

# Cleavage and Serum Reactivity of the Severe Acute Respiratory Syndrome Coronavirus Spike Protein

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Severe acute respiratory syndrome (SARS) coronavirus (SCoV) spike (S) protein is the major surface antigen of the virus and is responsible for receptor binding and the generation of neutralizing antibody. To investigate SCoV S protein, full-length and individual domains of S protein were expressed on the surface of insect cells and were characterized for cleavability and reactivity with serum samples obtained from patients during the convalescent phase of SARS. S protein could be cleaved by exogenous trypsin but not by coexpressed furin, suggesting that the protein is not normally processed during infection. Reactivity was evident by both flow cytometry and Western blot assays, but the pattern of reactivity varied according to assay and sequence of the antigen. The antibody response to SCoV S protein involves antibodies to both linear and conformational epitopes, with linear epitopes associated with the carboxyl domain and conformational epitopes associated with the amino terminal domain. Recombinant SCoV S protein appears to be a suitable antigen for the development of an efficient and sensitive diagnostic test for SARS, but our data suggest that assay format and choice of S antigen are important considerations.

A novel coronavirus (CoV), designated "SCoV," has been identified as the etiological agent of severe acute respiratory syndrome (SARS), a respiratory epidemic of humans identified originally in southern China [1–3]. Sequence analysis has shown SCoV to be a previously uncharacterized isolate distinct from the widely studied avian, porcine, and human CoVs [4–6]. Comparative sequence analysis of several human SCoV isolates, obtained in diverse geographic locations during the epidemic, has shown little genetic drift but a significant nucleotide change at position 22,222 in the spike (S) protein [7]. Comparison of SCoV sequences from animal and human sources also shows that most sequence changes occur in the S protein [8]. CoV S protein is the major surface glycoprotein of the virus

and is responsible for virus attachment to receptors, entry by fusion, and the development of neutralizing antibody [9]. Of interest, most of the residue changes identified within S protein lie in a region that, in other CoVs, causes significant alteration in tropism [10], suggesting that drift toward a virus more capable of using a human cell-surface receptor could occur. The structure and function of SCoV S protein is, therefore, an integral part of studies of viral tropism and of the development of a receptor-blocking antibody response. During the convalescent phase of SARS, antibody against the whole virus can be detected at day 10 and increases to a peak titer by day 28 [11]. However, the spectrum of the antibody response, its role in viral clearance, and its use as a correlate of protection has not been described. Because SCoV S protein is such a pivotal viral protein, we have applied a high-throughput expression strategy to the production of recombinant S protein for biochemical and antigenic characterization. Here, we describe high-level expression of 6 overlapping fragments of S protein on the surface of insect cells, a context that allows high-level production and maintains sensitive conformation [12, 13]. Recombinant S protein showed strong serum reactivity in con-

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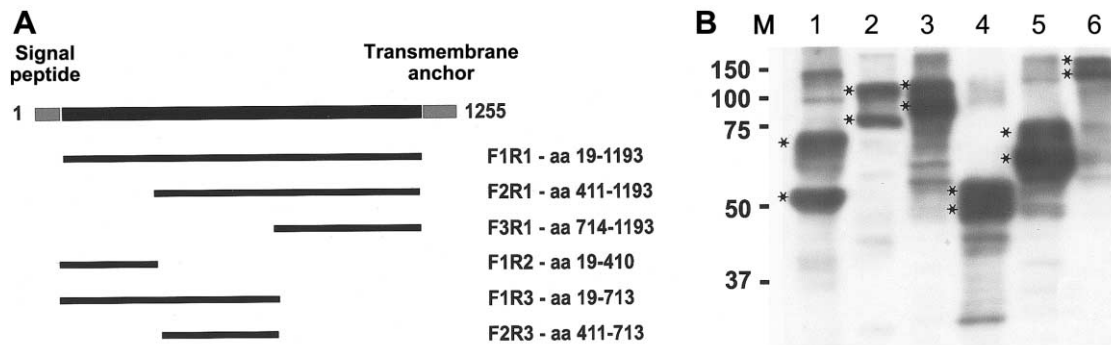
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**Figure 1.** A, Origin of the fragments used for the expression of severe acute respiratory syndrome coronavirus spike protein in insect cells. The boundaries were chosen after inspection of the protein sequence for secondary structure and fold propensity. B, Expression of each fragment in insect cells detected by use of Western blotting with an anti-vesicular stomatitis virus G serum that recognizes the membrane anchor sequence common to all fragments. The sample order is F1R2, F1R3, F2R1, F2R3, F3R1, and F1R1. Molecular mass markers (M) are kilodaltons. Asterisks (\*) indicate bands discussed in the text.

