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Clinical Immunology 226 (2021) 108730

Contents lists available at ScienceDirect

Clinical Immunology

journal homepage: www.elsevier.com/locate/yclim

A novel application of delayed-type hipersensitivity reaction to measure cellular immune response in SARS-CoV-2 exposed individuals

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ARTICLE INFO

Keywords:
SARS-cov2
DTH
Skin test
Delayed-type hypersensitivity
T-cell response
COVID-19
Cell response
Humoral response

ABSTRACT

Objective: To understand the anti-virus adaptive immune response occurring during SARS-Cov-2 infection is necessary to have methods to investigate cellular and humoral components. The goal of this study has been to investigate the utility of a specific spike-DTH test using a coronavirus recombinant protein in COVID-19 patients.
Methods: DTH studies were performed by intradermal injection of a commercial recombinant spike protein from SARS-CoV-2 along with conventional serology studies.
Results: Fifty-one COVID-19 patients were studied showing 84,3% of concordance with spike-DTH and anti-RBD-IgG. Spike-DTH was superior to identify seven more COVID-19 individuals. A high specificity was found with no positive spike DTH reactions in the non-sick individuals. The skin test also showed more stable results over time while specific anti-RBD-IgG decreased gradually. Clinical severity groups also showed a progressive gradient of larger positive spike-DTH.
Conclusion: Specific spike DTH test seems to be an easy method to study cell immune response.

1. Introduction

The measurement of the immune response against SARS-CoV-2 has been a hot topic since the emergence of the pandemic situation. During this year, a lot of research has been directed to dissect the humoral response [1] and big efforts have been done to develop antibody test detection methods that could correlate well with the status of the immune response in the infected individuals. From these studies, it is currently accepted that there are in the market many reliable standard serological ELISAs, some of which even correlate with virus neutralization titers [2]. But an understanding of the critical in vivo T-cell responses to the SARS-CoV2 virus is lacking, mainly due to the difficult task of development of cellular assays to investigate the T-cell compartment. Several reports with limited number of participants have proposed different relationships between these two sides of the adaptive immune response [3,4]. Both ELISAs antibody methods and in vitro cellular assays require the extraction of a blood sample from the patient, what complicates possible massive analysis in large populations. Particularly, technologies to study T-cell responses in vitro are too complex, tedious and time consuming to be applied to thousands of samples. For these reasons, an alternative method to evaluate the magnitude of anti-SARS-CoV2 cellular responses in vivo that could be easily accomplished in such high-throughput investigations is urgently needed.

Cutaneous antigen-recall models allow the study of human memory responses in vivo [5]. In this report we describe a feasible method, the classical delayed-type hypersensitivity (DTH) response to the intradermal injection of a recombinant protein representative of the SARS-CoV-2 virus to assess the T-cell mediated memory recall immune response. In our hands, the DTH reaction to the spike protein of SARS-CoV2 seems to be a good and simple tool to measure specific cellular response with a strong correlation with specific serological tests. DTH also seems to be highly specific and more stable over time after infection.

Abbreviations: COVID-19, Coronavirus disease 2019; DTH, Delayed-type hypersensitivity; ELISA, Enzyme-Linked Immunosorbent Assay; RBD, Receptor Binding domain; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2.

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https://doi.org/10.1016/j.clim.2021.108730
Received 5 March 2021; Received in revised form 13 April 2021; Accepted 14 April 2021
Available online 16 April 2021

2. Methods

2.1. Individuals

A total number of 65 individuals (43 female/22 male) with mean age was 47.9/46.7y-o were analyzed. Fifty-one individuals (36 female/15 male, mean age was 47.4y-o) were COVID-19-positive cases defined either by clinical or SARS-Cov-2 PCR-positive. Considering clinical phenotypes, 34 individuals were classified as asymptomatic/mild disease (group I), 13 moderate (group II) and 4 were severe/hospitalized (group III) with Pneumonia (E1). Fourteen individuals (7 female/7 male; mean age:49.2) were used as COVID-19-negative (non-infected) controls [6]. PCR was not performed in the controls (no clinical symptoms and negative serology).

