

# Antibody Responses Against SARS Coronavirus Are Correlated With Disease Outcome of Infected Individuals

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Most of the SARS-CoV-infected patients spontaneously recovered without clinical intervention while a small percentage succumbed to the disease. Here, we characterized temporal changes in N protein-specific and S glycoprotein-specific neutralizing antibody (Nab) responses in infected patients who have either recovered from or succumbed to SARS-CoV infection. Recovered patients were found to have higher and sustainable levels of both N protein-specific and S glycoprotein-specific Nab responses, suggesting that antibody responses likely play an important role in determining the ultimate disease outcome of SARS-CoV-infected patients. **J. Med. Virol.** 78:1–8, 2006. © 2005 Wiley-Liss, Inc.

**KEY WORDS:** SARS; neutralizing antibody; disease progression; vaccine

## INTRODUCTION

SARS, or severe acute respiratory syndrome, is a serious respiratory illness caused by a novel variant of coronavirus (SARS-associated coronavirus, SARS-CoV) [Drosten et al., 2003; Holmes, 2003a,b; Holmes and Enjuanes, 2003; Ksiazek et al., 2003; Kuiken et al., 2003; Lee et al., 2003; Marra et al., 2003; Peiris et al., 2003a,b; Poutanen et al., 2003; Rota et al., 2003; Tsang et al., 2003]. Most of the SARS-CoV-infected patients spontaneously recovered without clinical intervention while a small percentage succumbed to the disease. Age is one of the major factors in determining the fatality rate, demonstrated by a significantly higher rate (43.3%) for patients aged 60 years or older than for patients younger than 60 years (13.2%) [Donnelly et al., 2003]. The role of immune responses, however, is less clear. While there is no systematic evaluation of cytotoxic T-cell (CTL) activity in infected patients, there are some reports showing detection of binding antibodies to nucleocapsid

(N) and spike (S) glycoprotein after about 2 weeks, remaining detectable for as long as 210 days after the onset of symptoms [Shi et al., 2003, 2004; Huang et al., 2004; Leung et al., 2004; Liu et al., 2004; Nie et al., 2004a; Temperton et al., 2005]. The antibody isotypes reactive to N and S proteins are found to be IgM, IgG, and IgA, although there are kinetic differences among these three isotypes against viral antigens during the course of disease [Li et al., 2003; Shi et al., 2004; Woo et al., 2004b,c]. It has also been reported that patients who died of SARS did not produce any type of antibodies against N protein, whereas those who recovered from the infection did [Leung et al., 2004]. However, many of these studies were conducted using recombinant N and S proteins produced in bacteria *E. coli* [Huang et al., 2004; Li et al., 2003; Leung et al., 2004; Shi et al., 2003, 2004; Woo et al., 2004b,c], the conformation of which may or may not resemble those on the SARS-CoV, in particular for the highly glycosylated S protein. Recent studies by Nie et al. [2004a] and Temperton et al. [2005] have used a pseudotyped virus bearing S glycoprotein on the viral surface to evaluate the presence of neutralizing antibodies (Nab) in recovered individuals. They found that the majority of patients have detectable levels of S glycoprotein-specific Nab, which can last over 200 days after the onset of symptom [Nie et al., 2004a; Temperton et al., 2005]. In the study by Nie et al. [2004a], it is not known why the remaining 14.1% of patients had never developed Nab, or if they were simply not infected at the time of sampling. Based on their cross-sectional studies

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of 623 serum samples collected from these probable cases [Nie et al., 2004a], they found that Nab became detectable around 5–10 days, peaked around 20–30 days, and could sustain for well over 150 days after the onset of symptoms. However, Nab responses in those who unfortunately died of SARS-CoV infection have not been characterized [Nie et al., 2004a].

