthcare workers with symptoms compatible with COVID-19 or were close contacts and followed infected individuals. Therefore, we included 53 subjects infected with SARS-CoV-2 at different time points: before vaccination (n=18) and after the first (n=6), second (n=10) or third vaccine dose (n=19). All had mild COVID-19 based on the World Health Organization classification [10]. Subjects received full vaccination schedules of BBIBP-CorV (Sinopharm), Sputnik V (Gamaleya NRCEM) or ChAdOx1 nCoV-19 (University of Oxford/AstraZeneca). ChAdOx1 nCoV-19 vaccine was given as third dose (Table 1). Same vaccine platforms were administered on similar dates and with similar interval time between doses.

Blood and saliva samples were collected since the beginning of the pandemic to evaluate the immune humoral response over time. Blood was drawn before vaccination (T0) and 21-30 days after the first (T1), second (T2) and third (T3) doses. Blood was also drawn 21-30 days following SARS-CoV-2 infection in subjects infected after the first ($T1_{1/2}$), second ($T2_{1/2}$) or third ($T3_{1/2}$) vaccine doses. Saliva was collected at T3 and $T3_{1/2}$ from individuals infected after the third dose (n=17) in parallel with blood. Blood and saliva from uninfected subjects (n=18) matched for sex, age, vaccine platform and collection time were used as controls.

Plasma or serum were obtained after centrifugation of the peripheral blood and stored in aliquots at -20°C until used. For saliva sampling, individuals spat their first saliva of the day into a tube, without drinking, brushing teeth or eating, before collection. Saliva samples were centrifuged at 17,000 xg for 10 min (4 °C) and the supernatant was stored at -20°C until used.

This study was approved by the local Academia Nacional de Medicina Ethics Committee. Written informed consent was obtained from participants.

SARS-CoV-2 antibody ELISA

SARS-CoV-2 spike-specific IgG antibodies were measured by ELISA (COVIDAR-IgG). The plates of the assay are coated with a purified mixture of the spike protein and the receptor binding domain (RBD) of the SARS-CoV-2 (B.1 variant). Antibody concentrations in binding antibody units (BAU) per mL (BAU/mL) were obtained interpolating the OD 450 nm values of samples into a calibration curve constructed with the provided standard (400 BAU/mL). For serum samples, SARS-CoV-2 antibodies were determined following the manufacturer's instructions (Laboratorios Lemos S.R.L, Buenos Aires, Argentina) [3]. As antibody levels are lower in saliva than in blood, the necessary conditions for salivary measurements were setting, by partially modifying those used for blood samples. Basically, salivary SARS-CoV-2 IgG antibodies were determined as plasma ones, without performing the first sample dilution. Since pre-pandemic samples were unavailable, negative controls comprised PCR-negative saliva samples from early stages of the pandemic obtained before vaccination started in our country.

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Table 1. Characteristics of subjects infected with SARS-CoV-2 at different time points of vaccination.

	Infection before vaccination (n=18)	Infection after dose 1 (n=6)	Infection after dose 2 (n=10)	Infection after dose 3 (n=19)	Uninfected (n=18)
General characteristics					
Age (years)	41 (26-63)	38 (27-49)	39 (28-52)	44 (27-53)	44 (28-60)
Sex, *n* (%)					
Female/Male	7/11 (39/61)	4/2 (67/33)	5/5 (50/50)	12/7 (63/37)	11/7 (61/39)
SARS-CoV-2-related characteristics					
Time since infection to dose 1 (months)	6 (1-12)	-	-	-	-
SARS-CoV-2 infection after vaccine (days)	-	43 (31-50)	58 (18-308)	57 (22-77)	-
Vaccine platforms before/after infection					-
One doce					-
BBIBP-CorV, *n* (%)	7 (39)	4 (66)	-	-	-
Sputnik V, *n* (%)	10 (56)	1 (17)	-	-	-
ChAdOx1 nCoV-19, *n* (%)	1 (5)	1 (17)	-	-	-
Two doses					-
BBIBP-CorV x 2, *n* (%)	-	-	6 (60)	-	-
Sputnik V x 2, *n* (%)	-	-	4 (40)	-	-
Three doses					
BBIBP-CorV x 2 + ChAdOx1 nCoV-19 x 1, *n* (%)	-	-	-	13 (68)	12 (66)
Sputnik V x 2 + ChAdOx1 nCoV-19 x 1, *n* (%)	-	-	-	3 (16)	3 (17)
ChAdOx1 nCoV-19 x 3, *n* (%)	-	-	-	3 (16)	3 (17)