2.2. Study design

The patients were seen in the medical consultation during the months of November and December 2020. Demographic details and the time where the infection was diagnosed by clinical symptoms or by RT-PCR were collected from all participants. Three different clinical groups were assigned depending on the symptoms of the exposed individuals: asymptomatic/mild disease or group I, moderate or group II and serious/hospitalized or group III of patients.

Each subject that intends to enter the study was given a written document called "Patient Information Sheet," which contains relevant and necessary information for the patient to decide on their participation in the study. Treatment, communication, and transfer of personal data of all participating subjects comply with the provisions of Law 03/2018 of 5 de December, RG:2016/679 on protection of personal data. The protocol was approved by the ethical committee of the Hospital (CHUC_2020_92). The study is conducted in accordance with the requirements expressed in Law 737/2015 about biomedical research and the Declaration of Helsinki (revised Brasil, October 2013).

2.3. Serology studies

All serum samples from patients were sent to Immunology laboratory for SARS-CoV-2-IgG and IgA determination between November and December 2020 from outpatients and were frozen. Then, serum samples were thawed and analyzed at a 1:100 dilution. A commercial ELISA specific for the S1 protein of SARS-CoV-2 was used according to manufacturer's instructions (SARS-CoV-2 IgA and IgG immunoassay, Euroimmun, Lübeck, Germany). As recommended for the manufacturer, the results were expressed as Optical density (O.D.) ratios. OD ratios under 0.8 were considered negative, OD ratios between 0.8 and 1.1 were considered borderline positive and values greater than 1.1 were considered positive.

2.4. Skin DTH studies

The protocol was performed according to usual clinical practice and following the Allergology Procedures Manual and the Safety and Quality Recommendations in Allergology (RESCAL-2018) of the *Spanish Society of Clinical Allergology* (SEAIC) to carry out allergology procedures (E2). According to the manual, intra-epidermal and intradermal skin tests are at Level A defined as the "set of tests that meet the following criteria of low complexity, short duration (the patient must remain under observation for less than 2 h) and, finally, low risk of reaction. The tests were carried out in the area of diagnostic techniques of the Allergy Service according to the usual clinical practice. Intradermal tests were not performed in patients with a history of grade II or higher anaphylaxis.

After signing the informed consent and following the usual clinical practice, and after sterilization with alcohol in the volar part of the arm, a specifically trained nursing professional administered the amount of 25 μL for intradermal puncture (IDT) of each of the proteins with immediate reading after 15 min. The patients had been instructed to avoid oral antihistamines and corticosteroids at least 5 days before the DTH tests. The late reading was made at 12, 24 and 48 h with measurement of the reaction obtained to evaluate the kinetics of the immune response. The patients were instructed to take a photograph of the part of the arm at the agreed times (12 h, 24 h, and 48 h after injection) with the puncture as well as to add a measuring ruler next to it to have a reference [7], They were given a telephone number for assistance 24 h a day for consultation and evaluation if necessary. A positive response was considered in the case a positive cellular response function (function that would be considered intact in patients).

Two different intradermal injections of 25 μL total volume of each antigen preparation were performed in all subjects. A lyophilized SARS-CoV-2 recombinant protein of the receptor binding domain (RBD) was resuspended in a sterile water and 0,22 μm sterile filtered with a final concentration of 0.1 mg/mL following manufacturer's instructions under controlled sterilizing conditions. The final concentration was the same as that normally used in the tuberculin test. [8,9] A titration approach was carried out with the first patient and several controls, observing that the concentration employed was not irritating to the skin.