In this report, using both the ELISA-based and pseudotyped retrovirus-based neutralization systems, we characterized temporal changes in N protein-specific antibody and S glycoprotein-specific Nab responses in infected patients who have either recovered from or succumb to SARS-CoV infection. We have found that in those recovered patients, antibodies against N protein were detectable as early as 10–15 days, peaked around 20 days, and sustained high levels 87 days after the onset of symptoms. However, in those patients who died, N protein-specific antibodies were either never developed or initially developed during the first 2–3 weeks and then decreased dramatically in ensuing period. In regard to S glycoprotein-specific Nab, both recovered, and deceased patients developed detectable levels during the first 10–15 days after the onset of symptoms, although the actual titer is significantly lower in the former than in the latter group. The production of Nab in the deceased patients was surprisingly faster than in recovered patients, and reached highest levels on day 15 after the onset of symptoms while Nab levels in recovered patients were still rising. What set these two groups of patients apart are their subsequent levels of Nab during the ensuing period of infection. While recovered patients continue to have higher levels of Nab and peak around day 20 after the onset of symptoms, those deceased patients dramatically reduced their levels after 15 days of onset symptoms. These results suggest that high and sustainable levels of N protein-specific and S glycoprotein-specific Nab are correlated with recovery of SARS-CoV-infected patients. The underlying mechanism for significant and rapid loss of anti-N antibodies and anti-S glycoprotein Nab in the deceased patients is currently unknown. It could either be due to the over exhaustion of the immune system as a result of excess virus replication and production or specific loss or inhibition of antibody producing cells. The former hypothesis would agree with the immunopathologic effects of SARS-CoV on lung and spleen, and rapid and generalized lymphopenia in SARS patients during the acute phase of infection [Booth et al., 2003; Li et al., 2004; Leung et al., 2004; Nicholls et al., 2003; Peiris et al., 2003a,b; Poutanen et al., 2003; Wong et al., 2003; Woo et al., 2004a]. Future studies are warranted to dissect these possibilities.

## RESULTS

### Development and Characterization of a Pseudotyped Retrovirus Bearing S Glycoprotein

To study Nab responses to SARS-CoV, there is a great need to develop a sensitive, specific, and safe assay that

is dependent on the functionality of the S glycoprotein. While the culture system using live SARS-CoV can be explored, the safety concerns and technical requirements for performing such a culture with a live virus have hampered the broad use of such a system. We sought to overcome these technical hurdles by developing a pseudotyped retrovirus bearing native and full-length S glycoprotein of SARS-CoV. We first cloned a wild-type full-length S gene by PCR amplification directly from cDNA generated from fetal rhesus monkey kidney (FRhK-4) cells infected with a SARS-CoV HK-39 (Genbank accession number AY278491) [Zeng et al., 2003]. To improve the levels of protein expression, we also constructed a codon-optimized full-length S gene using synthetic overlapping oligonucleotides. The amplified products were then cloned into a eukaryotic expression vector pcDNA3.1 under the control of CMV promoter (Invitrogen, Inc., Carlsbad, CA). The full-length S gene sequence was confirmed by sequencing. To our surprise, the wild-type S gene did not express detectable levels of S glycoprotein despite multiple attempts whereas the optimized gene expressed high levels of S glycoprotein in the transfected 293 cells (data not shown), suggesting that a genetic feature of the wild-type S gene or the particular promoter and gene combination prevents efficient transcription and translation of these gene constructs. In any case, it is clear that the optimization approach has significantly increased the expression levels of S glycoprotein in mammalian cells, a critical step for the development of our pseudotyped retrovirus system. We have, therefore, used codon-optimized S gene to generate a pseudotyped retrovirus. Specifically, construct pNL43R-E-luciferase containing the vpr- and env-defective HIV-1 proviral genome and expression plasmid encoding the optimized full-length S glycoprotein were co-transfected into the 293 cells to generate a pseudotyped retrovirus (Fig. 1). A control plasmid expressing HIV-1 envelop glycoprotein JRFL strain was also co-transfected with construct pNL43R-E-luciferase. Forty-eight hr after transfection, culture supernatant was collected and tested for HIV-1 p24 antigen content (Aalto Bio Reagents, Dublin, Ireland) for standardizing retrovirus input in the subsequent neutralization assay.

To test the entry efficiency of the pseudotyped retrovirus bearing S glycoprotein, multiple cell lines were used including Vero, VeroC1008, Vero76, Huh-7.5, Hep3B, 293/ACE2, 293, BHK-21 Host-T4-X4 or R5, and 5.25m7. Figure 2 demonstrates the entry efficiency of the pseudotyped retrovirus into these cell lines. It is clear the pseudotyped retrovirus bearing S glycoprotein is able to enter Vero, VeroC1008, Vero76, Huh7.5, and most efficiently 293/ACE2, but not Hep3B, 293, BHK21, Host-T4-X4 or R5, and 5.25m7. The cell tropism of the pseudotyped retrovirus is, therefore, consistent with that of live SARS-CoV reported previously [Peiris et al., 2003b]. Entry efficiency into the 293/ACE2 cell line (293 cell line transduced with SARS-CoV receptor gene ACE2) is about 10–100-fold higher than that into other susceptible cell lines (Fig. 2). On the other hand, the

