Use of Antibody Avidity Assays for Diagnosis of Severe Acute Respiratory Syndrome Coronavirus Infection[▽]

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An indirect immunofluorescent assay (Euroimmun AG, Luebeck, Germany) was used to investigate the avidity of immunoglobulin G (IgG), IgM, IgA, and total Ig (IgGAM) antibody responses to severe acute respiratory syndrome coronavirus (SARS CoV) infections. Serial serum samples from eight patients collected during the first, third, and ninth months after the onset of infection were evaluated. It was found that low-avidity IgG antibodies were detected in 15/15 (100%), 1/5 (20%), and 0/8 (0%) serum samples collected during the first, third, and ninth months after the onset of symptoms, respectively. Low-avidity antibodies of IgA and IgM subclasses were detected in 14/14 (100%) and 3/14 (21%) serum samples, respectively, collected in the first month after the onset of infection. However, IgA antibodies remained low in avidity in a proportion of patients even during late convalescence. As a consequence, IgG antibody avidity assays gave better discrimination between acute-phase and late-convalescent-phase serum samples than IgM, IgA, or IgGAM assays. In two of these patients, sequential serum samples were also tested for IgG avidity against human CoV strains OC43 and 229E in parallel. While SARS CoV infections induced an anamnestic IgG antibody response to the 229E and OC43 viruses, these cross-reactive antibodies remained of high avidity from early (the first month) postinfection. The results showed that assays to detect low-avidity antibody may be useful for discriminating early from late antibody responses and also for distinguishing anamnestic cross-reactive antibody responses from primary specific responses. This may be useful in some clinical situations.

Severe acute respiratory syndrome (SARS), caused by the SARS coronavirus (SARS CoV), is a newly emergent infectious disease that caused a major threat to global public health (6, 12, 16). SARS CoV is now classified as a group 2b CoV (7). It rapidly spread to affect 29 countries across five continents and caused disease in 8,096 patients and death in 744 (22). Prompt and determined public health measures interrupted the spread of the human-adapted SARS CoV (23). However, the precursor virus remains in its animal reservoir with bats (13, 15), and small mammals such as civet cats within live game-animal markets in southern China are likely amplifiers of the virus and sources for interspecies transmission to humans (9). As it is possible that this precursor animal virus may again adapt to human-to-human transmission and pose a renewed threat to human health, it is important to maintain surveillance for a reemergence of SARS. In addition, lessons from the SARS outbreak are likely to be relevant in confronting future novel emerging infectious disease threats.

The diagnosis of SARS CoV infection in humans is dependent upon the detection of viral RNA using reverse transcription-PCR from clinical specimens (3, 18) and the detection of antibody responses in the blood (8, 10, 17, 21). Seroconversion by indirect immunofluorescence (IIF) or neutralization tests is regarded as a gold standard for the diagnosis of SARS CoV

infection (17, 19). However, previous studies showed that SARS CoV infection can stimulate anamnestic cross-reactive IF-antibody responses to one or more human CoVs (OC43, 229E, and NL63) in patients with prior antibody to these viruses (4). Conversely, while OC43 or 229E infections can boost the preexisting titer of IF antibody to the other virus, cross-reacting antibody to SARS CoV antibody was not elicited. This was possibly because these patients had no prior immunological memory of SARS CoV. It is possible, however, that patients with a past immunological memory of SARS CoV or the animal precursor of the SARS CoV who are subsequently infected with OC43, 229E, NL-63, or HKU-1 may indeed manifest an increase in antibody to the SARS CoV titer, giving rise to diagnostic confusion with significant implications for the global public.

While antibody responses are usually used as indicators of a host's immune response to a pathogen, sometimes the subclass or the quality of an antibody may provide additional useful information. For example, the immunoglobulin M (IgM) antibody is often used as an indicator of recent infection. However, in SARS, the IgM antibody to SARS CoV is still detectable at 7 months postinfection (4). Antibody avidity is the strength with which a multivalent antibody binds with a multivalent antigen, while affinity is the strength of a single antigen-antibody bond (20). Low-avidity antibody is usually produced during the primary response, and the strength of the avidity of an antibody increases over time with the maturation of the IgG antibody response (5). IgG avidity has been used to differentiate current from past infections with other viruses, such as Epstein-Barr virus, cytomegalovirus, and West Nile virus (1, 2,

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TABLE 1. Optimization of urea concentration for detection of IgG avidity by IIF