valescent-phase serum samples by use of both flow cytometry and Western blotting (WB), suggesting that it could be used in the development of SARS-specific diagnostic techniques. Reactivity was not equivalent between fluorescence-activated cell sorting (FACS) and WB, however, and was not equal on all fragments, providing evidence of a variety of antibodies in the host response.

## MATERIALS AND METHODS

**Cell culture.** *Spodoptera frugiperda* (Sf9) cells were routinely cultured in SF900-II medium (Life Sciences) at 28°C in suspension culture. For plaque assays, cells were allowed to settle onto polystyrene dishes for 1 h before virus inoculation. After virus adsorption, cells were sequentially overlaid with 1.6% low-melting-temperature agarose and then with SF900-II supplemented with 5% fetal calf serum and were cultured for 4 more days before staining with neutral red.

**Vector construction.** A cDNA encoding the full-length S protein minus the signal peptide and transmembrane (TM) domain (aa 18–1193) of the Hong Kong isolate of SCoV [7] was supplied by Andrew Davidson and Stuart Siddell (University of Bristol, Bristol, UK) as a reverse-transcription polymerase chain reaction–amplified fragment and was first cloned into pTOPO (Invitrogen). Several overlapping fragments of S protein, with end points chosen after bioinformatics analysis, were amplified from the original clone. All fragments generated were flanked by dissimilar restriction sites for the enzyme *Sfi*I. Each fragment was cloned into a set of 3 vectors (18 variants in all), to provide His-tagged, maltose binding protein–tagged, and cell surface–displayed forms of S protein. Only the cell surface–displayed cassette is described in detail here. The baculovirus display transfer vector pAcSVS<sub>GM</sub> [12], modified to include *Sfi*I sites at the junction of the signal peptide and the

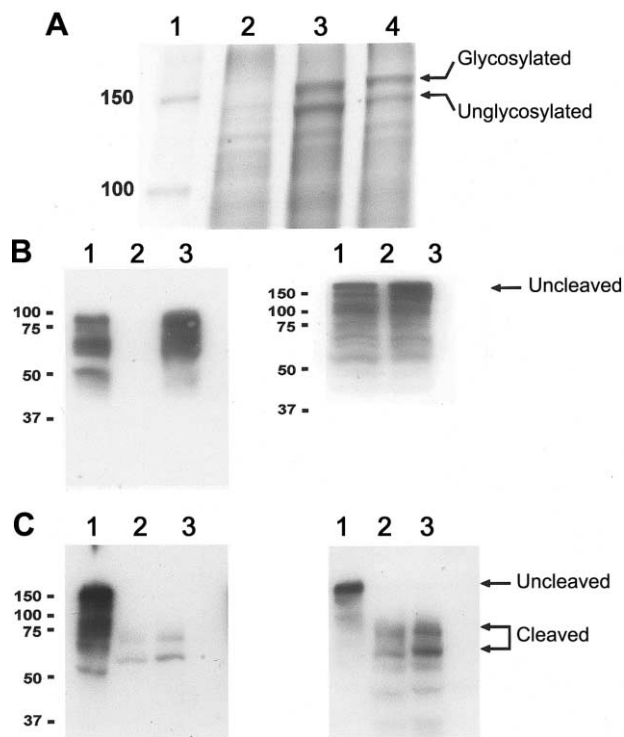
vesicular stomatitis virus (VSV) G protein TM domain, was used to provide expression at the cell surface.