Generation of a retrovirus pseudotyped with SARS S glycoprotein

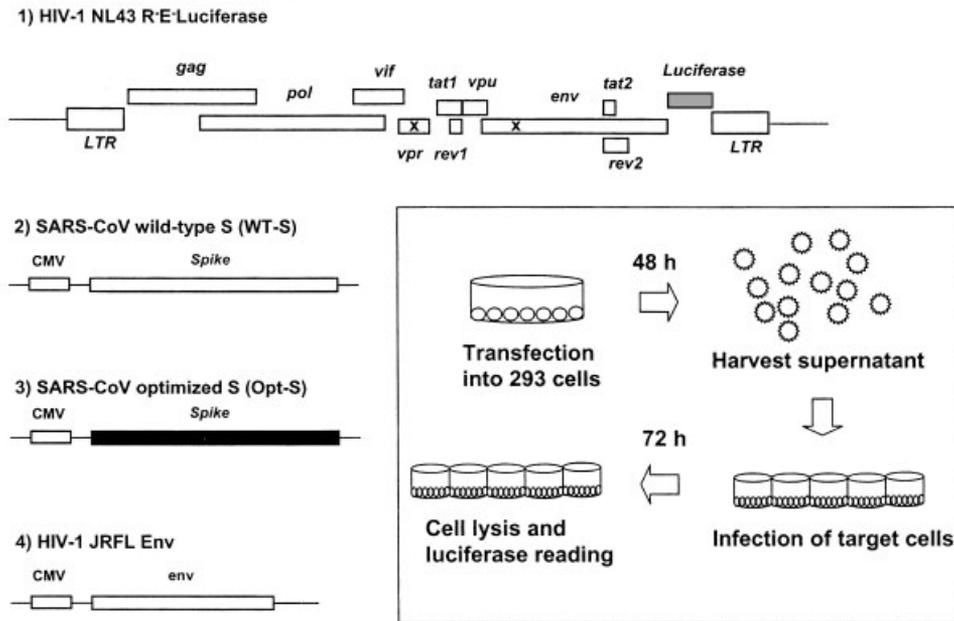


Fig. 1. Schematic representation of plasmid constructs used to generate pseudotyped retrovirus bearing either SARS S glycoprotein or HIV-1 JRFL envelop glycoprotein. The insert illustrates the time course for generating pseudotyped retrovirus and subsequent infection of target cells by the pseudotyped retrovirus.

pseudotyped retrovirus bearing the HIV-1 JRFL envelope is only able to infect cell lines 5.25m7 and Host-T4-R5, consistent with its cellular tropism and requirement for CD4 and CCR5 expression for entry, although the entry efficiency into Host-CD4-R5 is about a log higher compared to that into 5.25m7 (Fig. 2). To further confirm entry specificity through SARS-CoV receptor ACE2, the pseudotyped retrovirus infection of three susceptible cell lines (293/ACE2, Huh7.5, and VeroC1008) was tested in the presence of polyclonal anti-ACE2 antibodies (R&D systems, Minneapolis, MN) or soluble ectodomain of ACE2 proteins. As shown in Figure 3, entry of a pseudotyped retrovirus bearing S glycoprotein into these three cell lines was significantly inhibited in the presence of anti-ACE2 antibody (IC<sub>90</sub> ~ 4 µg/ml) or soluble ACE2 (IC<sub>90</sub> ~ 20 nM), suggesting that retroviral entry is mediated specifically through the receptor ACE2. These results have presented evidence that this pseudotyped retrovirus is capable of infecting target cell lines susceptible to live SARS-CoV infection but not non-susceptible ones. Furthermore, infection by this pseudotyped retrovirus can be effectively blocked by polyclonal antibodies specific to the receptor ACE2 or by soluble forms of ACE2, further confirming the structural and functional integrity of S glycoprotein on this pseudotyped retrovirus. These results are in complete agreement with reports published previously for other viral pseudotype system [Giroglou et al., 2004; Han et al., 2004; Moore et al., 2004; Nie et al., 2004b; Temperton et al., 2005].

