

False Positive Antibody Results Against Human T-Cell Lymphotropic Virus in Patients with Severe Acute Respiratory Syndrome

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Taiwan suffered from the outbreak of severe acute respiratory syndrome (SARS) in 2003. Our laboratory performed a series of virology and serology tests for SARS patients admitted to our hospital. Cross-reactivity was found when testing for antibody against human T-cell lymphotropic virus (HTLV) in one patient with SARS. Therefore, antibodies against HTLV were examined in paired-sera from 26 SARS patients. ELISA and a neutralization test were used to measure anti-SARS antibodies. Seroconversion for antibody against SARS-CoV was observed in all patients. Surprisingly, with the use of ELISA for HTLV, sera for 13 patients were positive for HTLV (50%), and seroconversion for HTLV was also observed in 10 patients (38.5%). Western blot for HTLV on those 26 paired-sera from 13 HTLV-positive patients displayed 5 positive results for HTLV-I, 7 positive results for HTLV-II, 1 positive result for both HTLV-I and II, 9 negative results for either HTLV-I or HTLV-II, and 4 "indeterminate" results. The findings that antibody to HTLV can be detected in blood samples collected from SARS patients provide important information for safe handling of blood products. Without such knowledge, blood products can be discarded mistakenly even though they contain anti-SARS-CoV antibodies that may be potentially valuable for SARS therapy. *J. Med. Virol.* 77:331–336, 2005. © 2005 Wiley-Liss, Inc.

KEY WORDS: severe acute respiratory syndrome (SARS); human T-cell lymphotropic virus (HTLV); cross-reactivity

INTRODUCTION

Severe acute respiratory syndrome (SARS) is a new infectious disease caused by a previously unrecognized coronavirus [Drosten et al., 2003; Ksiazek et al., 2003;

Poutanen et al., 2003; Rota et al., 2003; Peiris et al., 2003b]. SARS originated from Southern China and then spread to many countries and regions, including Taiwan [Chan et al., 2004]. The latter experienced a SARS outbreak from March to June of 2003 and suffered from 680 probable cases of SARS with 81 confirmed deaths [Wu et al., 2004]. In our hospital, a 23-year-old patient recovered from SARS and was ready for discharge on the 17th day after the onset of the disease. This patient donated blood for research use. For biosafety reasons, a series of routine tests were conducted to detect antibodies against human immunodeficiency virus (HIV), human hepatitis B, C virus (HBV, HCV), and human T-cell lymphotropic virus (HTLV). The result was HTLV positive by HTLV ELISA kit (Murex HTLV I+II, Abbott Laboratories, Chicago, IL). At the time of sera collection, antibody to SARS coronavirus (SARS-CoV) was also detected in the serum of this patient. An earlier serum collected from the same patient on the 3rd day after disease onset was retested, and it was found that the results were negative for both SARS-CoV and HTLV antibodies. The results suggested that anti-SARS antibodies might recognize HTLV antigens. We therefore examined the antibody responses using sera from additional 25 SARS patients.

MATERIALS AND METHODS

Patients and Sera

All 26 patients were diagnosed as probable SARS infections based on the clinical manifestations and

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contact history, as suggested by the WHO [2003a]. The age of the patients was ranged from 12 to 77 years old. Twenty-three of them are female and three are male. Sera were collected from April 2003 to February 2004.

Neutralization Test for Anti-SARS-CoV Assay [WHO, 2003b]

Serum was tested in twofold serial dilutions from 1:2 to 1:4,096 using 2% MEM. Moreover, 100 μ l of diluted serum was mixed with 50 μ l of 25 TCID₅₀ SARS-CoV in 96-well plate. After 2 hr incubation at 37°C, 100 μ l of 1×10^5 /ml Vero-E6 cells were added to each well. The plates then were incubated at 37°C for 3–5 days. The results were read when CPE appeared in 1TCID₅₀ virus in the control wells (at least five wells for controls were used per experiment). Three types of controls were also included in the neutralization test, namely the cell control: cell + MEM; serum control: serum + MEM + cell, and virus control: 100, 10, 1, 10^{-1} TCID₅₀ + MEM + cell.