SARS patient	Phase of serum sample (day postonset)[a]	IF intensity[b] at a urea concn (M) of:				
		0	3	4	5	6
A	Acute (22)	2+	1+	w+	w+	0
	Convalescence (220)	4+	4+	4+	3+	2+
B	Acute (24)	4+	3+	1+	1+	0
	Convalescence (207)	4+	4+	4+	3+	2+
C	Acute (28)	4+	2+	1+	1+	0
	Convalescence (206)	4+	4+	4+	3+	2+
D	Acute (19)	1+	w+	0	0	0
	Convalescence (221)	2+	2+	2+	2+	1+
E	Acute (20)	4+	2+	1+	1+	0
	Convalescence (258)	4+	4+	4+	3+	2+

[a] All serum samples were tested at a dilution of 1/10.
[b] The IF intensity was scored as follows: 0, no fluorescence; w+, very weak fluorescence intensity; 1+, weak fluorescence intensity; 2+, moderate fluorescence intensity; 3+, strong fluorescence intensity; and 4+, very strong fluorescence intensity.

14). In this study, we describe the avidity of antibody responses to SARS and other CoVs and investigate antibody avidity as an option for the serodiagnosis of recent SARS CoV infections.

MATERIALS AND METHODS

Patients and serum samples. Eight SARS patients from whom five to six sequential serum samples were available were investigated (4, 6, 16). The serum samples were collected mainly in the first month, but some serum samples were collected from the third and ninth months after the onset of SARS. Another five pairs of serum samples (acute phase and convalescent phase) from SARS patients were used for the optimization of urea concentration. The serum samples were aliquoted and stored at −80°C until use.

Preparation of CoV-infected smears. BNI 1 SARS CoV-infected Vero E6 cells (6) on Biochip slides (Euroimmun AG, Luebeck, Germany) were used in this study. Each field in a slide contains two Biochips, one with SARS CoV-infected cells and the second with uninfected cells. 229E-infected MRC-5 and OC43-infected BSC-1 cell smears were prepared according to the method described previously (4). Briefly, infected cells showing 60% to 70% infection were harvested, fixed in chilled acetone for 10 min at −20°C, and stored at −80°C until use.

Antibody avidity IF assay. Serum samples were tested for antibody avidity against SARS CoV-, 229E-, and OC43-infected cells using an IIF test as described previously (4, 14). Sequential serum samples from each patient were assayed in the same experiment to avoid interassay variation. Briefly, serial twofold dilutions, starting from 1/10, of each antiserum in phosphate-buffered saline (PBS) were added to duplicate reaction fields of a Biochip slide (Euroimmun AG, Luebeck, Germany), according to the manufacturer's instructions. After incubation at room temperature for 30 min, the cells were treated either with 4 M urea in 0.2% Tween 20 in PBS solution or with 0.2% Tween 20 in PBS for 10 min. After the cells were washed once in 0.2% Tween 20 in PBS for 5 min, fluorescein-labeled antihuman IgG, IgA, IgM, or total Ig (IgGAM), as appropriate, was added for 30 min. The avidity titration tests for 229E- and OC43-infected cells were carried out similarly, except the IF slides were prepared "in-house," as previously described (4), and antihuman IgG fluorescein isothiocyanate conjugate (Inova Diagnostics, San Diego, CA) was used. The IF-dye-stained cells were examined at a ×20 magnification under a UV fluorescence microscope. For each serum sample, the titer of antibody to CoV in infected cells treated and not treated with 4 M urea was determined, and a fourfold reduction in titer by 4 M urea was regarded as evidence of low-avidity antibody (2). In patients with a low SARS antibody titer (e.g., 1/10), it is not possible to determine avidity based on such a fourfold reduction in antibody titer.

Optimization of urea concentration. Concentrations of urea from 3 to 6 M (Euroimmun AG, Luebeck, Germany) were used to test, according to the method described above, acute-phase (at ~3 to 4 weeks) and convalescent-phase

TABLE 2. Detection of antibodies and avidity in patients with SARS CoV infection[a]

No. of days after onset of symptoms	No. of low-avidity samples/total no. of samples tested for indicated antibody (%)[b]			
	IgG	IgA	IgM	IgGAM
11–38	15/15 (100)	14/14 (100)	3/14 (21.4)	16/16 (100)[c]
83–86	1/5 (20)	2/4 (50)	0/4 (0)	3/5 (60)
206–269	0/8 (0)	1/4 (25)	0/2 (0)	2/8 (25)

[a] Serum samples from eight SARS patients were tested.
[b] As the titers and durations of SARS CoV antibody to each isotype differ between patients, the denominators of the isotypes tested for avidity may be different.
[c] Two SARS patients had detectable low-avidity IgGAM antibodies earlier than low-avidity IgG antibodies.