Candida albicans antigen consisted in a commercial standard extract for allergy testing and prepared for intradermal injection [10]

2.5. Statistical analysis

Continuous variables are expressed with means and standard deviations, and categorical variables with frequencies and percentages. Differences between the distributions of continuous variables were evaluated using the Mann-Whitney *U* test. Proportions between groups were compared with chi-square or Fisher exact tests, as appropriated. Association between variables were assessed with Pearson and Spearman correlation tests, as appropriated. All p value lower than 0.05 was considered statistically significant. Statistical analysis was carried out with SPSS v.25.0 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows. Armonk, NY).

3. Results

A total number of 65 individuals, 51 COVID-19-positive cases and 14 non-exposed controls, were analyzed (Fig. 1). COVID-19-positive cases were defined either by SARS-Cov-2 positive RT-PCR (48 patients) or clinically/serology suggested if PCR was not feasible at the time of the diagnosis (3 patients).

Among the 51 COVID-19-positive individuals, 37 were considered as positive specific-antiRBD-IgG (31 with values >1.2 O.D.ratio (60.8%) and six (11.8%) with values between 0.8 and 1.1O.D.ratio and 14 were negative (27.4%). The distribution of the O.D.ratio of specific-antiRBD-IgG showed an increasing tendency along the three different clinical phenotypes (O.D. ratio 2.1 in group I, 3.1 group II and 3.2 group III) (Fig. 1A**, red line**). All 14 non-exposed controls were negative for specific-antiRBD-IgG (mean O.D.ratio 0.2).

Among the COVID-19-positive individuals, 34 were considered as positive specific-antiRBD-IgA (26 with values >1.2 (51%); and eight (15.7%) with values between 0.8 and 1.1) and 17 (33.3%) were negative (Fig. 1A**, purple line)**. The mean O.D.ratio of controls was 0.3.

The 37 individuals with positive specific-antiRBD-IgG (72.5%) were distributed as follows 27 IgG+/IgA+ (53%), 10 IgG+/IgA- (19.6%) (Fig. 1C**)**. Seven out of 14 individuals with negative specific antiRBD-IgG (27.4%) had positive specific-antiRBD-IgA.

Candida albicans is the most frequently reactive recall antigen in DTH in normal individuals [11] in order to know the cellular immune competence of the analyzed individuals. Fifty out of 51 COVID-19-positive patients developed a positive skin test for *C. albicans*. The immediate reading from 15 min to the first 30 min was negative in all cases. The kinetic of the positive late reaction was in group I with 3.3 mm (3.5) at 12 h, 8.1 mm (5.4) at 24 h and 11.7 mm(6.2) at 48 h; in

