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Antibody response and viraemia during the course of severe acute respiratory syndrome (SARS)-associated coronavirus infection

Weijun Chen,[1,2]† Zuyuan Xu,[1,3]† Jingsong Mu,[4]† Ling Yang,[1,2] Haixue Gan,[2] Feng Mu,[1,2] Baoxing Fan,[5] Bo He,[2] Shengyong Huang,[2] Bo You,[2] Yongkui Yang,[2] Xiangjun Tang,[2] Ling Qiu,[6] Yan Qiu,[7] Jie Wen,[1,2] Jianqiu Fang[1,2] and Jian Wang[1,2]

Correspondence
Weijun Chen
chenweijun72@sohu.com
Jian Wang
wangjian@genomics.org.cn

[1]Beijing Genomics Institute, Chinese Academy of Sciences, Beijing 101300, China

[2]Beijing BGI-GBI Biotech Co. Ltd, Beijing 101300, China

[3]Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China

[4]Beijing 302 Hospital, Beijing 100039, China

[5]Beijing 301 Hospital, Beijing 100853, China

[6]Beijing Union Hospital, Beijing 100730, China

[7]Beijing Red Cross Blood Center, Beijing 100088, China

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To understand the time-course of viraemia and antibody responses to severe acute respiratory syndrome-associated coronavirus (SARS-CoV), RT-PCR and ELISA were used to assay 376 blood samples from 135 SARS patients at various stages of the illness, including samples from patients who were in their early convalescent phase. The results showed that IgM antibodies decreased and became undetectable 11 weeks into the recovery phase. IgG antibodies, however, remained detectable for a period beyond 11 weeks and were found in 100 % of patients in the early convalescent phase. SARS-CoV viraemia mainly appeared 1 week after the onset of illness and then decreased over a period of 1 month, becoming undetectable in the blood samples of the convalescent patients. At the peak of viraemia, viral RNA was detectable in 75 % of blood samples from patients who were clinically diagnosed with SARS 1 or 2 weeks before the test.

INTRODUCTION

An outbreak of atypical pneumonia, designated severe acute respiratory syndrome (SARS) by the World Health Organization (WHO), first emerged in Guangdong Province, China in November 2002 and was soon recognized as a global threat (Lee *et al.*, 2003). Until 5 July 2003, when WHO reported that the last human chain of transmission of SARS had been broken, it had infected over 8400 people and caused over 800 deaths in 33 countries and regions worldwide (WHO, 2003a, b). A novel coronavirus (SARS-CoV) isolated from SARS patients was identified as the causative agent of SARS (Drosten *et al.*, 2003; Ksiazek *et al.*, 2003; Peiris *et al.*, 2003a; Rota *et al.*, 2003; WHO, 2003c). The disease is characterized by fever, a non-productive cough, dyspnea, chest pain, lung infiltrates and fibrosis and a decreased lymphocyte count (WHO, 2003d).

SARS-CoV is detectable in the blood, faeces, urine and respiratory secretions of SARS patients (WHO, 2003e). Laboratory methods for SARS diagnosis are primarily PCR-based to detect viral RNA in patient's secretions/excretions and antibody-based to detect their immune responses, such as the titres of antibodies in the blood. Although seroconversion was found in all SARS patients (Li *et al.*, 2003), viral RNA detection rates are highly dependent on the type of specimen, and vary for the same type of specimen at different times following the onset of illness (Poon *et al.*, 2003). To understand the timing of SARS-CoV viraemia and the humoral immune responses, we performed a follow-up study on viral RNA and IgM and IgG antibodies of 376 blood samples from 135 SARS patients, including

†These authors contributed equally to this article.

Abbreviation: SARS-CoV, severe acute respiratory syndrome-associated coronavirus.

patients who were recovering from the illness nearly 2 months into their early convalescent phase.