Neutralizing Antibody Responses Against S Glycoprotein and N Protein During the Course of Infection With SARS-CoV

Since the 293/ACE2 cell line is the most susceptible to entry of the pseudotyped retrovirus bearing S

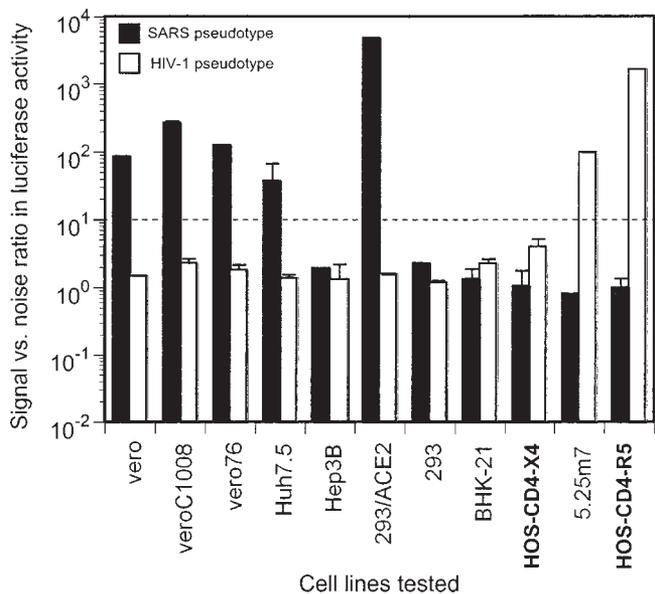


Fig. 2. Entry efficiency of pseudotyped retrovirus bearing either SARS S glycoprotein (filled boxes) or HIV-1 JRFL envelop glycoprotein (open boxes) into various cell lines.

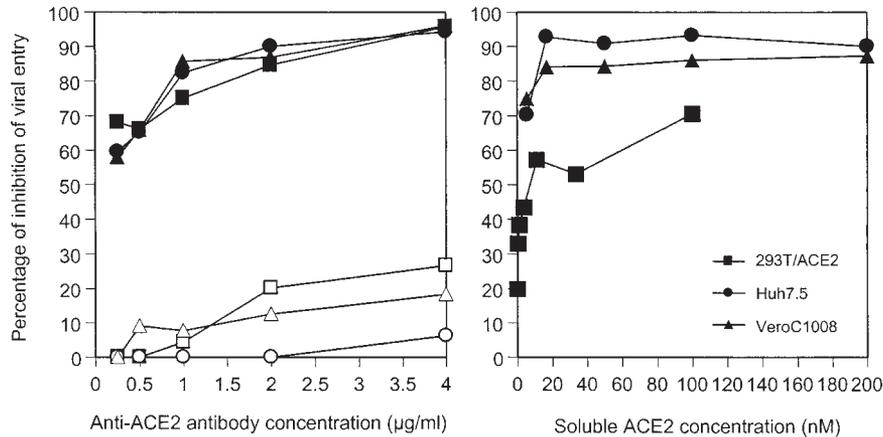


Fig. 3. Entry inhibition of pseudotyped retrovirus bearing S glycoprotein by ACE2-specific antibodies and soluble ACE2 protein. Percent of inhibition by ACE2-specific antibodies was exemplified in solid symbols whereas that for control antibodies were in open symbols (left panel). Right panel demonstrates potent inhibition of retroviral entry by soluble ACE2 protein. This figure collectively highlights the entry of pseudotyped retrovirus into target cell lines specifically mediated through the ACE2 receptor.

glycoprotein, we chose this cell line for studies of neutralization antibody (Nab) activities of serum samples collected from SARS-CoV-infected patients. Cross-sectional serum samples were collected from eight deceased and eight recovered patients of SARS-CoV infection. Sequential serum samples were also collected from three deceased and three recovered patients. All study patients were admitted to United Christian Hospital and Caritas Medical Centre between March 24th and April 28th in 2003. The study was approved by the local ethics committees and the IRB of the Rockefeller University. Diagnosis of coronavirus infection was made with nasopharyngeal swabs on admission and/or convalescent serum from days 14 to 28 after the onset of symptoms. The detailed reports on clinical manifestation of these patients during the course of infection have been published elsewhere [Chu et al., 2004].