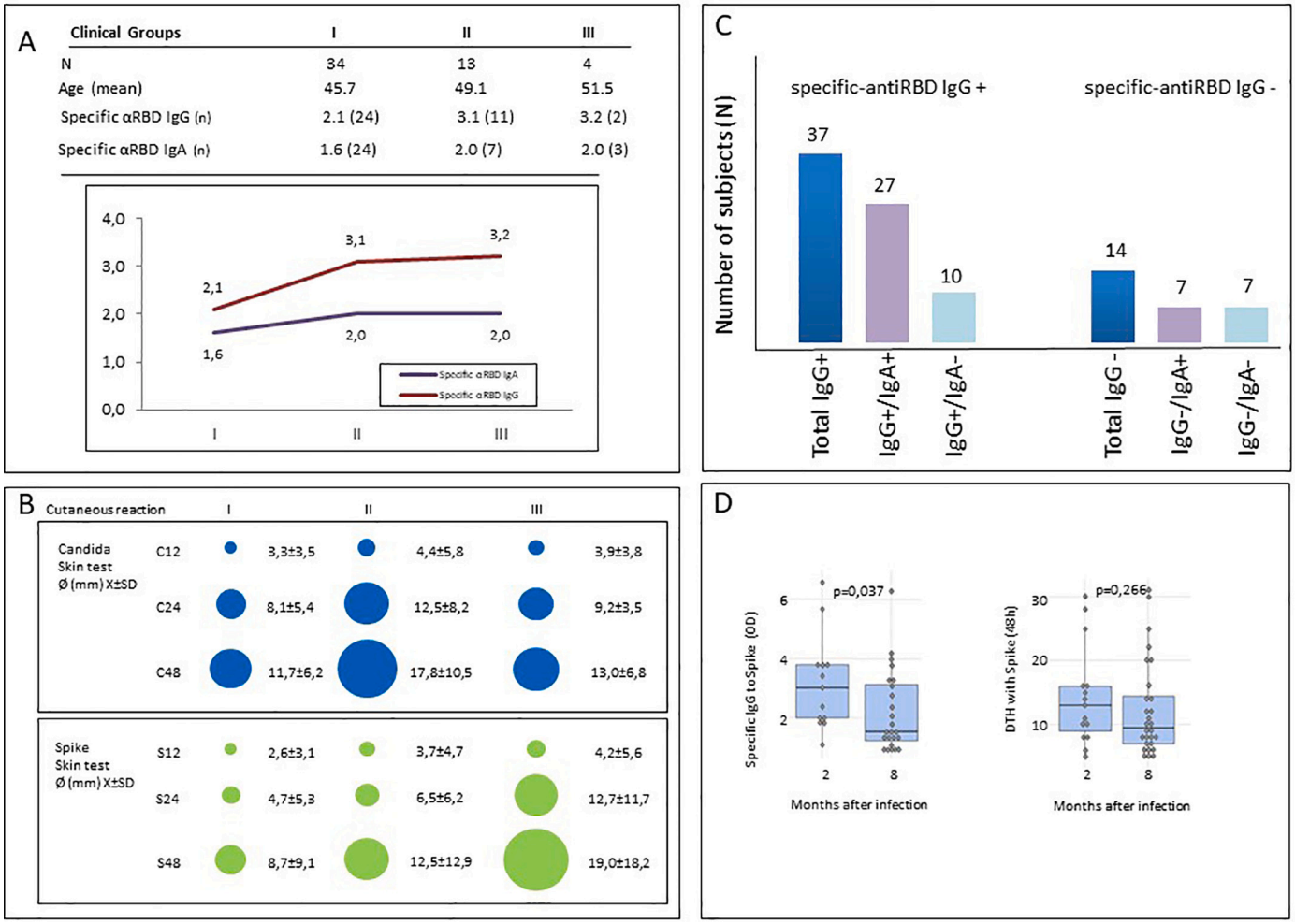

Fig. 1. A. The distribution of the levels of antibodies (in O.D. ratio) of specific-antiRBD IgG (**red line)** and specific-antiRBD IgA (**purple line)** in group I (asymptomatic/mild disease), group II (moderate disease) and group III (severe/hospitalized). **B.** Kinetics of the skin reaction (mean in mm) at 12 h, 24 h and 48 h after intradermal skin test with candida extract (**blue)** and with spike RBD protein of SARS-cov-2 (**green)** in group I, group II and group III. **C.** Distribution of number of patients according to the result of specific-antibodies IgG and IgA against RBD of SARS-cov-2. **D. A.** Humoral response by serology of specific-antiRBD-IgG (in O.D. ratios) in group of individuals after two months of infection (+2) and in group of individuals after 8 months of infection (+8). **B.** Cellular immune response by Spike-DTH skin test (in mm) after 48 h of intradermal test in group of individuals after two months of infection (+2) and in group of individuals after 8 months of infection (+8). Each diamond represents a single individual. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

group II 4.4 mm (5.8) at 12 h, 12.5 mm (8.2) at 24 h, 17.8 mm (10.5) at 48 h; in group III 3.0 mm (3.8) at 12 h, 9.2 mm (3.5) at 24 h, 13.0 mm (6.8) at 48 h **(**Fig. 1**B).** Twelve out of 14 control individuals were positive for the candida-DTH skin test. The kinetics of the positive ones were 2.6 mm (12*h*), 7.0 mm (24 h) and 8.8 mm (48 h) after intradermal application.