METHODS

SARS patients and sample collection. SARS patients were diagnosed in Beijing 302 Hospital and Beijing Union Hospital, based on clinical characteristics that satisfied the WHO case definitions (WHO, 2003f). The WHO case definitions were as follows: a person presenting with high fever, cough or breathing difficulty and close contact history with SARS patients; a suspect case with radiographic evidence of infiltrates consistent with pneumonia or respiratory distress syndrome on chest X-ray; a suspect case of SARS that is positive for SARS-CoV by one or more assays; a suspect case with autopsy findings consistent with the pathology of respiratory distress syndrome without an identifiable cause. Clinical data were collected from the medical records of the hospitals, including age, sex, therapy protocols and drugs, identified risk factors for SARS-CoV infection and time since fever onset. If patients had no fever records, time was counted according to the records of other clinical symptoms such as cough, breathing difficulty and close contact history. The mean age of the 135 patients was 38·6 years and ranged from 15 to 82. Seventy-five of the patients were male. Fever was present in 126 patients and all patients gave a positive contact history. Of the patients, 128 received steroids, the dose varying between 15 and 640 mg day^{-1}, for 1–32 days. Blood samples were collected between 10 : 00 and 12 : 00 each day and stored in 0·05 mM EDTA at 4 °C before RNA preparation within a few hours.

Early convalescent patients and controls. When patients recovered and were discharged from hospital, they were called for blood donation. The standard of SARS convalescence was defined based on the discharge guidelines (Ministry of Health, People's Republic of China, 2003). Ninety early convalescents from the same patient pool (1–7 weeks from the day when they were discharged from the hospital) were selected and their blood samples were collected in Beijing Red Cross Blood Center and Beijing 301 Hospital. Control blood samples were collected from two groups: 30 healthy volunteers who had no contact with SARS patients and 30 healthy doctors and nurses who had contact history in a hospital, but were without symptoms of SARS 14 days after the sampling.

ELISA method. Human plasma was prepared from patients' blood samples and assayed using SARS ELISA IgG and IgM kits (Beijing BGI-GBI Biotech). Briefly, a confluent layer of Vero-E6 cells were infected with SARS-CoV BJ01 strain. After 72 h, the cells were harvested and inactivated using β-lactone (Sigma). The cell lysate was obtained by freeze–thaw cycling, and cell debris were removed by centrifugation (10 000 ***g***, 30 min). Supernatants were concentrated to one-fifth of the volume using PEG20000, and then disrupted using an Ultrasonic Processor set at 350 W for 10 min (20 s sonication followed by 15 s pause on ice). One hundred microlitres of the lysate diluted in 50 mM $NaHCO_3$ buffer (pH 9·6) (1 : 2000) was used as antigen to coat each well of a 96-well microtitre plate. Coating was allowed to occur at 4 °C overnight. Each well was rinsed and blocked with PBS containing 0·05 % Tween-20 and 3 % BSA. After incubation at 37 °C for 1 h, the plates were washed five times with PBS-Tween. Diluted serum samples (1 : 10 with PBS) were added to the plates. The plates were incubated at 37 °C for 30 min and washed five times with PBS-Tween. Peroxidase-conjugated mouse anti-human IgG/IgM, diluted 1 : 2000 in PBS supplemented with 0·5 % Tween-20 and 1·5 % BSA, was added to each well followed by incubation at 37 °C for 30 min. The plates were washed five times with PBS-Tween before the addition of tetramethyl benzidine (TMB) substrate. The reaction was stopped with the addition of 2 M H_2SO_4. The $A_{450/630}$ value was measured in triplicate. A blank control, a negative control and a positive control were included on each plate. The cut-off values for IgG and IgM were 0·18 and 0·16, respectively, which were calculated as the mean + 2 SD of the readings given by 1000 blood donor control sera.