(at ~7 to 9 months) serum samples from another five SARS patients to determine the optimal concentration of urea for discriminating between high- and low-avidity antibodies. A serum dilution of 1/10 was used unless otherwise specified. After incubation at room temperature for 30 min, cells were treated separately with different concentrations of urea (3 M, 4 M, 5 M, and 6 M) mixed with 0.2% Tween 20 in PBS solution or with 0.2% Tween 20 in PBS without urea as the control for 10 min. After the cells were washed once with 0.2% Tween 20 in PBS for 5 min, fluorescein-labeled antihuman IgG was added for 30 min. The IF-dye-stained cells were examined at a ×20 magnification under a UV fluorescence microscope. A positive reaction appears as a distinct apple-green fluorescence of the infected cells mainly in the area of the cytoplasm, where fine- to coarse-granular structures containing viral material fluoresce. The degree of fluorescence intensity was scored as follows: no fluorescence was scored as 0, weak fluorescence, 1; moderate, 2; strong, 3; and very strong, 4. The presence of low-avidity IgG antibodies was inferred by the reduction in fluorescence of the cells treated with urea compared with that of the buffer-treated control cells. At a given dilution, a reduction in the score of the intensity of ≥2 was taken to indicate the presence of low-avidity antibodies. The highest concentration of urea that demonstrated the greatest discrimination between high- and low-avidity antibodies at a serum dilution of 1/10 was considered the optimum concentration of urea and was used throughout the study. The slides were read without knowledge of their content, and readings were performed twice.

RESULTS

The antibody avidity against SARS CoV was determined in acute-phase (19 to 28 days after onset) and late-convalescent-phase (206 to 269 days after onset) serum samples from five patients to determine the optimal concentration of urea that best discriminates acute- from late-convalescent-phase serum samples (Table 1). It was found that 4 M urea was optimal at discriminating IgG in acute- and convalescent-phase serum samples. Therefore, 4 M urea was used for all subsequent assays of antibody avidity in this study.

Low-avidity IgG antibodies were detected in 100% (15/15) of the serum samples collected during the first 38 days after the onset of symptoms (Table 2). In contrast, only one of five serum samples collected during the third month postinfection and none of eight serum samples collected 200 days after onset had low-avidity IgG antibodies. Similarly, low-avidity IgA antibodies were detected in 100% (14/14) of the serum samples collected during the first 38 days of illness, in two of four serum samples collected between days 83 and 86, and in one of four serum samples collected between days 206 and 269 after the onset of disease. In contrast, the low-avidity antibody IgM was not detected in serum samples at or after 3 months postinfection, and it was detected in only 3 of 14 serum samples even within the first 38 days of illness.

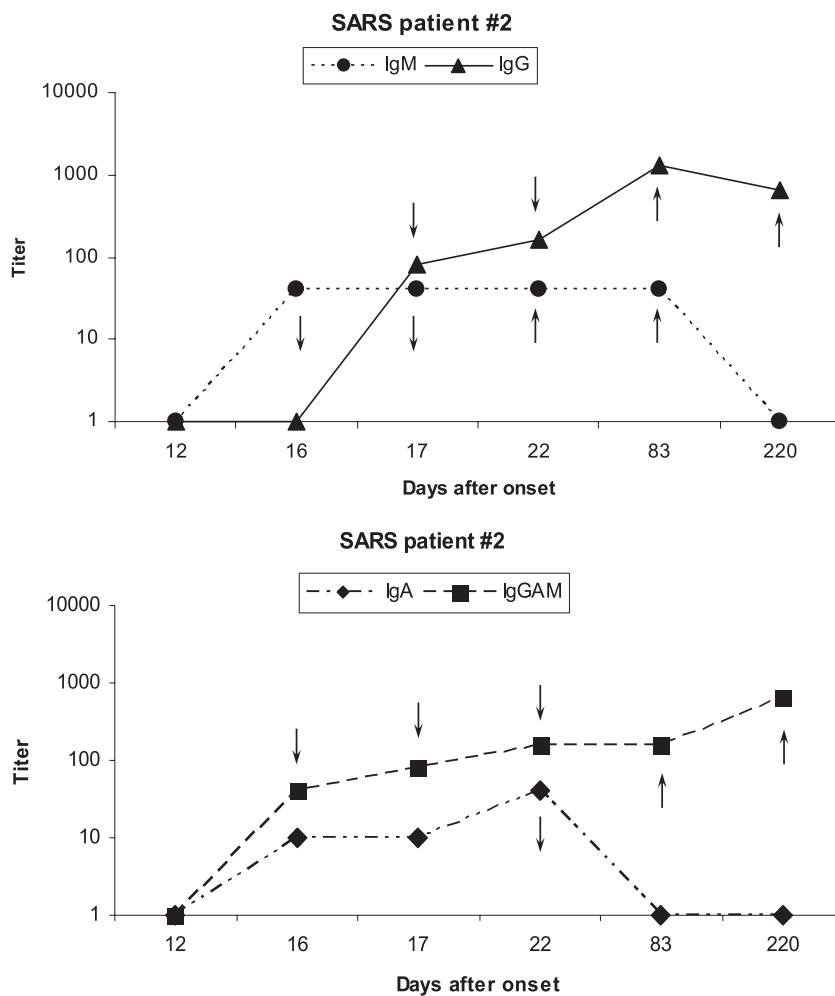The IgG, IgM, IgA, and IgGAM antibody responses and