ELISA for Detection of Anti-SARS-CoV Antibodies in Patients

The ELISA antigen used for anti-SARS-CoV antibody detection was the detergent-extracted and gamma-irradiated Vero E6 cells infected with SARS-CoV. A similar preparation for uninfected Vero E6 cells were used as the control. Patient sera were diluted ten times and added to the ELISA plates, and goat anti-human IgG antibody conjugated with horseradish peroxidase (DAKO, Cambridgeshire, UK) was added for enzymatic reaction. Following the addition of the substrate, *O*-phenylenediamine, the optical density (OD) value was measured under 450 nm wavelength. A cut off value of 0.150 was used for this ELISA. [Peiris et al., 2003a; Rota et al., 2003; WHO, 2003b; Chan et al., 2004]

ELISA for Detection of Anti-HTLV Antibodies in Patients

The ELISA commercial kit (Abbott Murex HTLV 1 + 2) used for anti-HTLV antibody detection was based on microwells coated with synthetic peptides representing immunodominant regions from the HTLV-I and HTLV-II envelope proteins and a recombinant transmembrane protein from HTLV-II [Rouet et al., 2001]. A cut off value of 0.250 was used for this ELISA.

Western Blot for HTLV

A commercial Western blotting kit (HTLV Blot 2.4, Genelab Diagnostics, Singapore) was used which is based on the nitrocellulose strips incorporated with HTLV-I and HTLV-II viral core and envelope proteins [Rouet et al., 2001]. The interpretation of results was according to the criteria described in the kit. Seronegative represents no reactivity to HTLV specific proteins. HTLV-I seropositive is the reactivity to GAG (p4 with or without p19) and ENV (GD21 and rgp46-I). HTLV-II seropositive is the reactivity to GAG (p19 and p24) and

ENV (GD21 and rgp46-II). HTLV seropositive is when the specific reactivity to GAG (p19 with or without p24) and ENV (GD21) were observed. "Indeterminate" indicates that HTLV specific bands were detected but did not meet criteria for HTLV-I, HTLV-II, or HTLV seropositive described above.

RESULTS

Serum conversion for antibody against SARS-CoV was observed in all 26 patients using both the SARS-IgG ELISA and SARS-neutralization tests (Table I). Surprisingly, 13 patients (13/26 or 50%) were seropositive for HTLV by ELISA (Abbott Murex HTLV-1/2 assay). Among these 13 patients (patient No. 1–No. 13), the OD values of HTLV ELISA from their second sera in nine patients (9/13 or 69.2%) were found greater than one. Seroconversion for HTLV was also observed in 10 these 13 patients (10/13 or 76.9%), except for patient No. 5, 7, and 10, where their first sera were found strongly positive (OD > 3.0) for antibody to HTLV than their second sera. All paired-sera for these 13 patients were further tested by Western blot analysis for HTLV (HTLV Blot 2.4, Genelab Diagnostics, Singapore). From Figure 1, all of the second sera for those ten SARS patients showing HTLV seroconversion as well as the first sera from patient No. 5, 7, 10 were reactive against the gag protein p24, except for the second serum of patient No. 13 (serum No. 13-2), which coincidentally had the lowest OD value among the second sera for those ten patients having seroconversion in HTLV ELISA (Table I). Reactivity against GD21 was found in all patients' second sera (except for serum No. 6-2) and first sera from patient No. 5, 7, 10. According to the seropositive definition described by the manufacturer (see Materials & Methods), the Western blotting for those 26 paired-sera from patient No. 1 to No. 13 displayed five positive for HTLV-I (serum No. 5-1, 5-2, 8-2, 9-2, and 12-2), seven positive for HTLV-II (serum No. 1-2, 3-2, 4-2, 7-1, 10-1, 10-2, and 11-2), one positive for both HTLV-I and II (serum No. 2-2), nine negative for either HTLV-I or II (serum No. 1-1, 2-1, 3-1, 4-1, 6-1, 8-1, 9-1, 12-1, and 13-1), and four for "indeterminate" (serum No. 6-2, 7-2, 11-1, and 13-2). Notably, in this HTLV Western blot assay, the intensity of the positive bands appeared in SARS patients were much weaker than those of truly HTLV-infected patients (in comparison with the positive controls in Fig. 1, lanes 1 and 2).