**Expression in insect cells.** For expression in insect cells, recombinant baculoviruses were constructed by use of a new, rapid recombination technique after cotransfection of each transfer vector with a linearized, modified form of bacmid DNA capable of growth only after recombination [14]. Coexpression of mouse furin or human calnexin was done by coinfection of viruses at MOIs >5 [15].

**WB.** Protein samples to be analyzed were separated on pre-cast 10% Tris-HCl SDS–polyacrylamide gels (Bio-Rad) and transferred onto Immobilon-P transfer membranes (Millipore). WB was performed with human convalescent-phase serum or rabbit serum to the VSV G protein TM domain (Research Diagnostics), followed by peroxidase-coupled secondary antibodies (Sigma). The membrane was finally developed by use of BM Chemiluminescence (Roche).

**FACS analysis (flow cytometry).** Cells were plated in a 6-well plate ( $1 \times 10^6$  cells/well) and overlaid with 2 mL of medium. After virus inoculation, cells were cultured for a further 2 days at 28°C, harvested, washed with PBS, fixed with CellFix (Becton Dickinson), and stained with serum and conjugate. Data acquisition and analysis were performed by use of a FAC-Scan flow cytometer and CellQuest software (both from Becton Dickinson).

**Human serum.** Serum samples from patients with SARS and human CoV 229E were obtained from the respiratory disease reference laboratory of the Health Protection Agency (Colindale, UK). Nine probable SARS cases occurred in the United Kingdom, but only 4 conformed to the clinical definition of SARS in use at the time. Serum samples from patients known to be positive for SARS were derived from the patients with clinically confirmed cases, whereas serum samples used as prob-



**Figure 2.** Biochemical characterization of full-length expressed severe acute respiratory syndrome coronavirus (SCoV) spike (S) protein. *A*, Coexpression of S protein with calnexin. *Lane 1*, Marker (kilodaltons); *lane 2*, cells expressing calnexin only; *lane 3*, cells expressing S protein only; and *lane 4*, cells coexpressing calnexin and S protein. Total infected cell profiles at 2 days after infection are shown after resolution by use of 10% SDS-PAGE. *B*, Coexpression of S protein with furin. *Lane 1*, Cells coexpressing furin and S protein; *lane 2*, cells expressing S protein only; and *lane 3*, cells expressing furin only. Shown are Western blots probed with anti-furin (*left*) and anti-vesicular stomatitis virus (VSV) G serum (*right*). *C*, Cleavage of cell-surface S protein with trypsin. *Lane 1*, S protein before addition of trypsin; *lanes 2* and *3*, whole-cell and pellet fractions, respectively, after treatment with trypsin. Shown are Western blots probed with anti-VSV G (*left*) and human SCoV serum (*right*). Molecular mass markers are kilodaltons.