Viral RNA extraction and RT-PCR method. Total RNA was extracted from whole blood with a QIAamp RNA blood mini kit (Qiagen) according to the manufacturer's instructions. The RNA was dissolved in 40 µl diethyl pyrocarbonate (DEPC)-treated water containing 1 U DNase I (Promega). A nested RT-PCR method was primarily used to ensure specificity. The PCR product was 131 bp covering the nucleotide positions 26241–26371 bp of SARS-CoV BJ01 strain (Qin *et al.*, 2003). cDNA was synthesized by reverse transcription from 6 µl RNA at 45 °C for 50 min in a 20 µl solution containing 50 mM Tris/HCl (pH 8·3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 100 ng random hexamer primers, 200 U Moloney murine leukaemia virus reverse transcriptase (MMLV, Promega), 25 U RNasin (Promega) and 0·5 mM dNTPs. The primary PCR was carried out in a 25 µl mixture containing 2 µl cDNA, 10 mM Tris/HCl (pH 8·4), 50 mM KCl, 2·5 mM $MgCl_2$, 100 µM dNTPs, 1 U *Taq* DNA polymerase (Promega), 0·25 µM forward primer (Coro1: 5′-TCACACTAGCCATCCTTACTG-3′) and 0·25 µM reverse primer (Coro2: 5′-TATTATGTACAAAAACCTGTTCC-3′). After 35 amplification cycles (94 °C for 30 s; 54 °C for 30 s; 72 °C for 30 s), the secondary PCR followed in a 25 µl mixture containing 1 µl of the first PCR product, 10 mM Tris/HCl (pH 8·4), 50 mM KCl, 2·5 mM $MgCl_2$, 100 µM dNTPs, 1 U *Taq* DNA polymerase, 0·25 µM inner forward primer (Coro3: 5′-CGTGAGTTTAGTAAAACCAA-3′), 0·25 µM reverse primer (Coro4: 5′-AATGTTAAAGTTCCAAACAGA-3′) and 0·25 µM TaqMan probe (5′FAM-AGAAGATCAGGAACTCCTTCAGA TAMRA3′) in a fluorometric thermal cycler (iCycler; Bio-Rad). Forty cycles of amplification (94 °C for 15 s; 58 °C for 1 min; fluorescence signals were recorded at 58 °C) were performed after denaturation at 95 °C for 4 min.

RESULTS

SARS-specific IgM and IgG antibodies and viral RNA in patients

We sampled 135 SARS patients at different stages of disease progression and combined the data when the numbers of specimens became significant. As a result, we have accumulated 286 blood samples, accompanied by basic clinical data, including age, sex, disease duration and drug treatment information. RT-PCR and ELISA tests were performed in parallel to evaluate viral RNA load (as an indicator of viraemia) and immune response for all patient samples (Table 1). It became clear that both IgG and IgM antibodies were present 1 week after diagnosis in more than half of the patients. The positive rate (samples detected as positive among total test cases) for IgM kept increasing until a month after diagnosis and then remained almost constant, at around 70 %. The positive rate for IgG reached 100 % after a month and remained higher than that of IgM. RT-PCR showed a higher sensitivity in detecting blood-borne viral RNA in the early phase of the disease than the ELISA method, but the positive rate was lower than that of antibody assays. Viral RNA was detectable in the blood samples 1 week after disease diagnosis and the positive rate peaked at around 2 weeks, reaching 75 %. The window to detect viral RNA in the blood is therefore much narrower than that to detect antibodies with the immunoassays.

Table 1. Detection of SARS-specific IgM and IgG antibodies and viral RNA

Figures in parentheses represent the percentage of patients who tested positive among total cases.

Course of disease (days)*	Samples (*n*)	Positives [*n* (%)]			
		IgM	IgG	IgM and/or IgG	RNA
1–7	13	0 (0)	0 (0)	0 (0)	1 (7·7)
8–14	32	14 (43·8)	15 (46·9)	17 (53·1)	24 (75·0)
15–21	51	37 (72·6)	41 (80·4)	43 (84·3)	30 (58·8)
22–28	45	35 (77·8)	41 (91·1)	41 (91·1)	23 (51·1)
29–35	54	38 (70·4)	50 (92·6)	52 (96·3)	20 (37·0)
⩾36	91	66 (72·5)	91 (100·0)	91 (100·0)	14 (15·4)
Total	286	190	238	244	174

*Days were counted from fever onset.