Values are expressed as median (range) or n (%).
BBIBP-CorV (Sinopharm); Sputnik V (Gamaleya NRCEM); ChAdOx1 nCoV-19 (University of Oxford/AstraZeneca)

Statistical analysis

Unpaired t test or Mann-Whitney test were used to compare the antibody levels between two groups. Categorical data were analyzed by the Chi-square test or Fisher's exact test. Spearman coefficient of rank correlation was used to assess the correlation between specific salivary and blood antibodies. For each time point, geometric mean concentrations (GMC) of specific antibody levels with 95% confidence intervals (95% CI) were calculated. A value of $p< 0.05$ was considered as a significant difference. Data analyses were performed using the GraphPad 9.1.2 Prism software (GraphPad Software, San Diego, CA, USA).

3. Results

Systemic antibody levels were analyzed in individuals infected with SARS-CoV-2 prior to vaccination (n=18) and after the first (n=6), second (n=10) or third vaccine dose (n=19).

All previously infected subjects seroconverted showing a GMC of 159.5 BAU/mL (95% CI: 80.4-316.5) before vaccination (T0). After one vaccine shot (T1), antibody levels significantly increased showing a 11-fold rise (GMC: 1775 BAU/mL; 95% CI: 1101-2861; $p<0.0001$) (Figure 1A).

Among subjects infected after the first dose, the GMC at T1 was 59.0 BAU/mL (95% CI: 18.2-191.5), although three of them showed no detectable antibodies (2 women vaccinated with BBIBP-CorV and 1 man vaccinated with Sputnik V). A sharp increase in antibody concentrations was observed following infection ($T1_{1/2}$), showing a GMC 64-fold higher than before infection (GMC: 3785 BAU/mL; 95% CI: 2014-7115; $p=0.005$) (Figure 1A).

The GMC at T2 in participants infected after the second dose was 167.5 BAU/mL (95% CI: 78.7-356.5) and increased 20-fold after infection ($T2_{1/2}$) (GMC: 3401 BAU/mL; 95% CI: 2135-5419; $p<0.0001$) (Figure 1A).

Anti-SARS-CoV-2 antibodies after the third dose (T3) were high (GMC: 1619 BAU/mL; 95% CI: 1085-2418) and increased even more following infection ($T3_{1/2}$) reaching a GMC 4.4-fold higher than before infection (GMC: 7099 BAU/mL; 95% CI: 5598-9001;

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p<0.0001) (Figure 1A). These antibody levels were higher than after one dose (T1) in subjects with prior SARS-CoV-2 infection (p<0.0001) or following infections after dose 1 ($T1_{1/2}$, p=0.04) or 2 ($T2_{1/2}$, p=0.01) (Figure 1A).

Antibody concentrations in saliva were analyzed at T3 and $T3_{1/2}$ in cases and in uninfected vaccinated control subjects. At T3, IgG specific salivary antibodies were present in 82% of cases (GMC: 40.9 BAU/mL; 95% CI: 15.9-105.1) and in 74% of uninfected controls (GMC: 29.2 BAU/mL; 95% CI: 10.5-81.4). At $T3_{1/2}$, salivary antibodies were detected in 100% of cases, showing a 14-fold increase in the GMC (GMC: 570.7 BAU/mL; 95% CI: 369.0-882.7; p<0.0001), while remaining detectable in 72% of controls (GMC: 21.7 BAU/mL; 95% CI: 10.0-80.4) (Figure 1B). Salivary antibody titers at $T3_{1/2}$ were also higher in cases (p<0.0001) than in uninfected control subjects (Figure 1B). A positive correlation between specific salivary and blood antibodies was observed (r= 0.61, p<0.0001).