It was speculated that some SARS-CoV peptides common to HTLV might be responsible for the false positive results observed in HTLV antibody detection. We retrieved and analyzed 6 SARS-CoV full genomes, as well as 15 HTLV full genomes (including 7 HTLV-I and 8 HTLV-II) from GenBank (as of May 2003). All the annotated coding sequences were cross-examined in searching for common peptides longer than six amino-acid residues between the SARS-CoV and HTLV. Four common peptides were found and listed in Table II. Among these, "RFPNITN" is located on the spike protein (S) of SARS-CoV and on the envelope protein (env) of HTLV-I, while "LALLLL" is located on the nucleocapsid

TABLE I. Results of 52 Paired-Sera From 26 Patients Associated with SARS

Patient no.-serum no.	Sample no.	Patient age	Serum collecting time (day after onset)	SARS-IgG ELISA (OD) ^a	SARS-neutralizing Ab (titer)	HTLV-I + II ELISA (OD) ^b
1-1	70s	23	4	0.012(-)	1:8	0.088(-)
1-2	765s		22	0.601(+)	1:512	1.766(+)
2-1	2s	42	5	0.001(-)	<1:8	0.047(-)
2-2	158s		26	0.834(+)	1:512	1.931 (+)
3-1	32s	21	11	0.348(+)	1:128	0.069 (-)
3-2	53s		16	0.949(+)	1:256	>3.0(+)
4-1	31s	35	11	0.334(+)	<1:8	0.064(-)
4-2	1087s		37	1.104(+)	1:64	1.705(+)
5-1	8s	47	3	0.113(-)	1:32	>3.0(+)
5-2	148s		17	0.635(+)	1:256	1.252(+)
6-1	24s	72	4	0.251(+)	1:8	0.059(-)
6-2	873s		27	1.110(+)	1:1024	0.376(+)
7-1	942s	34	5	0.025(-)	<1:8	>3.0(+)
7-2	2119s		26	0.535(+)	1:256	1.104(+)
8-1	10s	37	3	0.278(+)	1:8	0.066(-)
8-2	725s		24	0.751(+)	1:512	1.323(+)
9-1	7s	30	3	0.118(-)	<1:8	0.044(-)
9-2	1104s		28	0.911(+)	1:512	0.442(+)
10-1	951s	23	4	0.033(-)	<1:8	>3.0(+)
10-2	160s		20	0.468(+)	1:64	2.714(+)
11-1	26s	38	9	0.006(-)	<1:8	0.032(-)
11-2	85s		16	0.317(+)	1:64	>3.0 (+)
12-1	39s	42	3	0.003(-)	<1:8	0.073(-)
12-2	837s		20	0.643(+)	1:512	0.950(+)
13-1	49s	51	3	0.018(-)	<1:8	0.062(-)
13-2	1041s		16	1.056(+)	1:256	0.360(+)
14-1	141s	77	3	0.049(-)	1:32	0.081(-)
14-2	1488s		25	0.861(+)	1:1024	0.135(-)
15-1	2137s	55	7	0.105(-)	1:64	0.085(-)
15-2	1501s		26	1.148(+)	1:512	0.063(-)
16-1	2300s	41	11	0.305(+)	1:16	0.079(-)
16-2	236s		32	0.635(+)	1:128	0.059(-)
17-1	5s	14	3	0.035(-)	1:16	0.090(-)
17-2	2132s		26	1.028(+)	1:128	0.063(-)
18-1	008s	39	8	0.001(-)	<1:8	0.136(-)
18-2	2164s		22	0.437(+)	1:256	0.051(-)
19-1	504s	48	5	0.010(-)	<1:8	0.079(-)
19-2	1208s		26	0.752(+)	1:1024	0.062(-)
20-1	205s	70	9	0.284(+)	1:64	0.090(-)
20-2	SAB68		39	1.320(+)	1:1024	0.058(-)
21-1	91s	42	4	0.001(-)	1:8	0.080(-)
21-2	1053s		18	0.296(+)	1:1024	0.072(-)
22-1	527s	53	14	0.079(-)	1:1024	0.074(-)
22-2	736s		27	1.179(+)	1:1024	0.103(-)
23-1	544s	18	1	0.135(-)	1:512	0.052(-)
23-2	2161s		20	0.876(+)	1:1024	0.075(-)
24-1	2350s	51	10	0.059(-)	1:256	0.100(-)
24-2	SAB94		50	0.912(+)	1:512	0.075(-)
25-1	007s	24	3	0.005(-)	<1:8	0.057(-)
25-2	2363s		20	0.337(+)	1:256	0.059(-)
26-1	514s	12	5	0.008(-)	<1:8	0.093(-)
26-2	762s		27	0.923(+)	1:128	0.088(-)

^aThe cutoff value for SARS coronavirus was 0.150, which was the averaged OD values from 20 healthy persons.