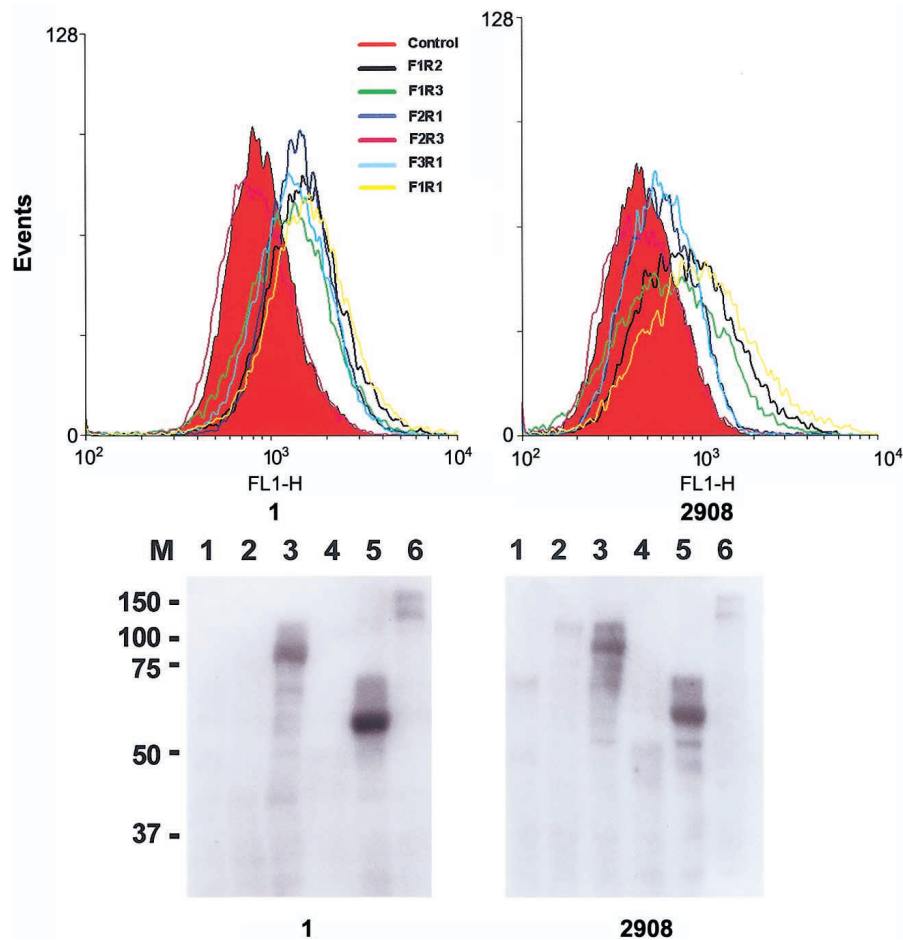
able but unconfirmed SARS cases were derived from the remaining 5 patients. All patients recovered from SARS. No human CoV OC43 serum samples were available for the present study, so the reactivity of CoV OC43 with SARS S protein could not be assessed. Similarly, only 2 serum samples from CoV 229E-infected patients were available. Serum samples were used in FACS and WB analyses at 1:50 and 1:400 dilutions, respectively, unless otherwise stated. The time-course serum samples, available from 1 of the patients with confirmed SARS, were assayed by use of ELISA using lentil lectin-captured, full-length S protein, and the 50% end-point titer was compared. The class of antibody bound was ascertained by use of class-specific, secondary anti-human antibodies (Sigma).

## RESULTS

**Expression and characterization of recombinant SCoV S Protein.** The SCoV S protein is ~1200 residues and has 23 glycosylation triplets clustered toward the amino and carboxyl termini. Because glycosylation and protein folding are often linked, we focused our expression studies on the use of a high-yielding but eukaryotic-based expression system: baculovirus-mediated expression in insect cells. We examined a number of biochemical features, to characterize the protein expression before use for antibody binding. The S protein signal peptide and TM domain were substituted for regions known to function well in this expression system—a signal peptide from the major baculovirus glycoprotein gp64 and a TM domain from the VSV G protein already present in pAcVSV G<sub>TM</sub> [12]. This vector efficiently displays proteins on the surface of insect cells and is a method of expression that maintains the conformational integrity of even problematic proteins [13]. In addition to the full-length S protein, fragments representing the predominantly  $\beta$ -sheet and  $\alpha$ -helical domains (residues 18–713 and 714–1193, respectively), a fragment from the N terminus (residues 18–410) predicted to form a distinct folded domain when analyzed by folding prediction software [16], and fragments representing residues 411–1193 and 411–713 were expressed in the same way (figure 1A). Abundant expression of all S protein fragments was achieved by use of this strategy, as shown by WB with a VSV G<sub>TM</sub> domain antibody that showed 2 predominant bands for each construct representing nonglycosylated and glycosylated proteins, with apparent molecular weights consistent with those predicted (figure 1B).

**Interaction with calnexin and S protein cleavage.** The S proteins of all CoVs are heavily glycosylated, and improper glycan trimming can result in poor surface-expression levels through retention of the viral glycoprotein by the glycoprotein chaperone calnexin [17, 18]. To assess whether the calnexin pathway was relevant to expression of S protein, we coinfecting recombinant full-length S protein with a virus-expressing calnexin and re-examined the level of glycosylated and nonglycosylated protein at 3 days after infection. Compared with expression of S protein only, coexpression of S protein with calnexin led to an increase in the ratio of glycosylated to nonglycosylated product, calculated by use of gel scan to be ~20% (figure 2A), showing that S protein interacts with calnexin during glycosylation. However, no overall increase in S protein yield was observed, despite improved glycoprotein processing.