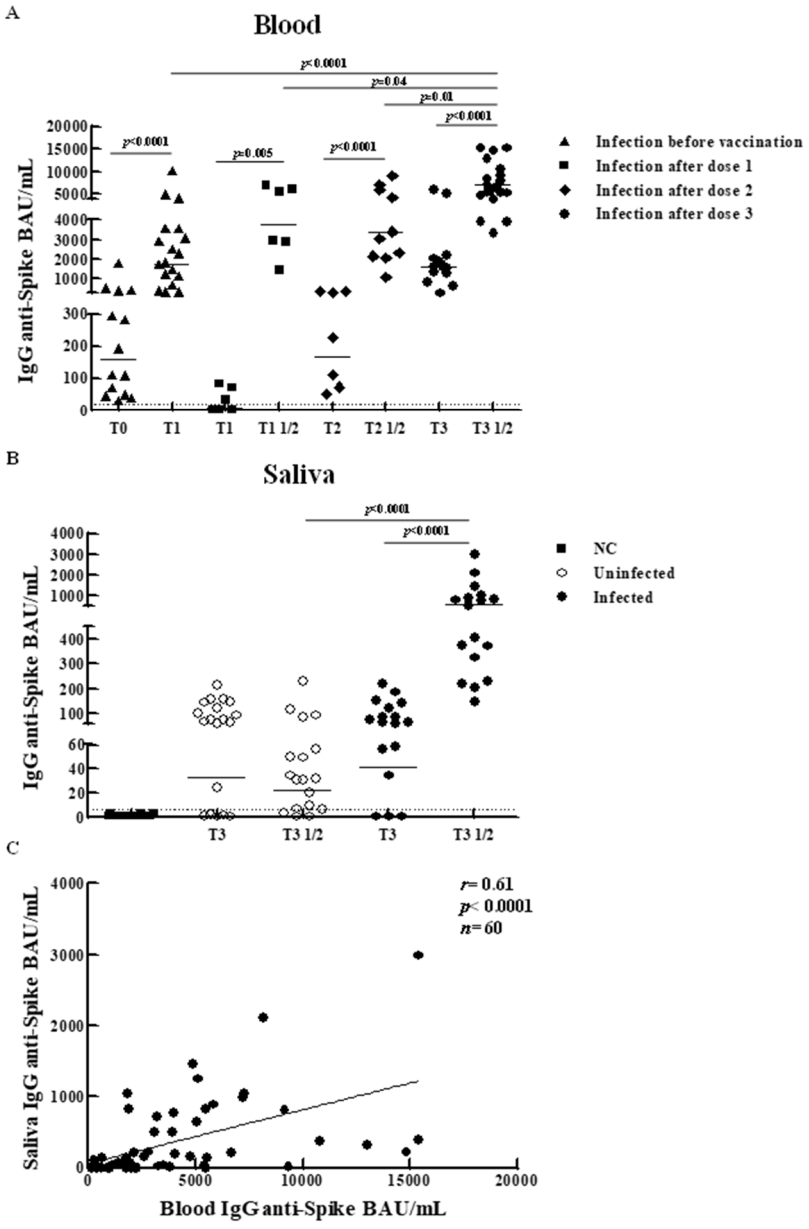

Figure 1. SARS-CoV-2 infection boosts humoral immunity in vaccinated individuals.

(A) Antibody concentrations in the blood of subjects infected with SARS-CoV-2 before vaccination or after the first, second or third vaccine doses. SARS-CoV-2 spike-specific IgG antibodies were measured before vaccination (T0) and 21-30 days after the first

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(T1), second (T2) and third (T3) doses. Antibody levels were also analyzed 21-30 days after SARS-CoV-2 infection in those subjects infected after the first ($T1_{1/2}$), second ($T2_{1/2}$) or third ($T3_{1/2}$) vaccine doses. IgG anti-spike antibody concentrations (BAU/mL) with geometric means are shown. Dotted line indicates the assay detection limit. *p* values were determined by Unpaired t test or Mann-Whitney test. (B) Salivary IgG to SARS-CoV-2 were measured at T3 and $T3_{1/2}$ in subjects with breakthrough infections after dose 3 and in uninfected vaccinated control subjects. PCR-negative saliva samples from the early stages of the pandemic were used as negative controls (NC) for reactivity. IgG anti-spike antibody concentrations (BAU/mL) with geometric means are shown. Dotted line indicates the assay detection limit. *p* values were determined by Mann-Whitney test. (C) Correlation between matched salivary and blood SARS-CoV-2-specific IgG responses. Blood and saliva sample pairs (n=60) were collected from the same individuals at the same time point and correlation between specific salivary and blood antibodies was analyzed by the Spearman rank correlation test. Spearman correlation coefficient (*r*) and *p*-value are indicated.

4. Discussion

The systemic antibody concentrations following vaccination in subjects with prior SARS-CoV-2 infection have been widely studied [2, 3]. A strong boost of the systemic humoral response after breakthrough infections in fully vaccinated individuals has also been reported [4, 5]. However, antibody concentrations in individuals infected with SARS-CoV-2 between doses or after third doses have been barely investigated.