^bThe cutoff value for HTLV was 0.250, which was calculated according to the suggestion from manufacturer.

protein (N) of SARS-CoV and on the envelope protein of HTLV-II. These four common peptides were synthesized and tested the cross-reactivity of antibodies in the sera of SARS versus HTLV-infected patients. Figure 2A shows the purities of the synthesized peptides conjugated with ovalbumin. In a preliminary analysis, one HTLV-infected patient was recruited in addition and one serum (second serum from patient No. 5 or serum No. 5-2) was selected from those SARS patients described earlier, for testing their sera using the synthe-

sized peptides. Negative sera provided by HTLV kit for control were also included. As shown in Figure 2B, serum from both the SARS patient and from the HTLV patient can react with the common peptides by Western blot, while no reactivity was found in the control serum.

DISCUSSIONS

While the prevalence rate for HTLV in Taiwan is approximately 0.05% [Lu et al., 2003], our study showed

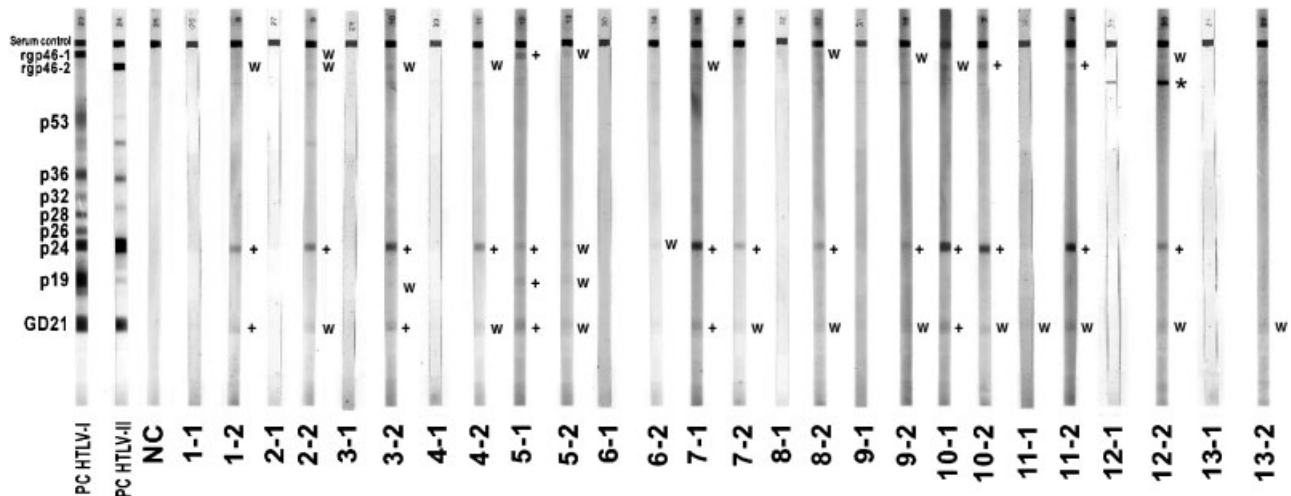


Fig. 1. HTLV Western blot analysis of immunoreactive sera from patients with SARS. First three lanes in the far left are HTLV-I-positive control, HTLV-II-positive control, and negative control. Results of 13 paired-sera with patient No. 1–13 were labeled 1-1 to 13-2 under the figure, with -1 the first serum and -2 the second serum. HTLV seropositive is the reactivity to GAG (p19 with or without p24)

and ENV (GD21); HTLV-I is the reactivity to rgp46-1, and HTLV-II is the reactivity to rgp46-2. The “+” or “w” (weak) displayed next to the lane presented the reactivity for specific band. A large part of rgp46-1, rgp46-2, and GD21 bands were exhibiting “w”. All patients have their first sera lacking of any specific bands except for 5-1, 7-1, and 10-1. Serum 12-2, exhibiting one extra nonspecific band, is shown as *.

a 50% HTLV seropositive rate in 26 SARS patients, suggesting that the antibodies against SARS-CoV might have cross-reacted with HTLV. Cross-examining the coding sequences for both pathogens revealed four common peptides, which might be responsible for the false positive results described. These four common peptides were synthesized further and tested for cross-reactivity of antibodies in the sera of SARS versus HTLV infected-patients. The results show that both sera from either HTLV or SARS patient can react with these four synthetic peptides (Fig. 2B). Since only one serum from HTLV and one for SARS-CoV infected patient was tested, the result obtained in this study is considered very preliminary.