Forty-three out of 51 positive-COVID-19 patients were positive for Spike-DTH skin test (Fig. 1B). The immediate reading from 15 min to the first 30 min was negative in all 51 cases. The kinetics of the positive cutaneous tests was: in group I, the mean was 2.6 mm (STD 3.1) at 12 h, 4.7 mm (5.3) at 24 h, and 8.7 mm (9.1) at 48 h; in group II, 3.7 mm (4.7) at 12 h, 6.5 mm (6.2) at 24 h, 12.5 mm (12.9) at 48 h; in group III, 4.2 mm (5.6) at 12 h, 12.7 mm (11.7) at 24 h, 19 mm (18.2) at 48 h after the intradermal injection. The 14 control individuals were negative for the Spike-DTH skin test.

The concomitant analysis of the 51 exposed individuals for both serological (anti-RBD specific IgG) and Spike-DTH skin test showed a concordance in 43 patients (84.3%). Thirty-six of them were positive for both methods and 7 individuals were negative for both methods. One individual was positive for IgG and negative in skin test and seven were negative for IgG with positive Spike-DTH skin test (Fig. 2). All 14 individuals belonging to the non-exposed group were negative for both specific anti-RBD IgG and Spike-DTH skin test showing a concordance of 100%.

Because the individuals had been infected in different periods of time, one corresponding to the first wave in Europe (March–April 2020) and another group of individuals corresponding to the second wave in Europe (September–October 2020), serology and cutaneous test response was divided in two different groups: those who had been infected with SARS-Cov2, eight months before (group "+8" with 34 individuals) and those who had been infected more recently, two months before (group "+2" with 17 individuals).

Disaggregated analysis of both groups showed that in group "+8" there were 24 individuals with positive IgG (70.6%) and 28 individuals with positive skin test (82.4%) and in group "+2" thirteen individuals had positive IgG (76.5%) and 15 individuals (88.2%) were positive for the cutaneous test. Moreover, if we compare the positive specific IgG ($n = 24$ in group "+8" and $n = 13$ in group "+2") vs positive-cutaneous test ($n = 28$ in group "+8" and $n = 15$ in group "+2"), the results showed that the cutaneous test positive individuals remains stable throughout the follow up whereas specific anti-IgG positive showed a decreased value (OD ratio) when compared between these two time points (Fig. 1D).