In this study, we analyzed the impact of infection timing on the magnitude of the systemic humoral response. We observed a sharp increase of systemic antibodies following infections after the first, second or third vaccine doses, reaching GMCs 64-, 20- and 6.8-fold higher than before infection. Moreover, antibody levels following breakthrough infections after dose 3 were higher than in subjects vaccinated and infected at any previous time point. These results agree with a study showing that increased number of exposures to SARS-CoV-2 antigens, through vaccination or infection, enhance serum specific antibody-binding titers [4]. Our results suggest that SARS-CoV-2 infection increases systemic antibody levels in vaccinated subjects, regardless of whether the exposure occurs before, during or after vaccination.

Antibody detection in saliva has been used to monitor exposure to pathogens, since the antibody profiles in saliva match well those found in blood [11]. Although IgA is the key immunoglobulin for mucosal immunity, evidence for salivary IgA specific against SARS-CoV-2 is inconsistent. Salivary IgG is derived primarily from circulating IgG through transudation, whereas salivary IgA can be produced locally [12]. Therefore, IgG antibody levels in saliva could reflect the systemic antibody response, although at lower concentrations. In agreement, it has been observed that the 100% of individuals receiving full vaccination schedules with SARS-CoV-2 mRNA vaccines showed IgG specific salivary antibodies, while only the 55% of subjects had IgA specific salivary antibodies [8].

In this report, we analyzed salivary antibody levels before and after SARS-CoV-2 breakthrough infections in cases and in uninfected vaccinated control subjects. We observed that most subjects showed IgG specific salivary antibodies at the time of infection; albeit at much lower levels than those in blood. Following infection, salivary antibody levels increased in cases, being significantly higher than before infection or in uninfected controls. These results show that SARS-CoV-2 breakthrough infections boost IgG antibody levels both in blood and saliva. Whether this boost of specific salivary antibodies could confer immune protection for future exposures remains to be determined. As previously reported [8, 9], we observed that the levels of specific antibodies in saliva and blood correlated positively. Although not analyzed in this study, immune boosting by the infection in subjects vaccinated with whole virus COVID-19 vaccines might also increase systemic and salivary antibodies against other SARS-CoV-2 proteins. It is important to note that the increase of systemic and salivary antibody levels after infection was observed

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in subjects receiving different vaccines, suggesting that it was independent of vaccine platform.

Limitations of this study include the small number of subjects within groups, lack of information on neutralization titers, cellular immune responses and viral variants involved in infections. Information about SARS-CoV-2 circulating variants in Argentina is available through the Argentine Interinstitutional SARS-CoV-2 Genomics Project (PAIS Project). In our study, subjects infected before vaccination got the disease at the time of Argentina's first wave, when the variants of interest (VOI) or concern (VOC) were not yet circulating. Infections after the first or second dose occurred when the circulating VOI/VOC alpha, gamma and lambda predominated. Breakthrough infections after third doses corresponded to the third wave in Argentina, when the few cases of the Delta variant were quickly fully replaced by the Omicron variant [13]. Besides, although salivary antibodies were not measured from the beginning of the study, our preliminary results show that four months after the third dose, salivary antibody concentrations in subjects infected at different time points of vaccination, are higher than in uninfected vaccinated subjects.

While an enhanced neutralizing activity against the Omicron variant was demonstrated after the administration of third doses [4], infections with this viral variant after third doses occurred [14], in agreement with our results. SARS-CoV-2 breakthrough infections were associated with waning immunity. However, in our cohort, infections after third doses happened despite high systemic antibody levels (GMC: 1619 BAU/mL; 95% CI: 1085-2418). These results lead to rethink current vaccination strategies, in order to analyze how many doses and vaccine type are necessary to control viral infections by emerging variants, and support the new vaccine candidates' development targeting these variants.

Saliva collection is an attractive alternative to blood testing, as it is non-invasive and allows home-based self-collection. This is important for studies in pediatric populations or where blood sampling is not possible. However, one limitation for the detection of specific salivary antibodies is that their levels are lower than in serum. In this report we showed that salivary antibody titers increased after SARS-CoV-2 breakthrough infections. Therefore, determination of salivary antibodies against SARS-CoV-2 could be a valuable tool in disease prevalence studies, for the follow-up of vaccinated individuals and to assist vaccination strategies against COVID-19, both in adult and pediatric populations, especially in settings where blood sampling cannot be fulfilled.

5. Conclusions

In summary, our results showed that the combination of SARS-CoV-2 infection plus vaccination, regardless of infection timing, produced a sharp increase in antibody levels, which was reflected both in the blood and the salivary compartment.

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