The viral spike protein, nucleocapsid protein, and envelope protein in many viruses are considered to possess antigenicity for serum antibodies. From Figure 1, it was noticed that only certain proteins were responsible for the cross-reactivity observed, for example, rgp46-1 for HTLV-I, rgp46-2 for HTLV-II, and GD21, p19, and p24 for both. Among the four common peptides being synthesized and tested, it was noticed that both “LALLLL” and “RFPNITN” are part of the transmembrane glycoprotein gp21, which is located next to GP46 on the product “env,” according to GenBank annotations. Moreover, “ALETPV” is part of the major core protein p19, which is located next to p24 on the product “gag”. Although it is not known whether the commercially used labels “GD21” and “rgp46” are synonyms to “gp21” and “GP46” we have found from GenBank annotations, it appears that these found common peptides are correlated to the displayed bands responsible for such cross-reactivity.

It has been reported that cross-reactivity to HTLV occurs with samples from patients infected by other pathogens, such as *Plasmodium falciparum* [Hayes et al., 1991]. There were 30% of the residents of Napsan,

Philippines, a site endemic for *Plasmodium falciparum*, found positive for HTLV by ELISA. Western blot immunoreactivity with two or more HTLV proteins was present in 81% of the ELISA positives sera. All of the ELISA positive sera reacted with p19 and 39.6% with p24; however, there was no reactivity with env proteins. Our findings show that 50% (13 of the 26) of the SARS patients were positive for HTLV by ELISA. Western blot immunoreactivity with two or more HTLV proteins was present in 85% (11 of the 13 HTLV-seropositive patients) of the ELISA positive reaction. Only 15% (2 of 13) ELISA positives have reactivity with p19 but 92% (12 of 13) with p24. In contrast to the study of *plasmodium falciparum*, 92% (12 of 13) of the ELISA positive patients have reactivity with env protein, GD21.

Note that some earlier collected samples since the disease onset (Table I, serum No. 5-1, 7-1, and 10-1) showed seropositive with OD values greater than 3.0 in the anti-HTLV test, yet were all negative in the anti-SARS test. Among the antibodies in the sera of SARS patients, those antibodies against the peptide “LALLLL” (located on N proteins of SARS-CoV) might develop earlier than other antibodies against the inactivated virus. Differential sensitivities of the tests used in the study might have caused this result. However, the possibility of something more specific to HTLV (but not anti-SARS-CoV antibody) developed earlier in these SARS patients responsible for the cross-reactivity cannot be excluded.

When designing the synthetic peptides for diagnostic reagents, bioinformatics approach combined with an informative sequence database would provide a useful tool for avoiding possible cross-reactivity among pathogens. Consequently, it is important to maintain a comprehensive sequence database for infectious microorganisms. Moreover, many reagents used currently should be reexamined with those newly emerged viruses

TABLE II. Common Peptides Found Between SARS Coronavirus and HTLV

Peptides	ALETPV	RFPNITN	LALLLL	PLQSLQ
Location for SARS genomes	765..770 for all six SARS genomes	315..321 for all six SARS genomes	220..225 for all six SARS genomes	1136..1141 for all six SARS genomes
Annotated product names for SARS genomes	“putative MHV p65 protein,” “ORF 1a,” or “nonstructural polyprotein ppla”	“Surface spike glycoprotein,” or “S protein,”	“nucleocapsid protein,” or “N protein”	“putative nsp1,” “ORF 1a,” or “nonstructural polyprotein ppla”
Location for HTLV genomes	53..58 for six HTLV-I genomes except j02029	401..407 for one HTLV-I genome j02029	446..451 for seven HTLV-2 genomes except G2	930..935 for one HTLV-II genome m10060
Annotated product names for HTLV genomes	“gag,” “gag polyprotein precursor,” “gag protein,” or “Pr55”	“env”	“envelope glycoprotein”	865..870 for four HTLV-II genomes (G2, SP-WV, RF329, k96) “pol,” “pol polyprotein,” or “reverse transcriptase”

Six SARS coronavirus genomes are IW1/ay291451, CUHK-W1/ay278554, TOR2/nc_004718, CUHK-Su10/ay282752, BJO1/ay278488, and Urbani/ay278741. Seven HTLV-I genomes are af033817, RKI3-Ger/af042071, af139170, WHP/af259264, d13784, j02029, and ATL-YS/ut19949. Eight HTLV-2 genomes are G2/af074965, SP-WV/af139382, RF329/af326583, k96/af326584, af412314, G12/111456, m10060, and y13051.