We first studied the Nab activities in cross-sectional samples. Serum samples were serially diluted 100-, 200-, 400-, 800-, 1,600-, and 3,200-fold prior to mixing with the p24 normalized pseudotyped retrovirus bearing the S glycoprotein and incubated for 1 hr at 37°C before adding to the 293/ACE2 cell line. Seventy-two hr later, cells were lysed with 50 µl/well of cell lysis buffer (Promega, Madison, WI) for 30 min at RT, and 30 µl of lysate was then tested for luciferase activity. Figure 4 demonstrates the serum Nab activities against the pseudotyped retrovirus in both deceased (A) and recovered (B) groups. In the deceased group, Nab activities were detected in all patients, though significant differences exist among different patients. Serum samples from patients D02, D03, and D08, for instance, retained over 90% neutralizing activities despite a 3,200-fold dilution. The same fold dilution for other serum samples, however, have significantly reduced Nab activities from 20% to 60% for patients D06, D04, D05, D01, and D07 (Fig. 4A). In the recovered group,

Nab activities were also detected in all patients with variable levels. In four (R11, R12, R13, and R14) of the eight patients, dilution of 3,200-fold had minimal effect on serum Nab activities, while the same fold dilution has reduced Nab activities from 20% to 55% for the rest of the patients (Fig. 4B). Furthermore, comparing overall Nab activities between the deceased and the recovered group, there is a clear trend that the recovered group has relatively higher levels compared to that of the deceased group (Fig. 4C), although none of the comparisons at a given dilution yield any significant differences between the two groups. Finally, there are clear dose-dependent Nab activities for all samples tested, reinforcing the structural and functional integrity of codon-optimized S glycoprotein on the surface of pseudotyped retrovirus (Fig. 4).

The same set of serum samples were also tested for their antibody binding activities to SARS-CoV N protein. We first cloned full-length N gene into the bacteria expression vector pET19b (Novagen, Inc., Madison, WI) through two unique restriction enzyme sites, Nde I and BamH I artificially inserted to the 5'- and 3'-end of the gene, respectively. The 3'-end of the N gene lacked the termination codon and fused to His-tag, which was used to purify recombinant N protein from *E. coli* strain BL21 (DE3) (Novagen, Inc.) through interaction with Ni-NTA agarose (Qiagen, Inc., Valencia, CA). Serum antibody binding activities to N protein was evaluated by quantitative ELISA. Figure 5 shows the relative titer of N protein-specific binding antibodies in the deceased (A) and the recovered (B) groups. For each serum sample, antibody-binding activities were tested for serial dilutions at 200-, 400-, 800-, 1,600-, 3,200-, and 6,400-fold. For significant numbers of patients in both groups, detectable levels of anti-N binding antibodies were found, although a large degree of variation existed among different patients. Overall, the average level of binding antibodies in the recovered group seems higher

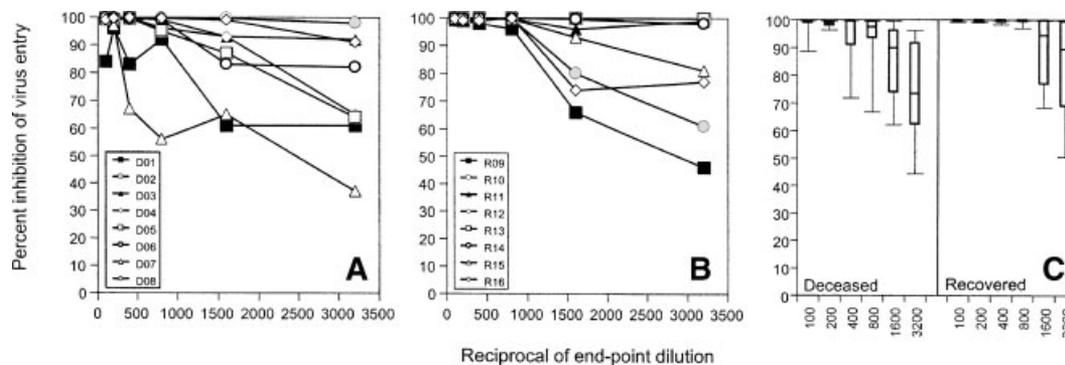


Fig. 4. Cross-sectional studies of neutralizing antibody (Nab) activities in patients either deceased (A) or recovered (B) from SARS-CoV infection. The box plot (C) compares overall Nab activities in two patient groups. The top and bottom of each rectangular box denote the 75th and 25th percentiles, respectively, with the median shown inside the box. Horizontal bars extending from each box represent the 90th and 10th percentiles.

than that in the deceased group (Fig. 5C). Four patients (D01, D04, D06, and D08) in the deceased group, for example, had OD reading at 450 nm less than 1 at dilution 200, while there is only one such patient (R16) in the recovered group (Fig. 5A,B). However, none of the comparisons at any given dilution revealed statistical differences between these two groups, suggesting that binding antibodies to N protein, like S glycoprotein-specific Nab activities studied above, may not be associated or immunologic surrogate for disease outcome in SARS-CoV infection.