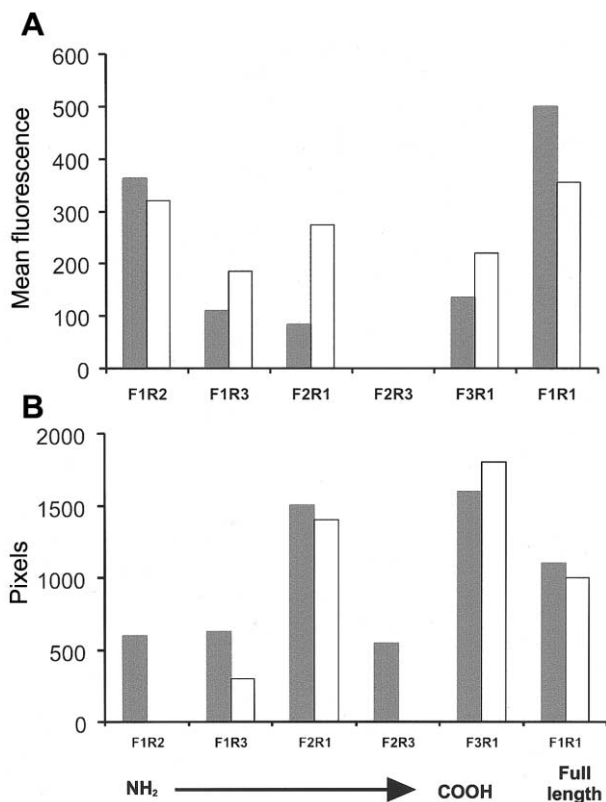
Some, but not all, CoV S proteins are cleaved around the center of the molecule to form the receptor-binding outer domain S1 and the inner-membrane fusion domain S2 [19, 20]. The full-length S protein was not cleaved in insect cells, with the fully glycosylated form having an apparent molecular weight of ~180 kDa (figure 1B). We assessed the potential for S protein



**Figure 3.** Reaction of infected human serum with severe acute respiratory syndrome coronavirus spike (S) protein. Shown are fluorescence-activated cell-sorting profiles (*top*) and Western blots (*bottom*) for 2 serum samples with each of the S protein fragments expressed. Of the 2, serum sample 1 was typical of 3 other serum samples tested, whereas serum sample 2908 represented the strongest responder. Lanes in the Western blots are as follows: 1, F1R2; 2, F1R3; 3, F2R1; 4, F2R3; 5, F3R1; and 6, F1R1. Molecular mass markers (M) are kilodaltons.

to be cleaved by 2 proteases, the subtilisin-like furin (provided by coinfection, as described elsewhere [15]) and by trypsin (added exogenously). Furin is a classic viral glycoprotein maturation enzyme [21]. The consensus furin cleavage site does not occur in the S protein sequence, but several dibasic sites, which also can be recognized by the enzyme [15], are present, allowing the possibility of furin cleavage. Recombinant S protein produced in the presence of coexpressed furin showed no evidence of cleavage (figure 2B), suggesting that furin is not associated with SCoV envelope maturation. S protein on the surface of infected insect cells was also treated with trypsin under conditions found to cleave murine CoV S [19] and was reanalyzed after the cleavage reaction, by WB using the VSV G<sub>TM</sub> domain and patients' antibody. Treatment with trypsin caused the loss of the ~180-kDa glycosylated S protein band and concomitant appearance of 2 new bands, the most intense of which was at ~70 kDa and whose mobility was indistin-

guishable from that shown by S-F3R1 (figures 1B and 2C). The F3/R3 junction, which was chosen wholly on the basis of predicted secondary structure, is at residue 713, and a single Lys residue occurs at position 714. On the basis of these data, we suggest that trypsin can access the S protein under nondenaturing conditions, to cleave at Lys 714 and produce a  $\beta$ -sheet-rich S1 domain and a  $\alpha$ -helix-rich S2 domain. Interestingly, molecular modeling of the SCoV S protein has also suggested that the S1–S2 junction lies within the amino acid sequence 680–727 [22]. No S protein was found in the nonpellet fraction after the addition of trypsin, suggesting that the 2 S protein domains remain associated even after cleavage (data not shown). When probed with patients' serum samples, the presumed S2 domain at ~70 kDa was also highlighted, but there were also a number of smaller breakdown products. No distinct band at the predicted size of the S1 domain was visible, suggesting that it is degraded by trypsin or is not detected efficiently