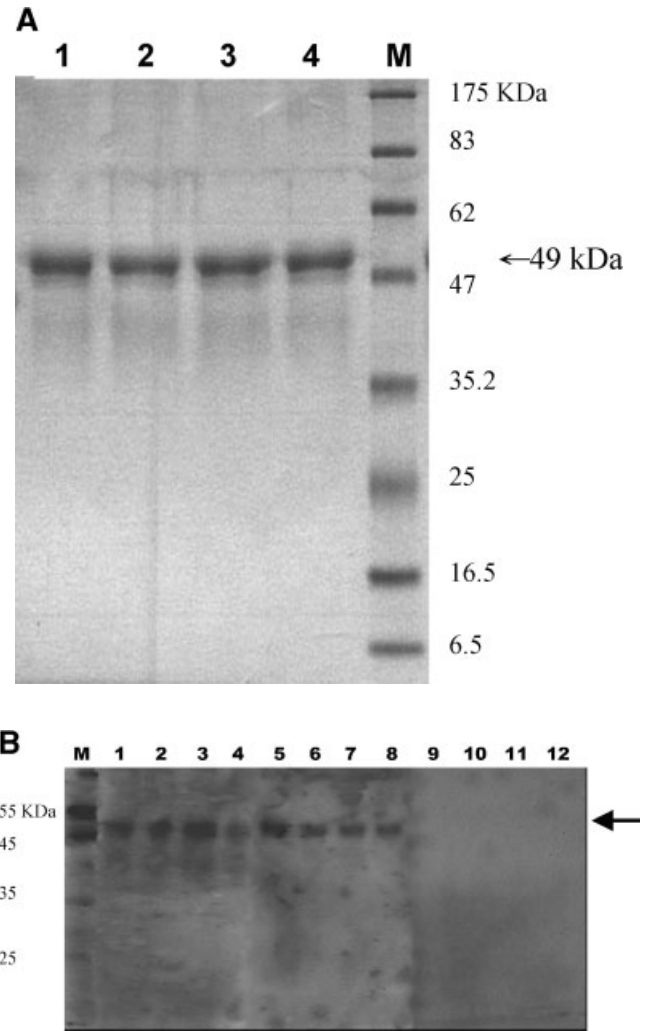


Fig. 2. **A:** Electrophoresis of synthetic peptides. Four common peptides of SARS coronavirus and HTLV were synthesized and conjugated with ovalbumin. SDS-PAGE (12%) was used to check each peptide and stained with coomassie blue. The size of each synthetic peptide is 49 kDa as shown with arrow. **Lane 1** is peptide of ALETPV, **lane 2** is LALLLL, **lane 3** is PLQSLQ, **lane 4** is RFPNITN, and M is the size marker. **B:** Western blotting of patient sera with synthetic peptides. Four synthetic peptides were run in 12% SDS-PAGE and hybridized with SARS patient serum No. 5-2, HTLV positive serum, and negative control serum. Lane 1 to lane 4: different peptides hybridized with SARS patient serum No. 5-2; **lane 5–lane 8:** different peptides hybridized with HTLV positive serum; **lane 9–lane 12:** different peptides hybridized with negative control serum. Arrow indicates the 49 kDa band.

at the sequence level, to prevent potential false positive results in laboratory diagnosis.

According to the WHO recommendations, SARS patients can donate blood after 3 months from disease onset. We have traced both anti-SARS and anti-HTLV antibodies in five SARS patients for a prolonged period of time. The antibody titers against SARS were still high (OD > 0.5) after 100 days of disease onset, while the antibody titers against HTLV have mostly declined (OD < 0.1). This finding suggests that among the antibodies in the sera of SARS patients, those with cross-reactivity with HTLV peptides might disappear

earlier. Notably, one of these five patients were still seropositive for HTLV after 100 days (data not shown). While waiting for an effective drug or vaccine treatment to be developed, SARS antibody found in patients who had already recovered from the disease represents a valuable resource for potential therapy for future SARS patients. Without noting that the anti-HTLV reactivity detected from SARS patients could be due to cross-reactivity as reported in this study, valuable blood products can be unnecessarily discarded.

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