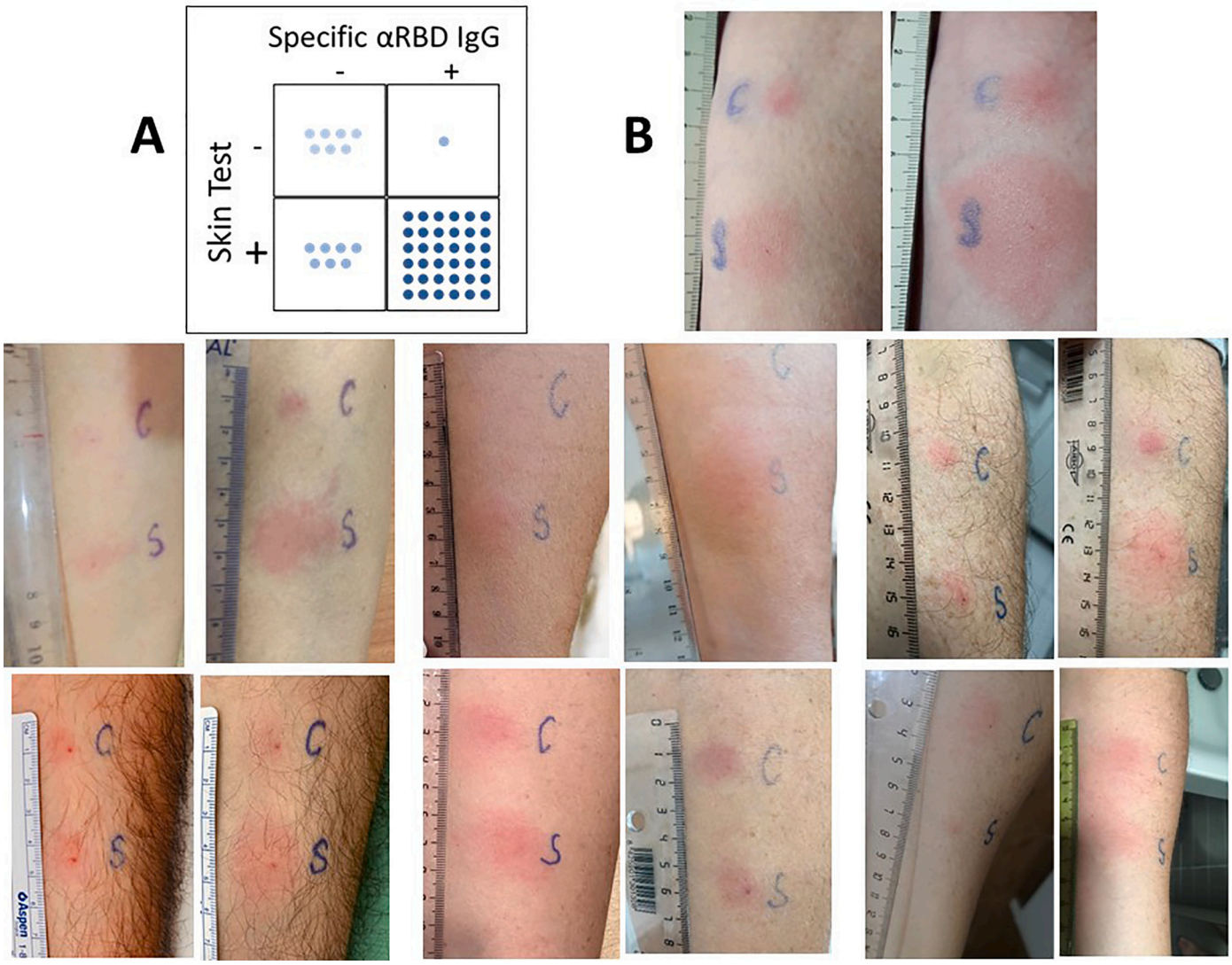

Fig. 2. A. Results of skin test and antiRBD-IgG in all 51 individuals with SARS-cov-2 infection. Each dot corresponds with a single individual. **B.** Results of skin test at 24 h and 48 h in six positive individuals showing reaction (upper right: woman 72y-o group III; middle left, woman, 51y-o group III; Middle center woman, 56y-o group I; Middle right, male 65y-o group II; lower left, male, 57y-o, group II; lower middle, woman 50y-o group I, lower right, woman 60y-o, group I) C: Candida DTH skin test; S: Spike DTH skin test.

4. Discusion

DTH studies has been used in the past to assess immune function on HIV-infected patients [12]. The goal of this study has been to investigate the utility of a specific spike-DTH test in the COVID-19 infection and consequently the correlation with the anti-RBD serology. The RBD recombinant antigen has been decided based on published data that consider the receptor binding domain of the viral spike protein as the immunodominant target of antibodies in SARS-CoV-2 patients [13,14].

As reported by others [15], there is a clinical gradient of immune activation among the 51 exposed individuals. The results showed an increased value of specific anti-RBD IgG O.D.ratio on clinical groups I, II and III. The low number of individuals in clinical group III precludes any strong conclusion. This gradient is also present on the results of Spike-DTH skin test expressed as measurement (mm) of the diameter of the cutaneous reaction on three (12–24-48 h) different times after the intradermal application of the antigen (Fig. 1).