**Figure 4.** Quantitation of serum reactivity to severe acute respiratory syndrome coronavirus spike (S) protein. Fluorescence-activated cell sorting profiles (A) and Western blots (B) were quantified by use of mean fluorescence and densitometry (pixel density), respectively. Gray bars, serum sample 2908; white bars, serum sample 1. The trend in fragment-sequence coverage is shown at the bottom of the figure.

by the available human serum samples (see below). Whether S protein is cleaved before or during SCoV cell entry remains undetermined.

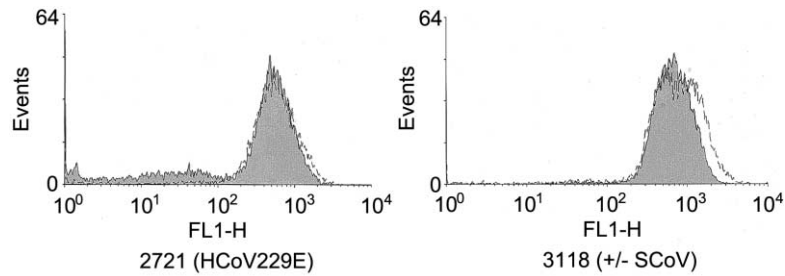
**Reactivity with human serum samples.** The abundant, stable, and characterized surface expression of a suite of S protein-related fragments prompted us to use these reagents to investigate the antibody response to S protein in the serum samples from infected individuals. Serum samples used were obtained from several UK patients (for patients' details, see Materials and Methods); in addition, serum samples representing a time course from disease onset were obtained from 1 reference patient. We assessed the reactivity of each serum sample with each fragment by use of flow cytometry and WB, to represent native and denatured sources of antigen, respectively. Cytometry profiles showed the greatest reactivity of patients' serum samples with the protein S-F1R2 and S-F1R1, but all other fragments also reacted, with the exception of S-F2R3 (aa 411–713), which represents the middle section of the S coding region and failed to react significantly with most patients' serum samples, despite high levels of expression (figure 1B). The same

general pattern of binding was apparent for all the patients' serum samples, although the exact degree of reactivity with each fragment varied somewhat (of the 2 serum samples shown in figure 3, the data for serum sample 1 were the more typical pattern). When serum reactivity was assessed by use of WB, however, the pattern of binding was substantially different. Reactivity was seen with the full-length protein S-F1R1, but strong binding was also seen with S-F2R1 and S-F3R1 (figure 3, bottom). One serum sample (2908, the most broadly reactive of all the samples) showed some reactivity with S-F1R2, S-F1R3, and S-F2R3 but was very weak, compared with fragments spanning the C-terminal half of the S protein, and was wholly absent in other serum samples (typified by the WB using serum sample 1 in figure 3, bottom). Quantitation of the serum response to S protein by densitometry (WB) and relative fluorescence (FACS) gave the reactivity orders  $F3R1 > F2R1 > F1R1 \gg \text{others}$  and  $F1R1 = F1R2 > F1R3 = F2R1 = F3R1$ , respectively (figure 4). Thus, there was a clear shift in reactivity with the same serum samples, depending on the assay format.