The lack of association between the levels of antibody responses against S glycoprotein and N protein and the disease outcome could be due to the insufficient number of patients studied and/or the actual timing of cross-sectional sampling since antibody responses against both of these proteins are a dynamic and evolving process during the course of infection [Li et al., 2003; Nie et al., 2004a; Shi et al., 2004; Temperton et al., 2005; Woo et al., 2004b]. To further study the relationship between antibody responses and disease outcome in a temporal

perspective, we collected sequential serum samples from three deceased (d1, d2, and d3) and three recovered (r1, r2, and r3) patients of SARS-CoV infection. Four to seven sequential samples were collected from each patient from day 9 to day 22 for the deceased patients and from day 4 to day 87 for the recovered patients after the onset of symptoms. The serum samples were serially diluted at 100-, 200-, 400-, 800-, 1,600-, and 3,200-fold, mixed with pseudotyped retrovirus bearing the S glycoprotein, and assayed for their Nab activities. Figure 6 shows the sequential serum Nab activities from the three deceased and three recovered patients based on the luciferase activities measured 72 hr after infection with the pseudotyped retrovirus. Three deceased patients had detectable levels of Nab activities on day 9–11, which rapidly peaked and reached plateau between days 12 and 16 after the onset of symptoms (Fig. 6, upper panel). However, dramatic drops in Nab activities were found in all three deceased patients approximately 5 days afterwards, which coincided with their clinical deterioration (Fig. 6, upper panel) followed

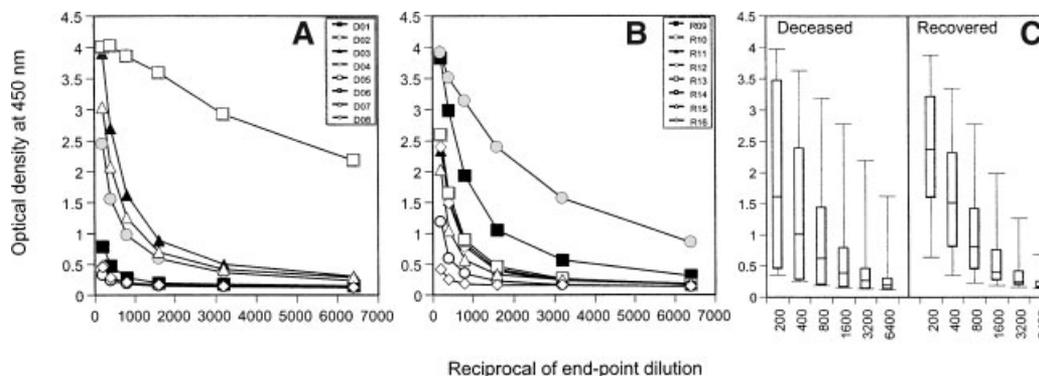


Fig. 5. Cross-sectional studies of N protein-specific antibody responses in patients either deceased (A) or recovered (B) from SARS-CoV infection. The box plot (C) compares overall N protein-specific antibody responses in two patient groups. The top and bottom of each rectangular box denote the 75th and 25th percentiles, respectively, with the median shown inside the box. Horizontal bars extending from each box represent the 90th and 10th percentiles.