Comparisons between specific anti-RBD IgG and Spike-DTH cutaneous test to identify the exposed individuals showed a concordance number of 43 (84,3%) (36 doble positive vs 7 doble negative). Spike-DTH showed a superior capacity to identify exposed individuals because there are 7 that are positive for the cutaneous test but negative for anti-RBD specific IgG, whereas just only one individual positive for anti-RBD specific IgG was found negative for the Spike-DTH (candida skin test used as control of cellular immune competence was positive) (Fig. 2A). There are 7 exposed individuals that showed negative results for serology and skin test. Serum from these individuals were studied and 6 have at least one different antigen (anti-nucleocapsid protein) (data not shown) or isotype (IgA) positive antibody response (Fig. 1C). Specific anti-spike IgA determination in all participants was included, given the relevance of this isotype in the control of the virus at the respiratory level. To improve the cutaneous test, it can be implemented in the future the use of other antigen specificities that could cover these "false" negative results. Others have suggested a CD4+ T-cell response against epitopes located in the small M protein [16], that could be one of the reasons for the cutaneous negative individuals. An already ongoing large study (including more individuals and other recombinant proteins of the SARS-Cov-2 virus has started to fill these knowledge gaps.

Seroconversion to SARS-CoV-2 range from 91 to 99% in large studies [17,18], but little is known about the kinetics of virus-specific T-cell responses during SARS-CoV-2 infection and their behavior in different grades of disease severity. Reports about correlation between T-Cell and

anti-IgG showed contradictory results, some demonstrating a robust correlation [16,19], while others showed poor correlation [6].

To the best of our knowledge, this report is the first one showing a good correlation among cellular in vivo measurement/specific IgG response. It can be hypothesized that the use of an in vivo method, although less sophisticated it may reproduce a more real situation on these exposed individuals. Moreover, we have not found any positive DTH to SARS-CoV-2 S protein on the unexposed individuals, showing the high specificity of the method. However, PCR-confirmation of these individuals was not done to rule out the remote possibility of some asymptomatic and negative serology individual. Another interesting finding is that while the serology tends to fall after few months (especially in the less severe group of patients), the cutaneous test remains stable (Fig. 2), reflecting a more homogenous in vivo T-cell response measurement. Possibly this reflects a shorter life span of the antibody producing plasma cells compared to the circulating anti-S T-cells.

In this report we have demonstrated that assessment of cellular immune activation through delayed hypersensitivity using a recombinant protein of the virus is an easy, affordable, and suitable method to study this new coronavirus infection. An extended use of this test in vaccinated population opens a new horizon for massive test of large populations that can be used as a screening method for assessment of cellular immunity to evaluate the efficacy of vaccination.

Funding

This research was self-funding and did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Disclosure statement

Y·B and V.M. have filed provisional Utility Model applications related to DTH tests for cellular immunity against SARS-CoV-2. All authors declare that they have no competing interests.

Authorship

Y·B and V.M. participated in the conception of the idea and designed the study and drafted the manuscript. ISM, AF, PPG, RGP participated in analysis and interpretation of data and revised the manuscript critically. All authors approved the final version to submit.

Ethical committee

All included subjects received full written and informed consent. The study was approved by the Ethical Committee with the code CHUC_2020_92.

Acknowledgements

We would like to thank to patients and Dr. Fernando Diaz-Espada, former Chief of Immunology Department in Hospital Clínica Puerta de Hierro for rich discussions to improve the project.

The authors want to thank the Immunology Laboratory Technicians (Tamara MJ Placer, Gloria Camacho, María Romera, Montserrat Padilla) and Allergy registered nurses (Sofia García, Antonio Pérez-Granados, Lila Pérez) for their help with the procedures and dedication, Alejandro Jiménez for statistical analysis and Miriam Hernández from Microbiology Service for helping with some negative patients.

Y·B and V.M. have filed (79241/P8547) Utility Model application related to DTH tests for cellular immunity against SARS-CoV-2.

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