**Specificity of serum reactivity with S protein fragments.**

The possible use of insect cell–displayed S protein for diagnostic application was assessed by examining fragment reactivity with serum samples from patients infected with human CoV 229E and also with serum from a patient with suspected but clinically unconfirmed SARS (serum sample 3118). Preliminary data have indicated that serum samples from some CoV 229E–infected individuals can cross-react with purified SCoV nucleocapsid protein (P.H. and M.Z., unpublished data), so the use of a more specific test for seroconversion, based on S protein, may be valuable. Serum samples from 2 patients with confirmed CoV 229E infection did not react with S-F1R2 by use of FACS (figure 5), whereas the serum sample from a patient with suspected SARS showed a weak but clear shift in fluorescence. WB could also distinguish the serum samples, although the discrimination between the samples was not as good as that achieved by use of FACS (data not shown), suggesting that the most discriminatory test in uncertain cases should use optimized S protein fragments presented in a nondenaturing assay format.

**Time course of antibody response.** A set of serum samples representing a time course from 6 to 40 days after the onset of SARS for 1 patient was obtained and used in an S protein–specific ELISA, to determine the increase in S protein titer over time. The titer of S protein antibodies was significant from day 10, similar to the serum responses reported for whole infected cells [11] and isolated N protein [23], and increased to the latest time point (40 days after onset) (figure 6). Serum samples were also used in WB assays to examine the changes of reactivity with each individual S protein fragment over time. The earliest WB–positive serum sample (10 days after onset) gave a pattern of binding similar to that of the other serum samples tested (figures 3 and

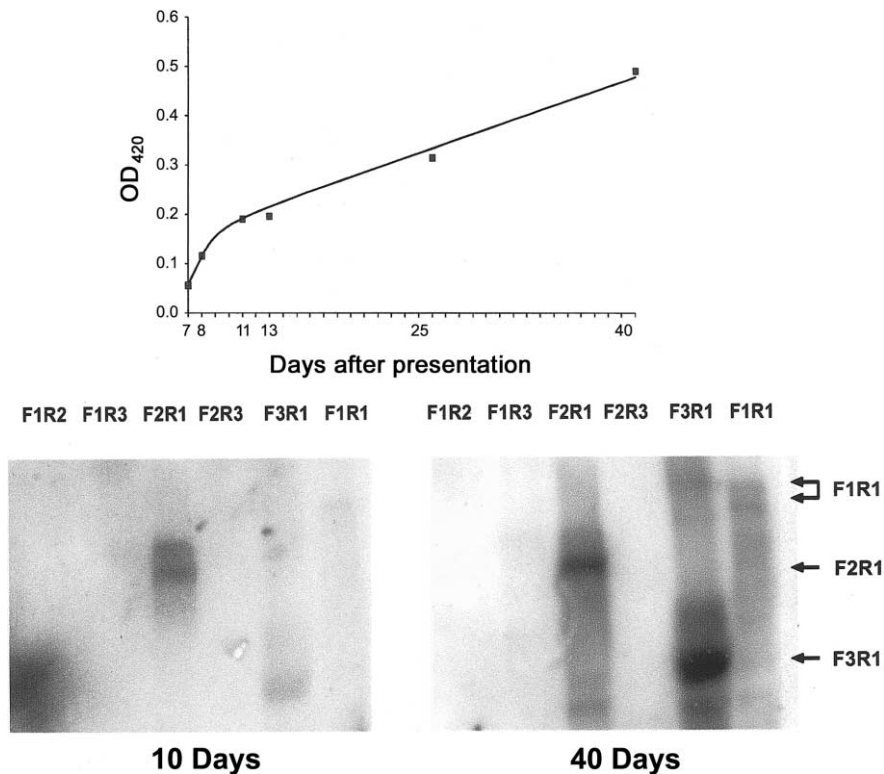


**Figure 5.** Specificity of severe acute respiratory syndrome (SARS) coronavirus (SCoV) spike (S) protein as a diagnostic antigen. Cells displaying S protein F1R2 were incubated with serum from a patient with human coronavirus 229E infection and with serum sample 3118, which was obtained from a patient with suspected but clinically unconfirmed SARS. *Filled histograms*, nonstained cells; *dotted lines*, stained cells.

6). The 40-day time point provided a much stronger signal, but the pattern of binding was not altered (figure 6). Blots were also probed with isotype-specific conjugates, and the results were compared with blots probed with an IgG-specific conjugate. We found evidence for the development of some IgM response late in the recovery period, but it was weak, compared with IgG (data not shown). Only serum antibodies were available for the present study, and it remains possible that secreted antibodies are also present in respiratory secretions early during the infection.