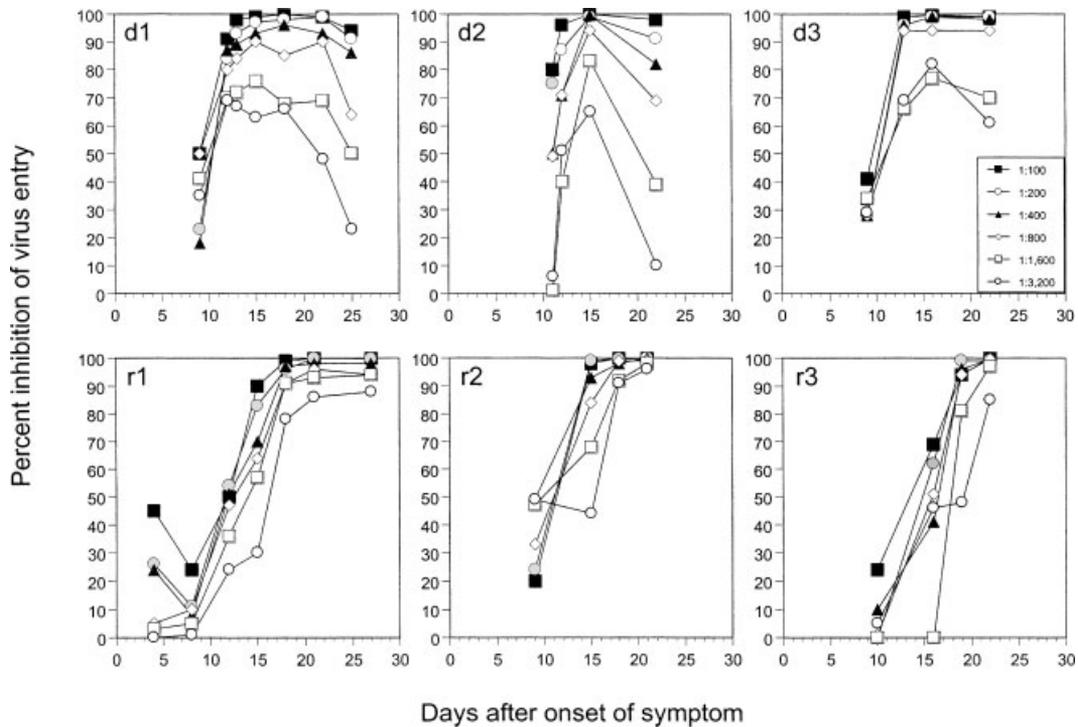


Fig. 6. Sequential analysis of Nab activities in patients either deceased (upper panel) or recovered (lower panel) from SARS-CoV infection. All serum samples were serially diluted (100-, 200-, 400-, 800-, 1,600-, and 3,200-fold) prior to neutralization studies.

by their death on day 25, 26, and 33 after the onset of symptom for d1, d2, and d3, respectively [Chu et al., 2004].

The temporal changes in Nab activities in recovered patients are distinctly different from that of the deceased patients. Although Nab activities were found in all three patients on day 4–10 after the onset of symptoms, the actual titer is significantly lower compared to that in the deceased patients during the same time period ( $P < 0.007$ ), in particular for patients r1 and r3 (Fig. 6, lower panel). Furthermore, it took an average of 20 days for the recovered patients to reach their peak of Nab activities, as opposed to only 14.7 days for the deceased patients (Fig. 6). Finally, there was no significant reduction in Nab activities after the peak Nab activities were reached, and in one patient (r3) Nab activities were retained at the highest levels, 87 days after the onset of symptoms (data not shown).

Sequential antibody response against N protein has also been evaluated for the two groups of patients. As shown in Figure 7, the three deceased patients either never developed any anti-N protein antibodies (d2 and d3), or initially developed some levels and then gradually dropped 25 days (d1) after the onset of symptoms (Fig. 7, upper panel). The recovered patients, however, started to develop anti-N protein antibodies between days 10 and 15 after the onset of symptoms, and gradually increased their titers at least 8–16-folds compared to the baseline levels 20–25 days after the onset of symptoms (Fig. 7, lower panel). Those results

clearly demonstrated that there is strong correlation between strong antibody responses to N and S proteins and the survival of SARS-CoV infection patients.

## DISCUSSION

In this report, we characterized temporal antibody responses against N protein and S glycoprotein during the first month of infection with SARS-CoV. Using pseudotyped retrovirus bearing S glycoprotein and ELISA-based method, we compared specific Nab and binding antibody activities in both cross-sectional and sequential samples collected from infected patients. In cross-sectional studies, we found a trend that recovered patients tended to have higher levels of Nab against S glycoprotein and binding antibodies against N protein than that in deceased patients. This trend was further confirmed by the studies of sequential samples. Although the number of patients studied in this report is quite small, the consistent results particularly from sequential studies suggest that higher levels of antibody responses are correlated with patients' recovery, and may play an important role in clearing and removing SARS-CoV in infected patients. It is interesting to note that there are considerable differences in the dynamics of antibody responses between the two groups of patients. While Nab activities in the deceased patients were higher and developed faster compared to that in the recovered patients during the first 15 days after the onset of symptoms, such activities, however, lacked the sustainability