FIG. 1. Serological profile of antibody avidity in sequential serum samples from one illustrative patient with SARS. Titers of antibody of each Ig subclass and of IgGAM to SARS CoV are shown. The low avidity of the antibody is denoted with a downward-pointing arrow, and the high avidity of the antibody is denoted with an upward-pointing arrow.

antibody avidity in sequential serum samples from one representative patient with SARS is shown in Fig. 1. Furthermore, the sequential serum samples of two patients were also tested for IgG avidity against the human CoVs OC43 and 229E. While these two patients had a ≥4-fold rise in their titers of antibody to 229E and/or OC43, these antibodies remained high in avidity from early in the illness (days 16 to 29 after onset). In contrast, the IgG antibody response to SARS CoV in these patients was of low avidity during this early postinfection period and became high only at 80 days postinfection or beyond (Table 3).

DISCUSSION

In this study, all eight patients with SARS were found to have low-avidity IgG antibodies for SARS CoV in their serum samples collected within 1 month of infection. While some of these patients may also have had an anamnestic boost in their titers of antibodies to other human CoVs (e.g., OC43 or 229E), such anamnestic antibody responses are of high avidity even early in the course of the infection, providing a means for discriminating a primary recent antibody response from an

anamnestic boost in antibody titer. Because patients with non-SARS CoV infections did not have a prior immunological memory of SARS CoV, they did not have a serological response against SARS CoV (16), and it was not possible to

TABLE 3. Detection of antibodies (without urea treatment) and avidity for CoV strains in SARS patients

SARS patient	No. of days after onset of symptoms	Reciprocal IgG titer (avidity) for[a]:		
		SARS CoV	229E	OC43
10	16	10 (NA)	80 (high)	40 (high)
	29	320 (low)	640 (high)	160 (high)
	87	1,280 (high)	160 (high)	160 (high)
	227	320 (high)	80 (high)	40 (high)
2	16	<10 (NA)	20 (high)	ND
	17	80 (low)	40 (high)	ND
	22	160 (low)	80 (high)	ND
	83	1,280 (high)	80 (high)	ND
	220	640 (high)	160 (high)	ND

[a] NA, not applicable. In patients with a SARS CoV antibody titer of 1/10 or lower, it is not possible to determine avidity based on the criterion of a fourfold reduction of antibody titer following urea treatment. ND, not detected.

assess whether this strategy also would be applicable in the converse situation, viz., in differentiating an anamnestic boost of SARS CoV antibody in a patient with another CoV infection. But we hypothesize that this is likely to be true in such instances where a person with prior infection with SARS CoV or a closely related animal virus is infected with another endemic CoV (e.g., 229E) (9). Cross-reactive boosts of antibody responses in such persons in response to a human CoV infection may well lead to an anamnestic response to SARS CoV, a potential diagnostic dilemma of major global public health consequences. Thus, antibody avidity may be of use in investigating a patient who has a rise in his titer of antibody to SARS CoV (rather than a seroconversion, which is less likely to be due to a cross-reacting serological response). In such instances, in addition to attempting to detect the pathogen RNA by reverse transcription-PCR tests, we suggest that antibody avidity may help clarify the diagnosis.

Due to its pentameric structure, the IgM antibody exhibits higher avidity than IgG or IgA (11). This is in agreement with our results showing that, even early in the course of the SARS CoV infection, low-avidity IgM antibodies were detected only in 21.4% (3/14) of serum samples. In contrast, low-avidity IgA antibodies for SARS CoV were persistently found (25%) even after 9 months. The IgGAM antibody reflects a composite of these complex dynamics, and this antibody is thought to be the earliest antibody detected; its avidity may remain low in 25% (2/8) of patients for over 6 months.

In summary, the determination of IgG avidity provides additional diagnostic certainty in differentiating between recently acquired and previous infections of SARS CoV and other human CoVs. Therefore, if the first available serum from a patient already has detectable antibody to SARS CoV, a rise in the titer of IF antibody to SARS CoV may not necessarily confirm SARS CoV infection.

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