## DISCUSSION

Our data provide the first profiles of antibody binding to the SCoV S protein. Efficient expression of the S protein as a specific source of antigen was achieved by use of a highly productive expression system, recombinant baculoviruses, combined with the use of signal and TM sequences proven to efficiently direct proteins to the surface of the expressing cell [12]. This format offered assays based on antibody binding to



**Figure 6.** Development of the anti-severe acute respiratory syndrome coronavirus spike (S) serum response over time. *Top*, S protein titer over time determined by use of S protein F1R1 released from infected cells and used as a capture antigen for an ELISA. OD<sub>420</sub>, optical density at 420 nm. *Bottom*, Western blots with the serum at 10 (*left*) and 40 (*right*) days after onset. Note that the reactivity pattern, indicated on the right and similar to that shown by serum sample 1 (figure 3), does not vary with time.

the cell surface, mimicking infected cells but with singularly high levels of expression of S protein, as well as denatured antigen prepared by cell lysis. Characterization of the expressed products provided some evidence for potential interaction with a known glycoprotein chaperone (calnexin), although the stimulation in glycan processing was marginal (20% of the total), and for cleavage of S protein by trypsin-like, but not furin, proteases. Cleavage of surface glycoproteins is a key factor in pathogenesis for some viruses [24, 25], and it will be interesting to evaluate the role of S protein cleavage, if any, in SCoV infectivity in both animal and human hosts. In the present study, full-length S protein on the surface of insect cells did not bind a variety of species of red blood cells, suggesting that it does not hemagglutinate. S protein released by detergent lysis also failed to bind to any discrete proteins in far-western blot assay of Vero cell membranes (data not shown). Accordingly, our data do not address the nature of the receptor for SCoV, in particular the early suggestion that it may be CD13 [14]. Recent data show angiotensin-converting enzyme 2 to be at least 1 functional SCoV receptor [26].

Efficient sources of several recombinant S protein fragments allowed us to evaluate the predominant antibodies present in convalescent serum samples. Interestingly, the patterns of S reactivity with available serum samples was similar within each assay format, suggesting that the immune response to S protein varies only quantitatively between individuals. This result would be in keeping with the apparently low immunological pressure suggested from the sequence variation observed in several isolates [7]. Of the 2 assay formats we used, nondenatured S protein present on the cell surface provided the most sensitive detection of antibodies, with clear shifts in fluorescence for serum samples from patients with suspected but clinically unconfirmed SARS. No shift was apparent with human CoV 229E serum samples, suggesting that this format is highly specific. When reactivity of positive serum samples to individual S protein fragments was compared, we observed a strong differential binding, depending on the assay used. Thus, although FACS showed the highest reactivity with fragments, including the amino terminus of S protein, WB with the same serum samples showed preferential reactivity to the carboxyl terminal half of the molecule. Differential antibody binding was not influenced by the high-mannose glycans present on insect-derived glycoproteins, because S protein prepared in Mimic cells (Invitrogen), which add complex glycans to the polypeptide [27], showed the same pattern of reactivity with the serum samples used (data not shown).

Our data, which were obtained with only the few serum samples from patients with SARS available in the United Kingdom, clearly require confirmation with larger sets of serum samples but would be consistent with a level of conformational antibody present in the serum samples preferentially directed toward the globular N terminus of the protein and detected by

use of FACS but not by use of WB. This class of antibody includes those most useful for diagnosis (see above), suggesting that the most suitable assay format for a final diagnostic will be best configured with authentically folded protein, rather than, for example, peptides. Our preliminary work with purified soluble full-length S protein suggests that sensitivity is maintained with the soluble protein, indicating that assay formats simpler than flow cytometry will be feasible. Confirmation that the class of conformational antibody directed at the globular S1 domain includes neutralizing antibody, and whether it has any bearing on the outcome of infection, will be important if recombinant S protein is also to be considered as part of a possible vaccine for SCoV infection.

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