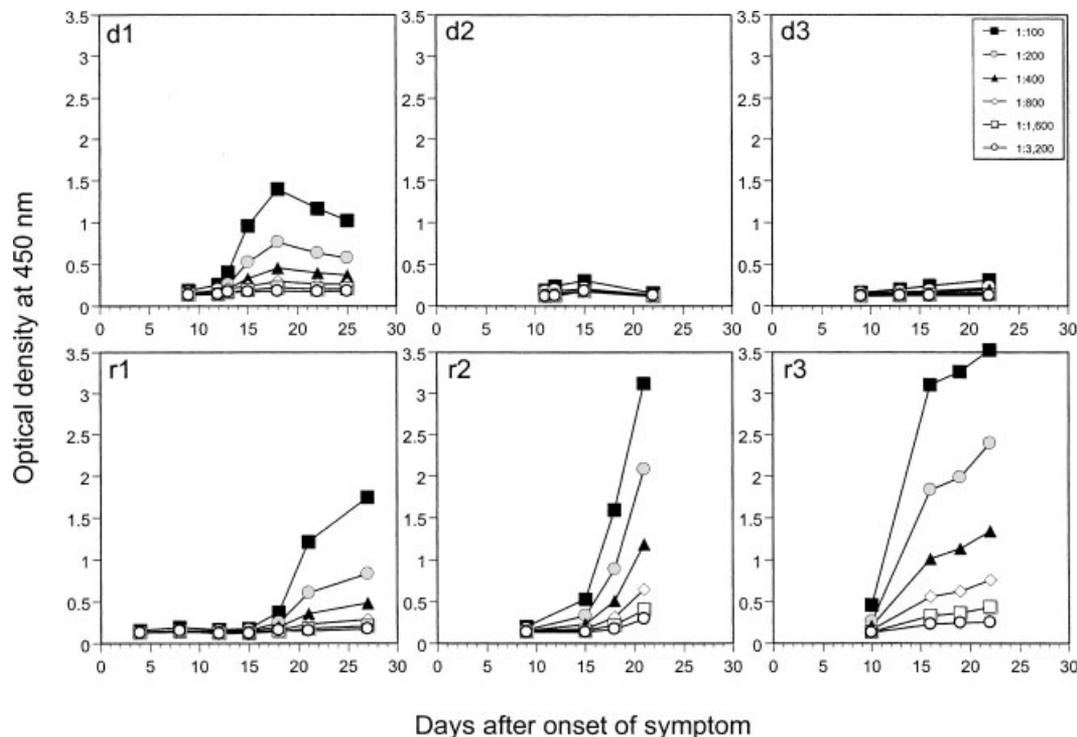


Fig. 7. Sequential analysis of N protein-specific activities in patients either deceased (upper panel) or recovered (lower panel) from SARS-CoV infection. All serum samples were serially diluted (100-, 200-, 400-, 800-, 1,600-, and 3,200-fold) prior to ELISA analysis.

and diminished rapidly in the ensuing period of infection. Nab activities in recovered patients, on the other hand, developed slowly and were able to maintain high levels for an extended period of time. In fact, many of the recovered patients have sustained high levels of Nab activities even 300 days after the onset of symptoms (Taisheng Li, personal communication). The mechanism underlying such dichotomy in two groups of patients is currently unknown. It is possible that excessive and “overly-quick” immune activation in those deceased patients had triggered systematic breakdown of the immune system. Severe inflammation and significant higher levels of pro-inflammation cytokine found in SARS patients are consistent with this hypothesis [Booth et al., 2003; Lee et al., 2003; Nicholls et al., 2003; Peiris et al., 2003a,b; Poutanen et al., 2003; Tsang et al., 2003; Wong et al., 2003]. However, systematic breakdown of the immune system could also be the direct consequences of dysfunction or loss of function of other bodily systems such as respiratory, since air-space consolidation is distinct manifestation of SRAR patients [Booth et al., 2003; Lee et al., 2003; Nicholls et al., 2003; Peiris et al., 2003a,b; Poutanen et al., 2003; Tsang et al., 2003; Wong et al., 2003]. Splenic atrophy and rapid and generalized lymphopenia observed in SARS-CoV-infected patients could also lead to reduced levels of antibody production [Booth et al., 2003; Ksiazek et al., 2003; Li et al., 2004; Nicholls et al., 2003; Peiris et al., 2003a,b; Poutanen et al., 2003; Wong et al., 2003]. Future studies would be needed to dissect out these possibilities.

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