Viral RNA and SARS-specific IgM and IgG antibodies in SARS patients in early convalescent phase

We also followed the majority of patients (90 out of 135; Table 2) into their early convalescent phase after SARS (1–7 weeks from the day when they were discharged from hospital). As expected, all samples tested negative for the viral RNA. In contrast, serum antibodies were not only readily detectable in this phase but also remained at a high positive rate, especially those of the IgG class, which were present in all patients even after 11 weeks. IgM antibodies diminished from week 5 and became untraceable after 11 weeks. We also performed control samples to ensure that the patient immune response was not due to contact in a hospital environment. The controls consisted of blood samples from 60 healthy individuals, 30 of which were a random sampling from non-hospital workers and 30 of which were doctors and nurses. All control samples were negative for IgM, IgG and RNA.

Table 2. Detection of SARS-specific antibodies and viral RNA in early convalescent patients

Figures in parentheses represent the percentage of patients who tested positive among total cases.

Course of disease (days)*	Cases (*n*)	Positives [*n* (%)]		
		IgM	IgG	RNA
49–56	18	4 (22·2)	18 (100·0)	0 (0)
57–63	17	3 (17·7)	17 (100·0)	0 (0)
64–70	24	3 (12·5)	24 (100·0)	0 (0)
71–77	12	2 (16·7)	12 (100·0)	0 (0)
78–84	13	0 (0)	13 (100·0)	0 (0)
⩾84	6	0 (0)	6 (100·0)	0 (0)
Total	90	12	90	0

*Days were counted from fever onset.

DISCUSSION

In this study, we have analysed 376 clinical blood specimens from 135 SARS patients, of which we followed up 90 patients who were in the convalescent phase after successful disease treatment. All patients in the study demonstrated seroconversion to SARS-CoV, thereby confirming the diagnosis. Although the samples were not collected in series from single patients due to management difficulties and other technical problems, the large number of samples and subjects made this analysis significant. The control groups were selected to determine whether covert infection exists in non-contact and close-contact people in epidemic areas; 60 samples of healthy people (30 non-contact persons and 30 close-contact persons) were tested. No SARS-specific antibodies or RNA were detected. This suggests that covert infection does not exist in our test samples. This result also shows that the measures of separation and prevention taken during the SARS epidemic period were valid for the control of SARS.

The two tests used for the diagnostic procedures were very consistent, ensuring reproducible data, and these results were consistent with published data (Li *et al.*, 2003). The kits for IgG and IgM tests were commercial products used in laboratories and hospitals in China, Hong Kong and Taiwan, and have reputable sensitivities and specificities (Gao *et al.*, 2003) as also shown in this study. The RT-PCR-based test is also reliable, in that a primer-nesting procedure and fluorescent detection method were used to ensure specificity. We did devise similar tests with multiple primer groups based on the sequence from different regions of the viral genome but did not improve either sensitivity or specificity, because, in most cases, only one or two PCR products were positive when we used the blood samples (our unpublished data). However, better results were obtained with other secreted/excreted liquids. We have also tried other methods to improve the specificity, such as to sequence the PCR products directly and to use hybridization schemes for specificity tests. We believe that the blood-borne SARS-

CoV load may be quite low even when viraemia occurs. Alternatively, some of the patients may not even develop viraemia since most of the virus propagates in epithelial tissues, such as the lung. Therefore, the fact that we have detected the virus in 75 % of the patient samples at the peak of viraemia is considered a realistic result. This is slightly later than the detection of virus in respiratory secretions (Poon *et al.*, 2003), and not an effect of false-negative experimental results.

Our results also suggest that the IgM antibody to SARS-CoV persists for a much shorter time than the IgG antibody, as basic immunological principles imply. However, it is possible that our inability to detect an IgM response in the later sera was due to the structure of the IgM ELISA, as high titres of antigen-specific IgG could out-compete the binding of antigen-specific IgM in this format. The prolonged IgG production may suggest that not only does it play a principal role in the humoral immune response against acute SARS-CoV infection but also exerts crucial actions in cleaning up the residue of virus foci in the recovering phase. This is an essential subject for further investigation.

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