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Inhibition of the interaction between the SARS-CoV Spike protein and its cellular receptor by anti-histo-blood group antibodies

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Severe acute respiratory syndrome coronavirus (SARS-CoV) is a highly pathogenic emergent virus which replicates in cells that can express ABH histo-blood group antigens. The heavily glycosylated SARS-CoV spike (S) protein binds to angiotensin-converting enzyme 2 which serves as a cellular receptor. Epidemiological analysis of a hospital outbreak in Hong Kong revealed that blood group O was associated with a low risk of infection. In this study, we used a cellular model of adhesion to investigate whether natural antibodies of the ABO system could block the S protein and angiotensin-converting enzyme 2 interaction. To this aim, a C-terminally EGFP-tagged S protein was expressed in chinese hamster ovary cells cotransfected with an α1,2-fucosyltransferase and an A-transferase in order to coexpress the S glycoprotein ectodomain and the A antigen at the cell surface. We observed that the S protein/angiotensin-converting enzyme 2-dependent adhesion of these cells to an angiotensin-converting enzyme 2 expressing cell line was specifically inhibited by either a monoclonal or human natural anti-A antibodies, indicating that these antibodies may block the interaction between the virus and its receptor, thereby providing protection. In order to more fully appreciate the potential effect of the ABO polymorphism on the epidemiology of SARS, we built a mathematical model of the virus transmission dynamics that takes into account the protective effect of ABO natural antibodies. The model indicated that the ABO polymorphism could contribute to substantially reduce the virus transmission, affecting both the number of infected individuals and the kinetics of the epidemic.

Keywords: ABO/cellular receptor/histo-blood group antigens/natural antibodies/SARS

Introduction

Severe acute respiratory syndrome (SARS) is characterized by an acute respiratory disease, often accompanied by gastroenteritis, which is fatal in approximately 10% of infected individuals (Gu and Korteweg 2007). The etiological agent is a novel coronavirus (CoV), designated as SARS-CoV, which emerged from an animal reservoir during the winter 2002–2003 when it infected over 8000 humans worldwide. It emerged again the next winter and since then, no SARS outbreak has been recorded (Chen and Subbarao 2007). Yet, a serious possibility of reemergence of SARS-CoV or of the introduction of other related viruses from an animal reservoir remains.

Spike (S) proteins of coronaviruses are large transmembrane heavily *N*-glycosylated proteins that mediate association with a cell surface receptor (Li et al. 2006). The SARS-CoV S protein possesses 23 *N*-linked glycosylation sites distributed in three clusters. The glycosylation of 13 of these sites has been confirmed (Krokhin et al. 2003; Ying et al. 2004; Chakraborti et al. 2005). Receptor binding domains (RBDs) have been identified in the S1 domain of a number of coronaviruses, and a fragment of the SARS-CoV S1 domain, from residues 318–510, binds human angiotensin-converting enzyme 2 (ACE2) with high affinity (Xiao et al. 2003; Babcock et al. 2004; Wong et al. 2004). It has additionally been demonstrated that ACE2 constitutes an obligate cellular receptor although other receptors may participate in the infection process (Li et al. 2003; Chen and Subbarao 2007). The structure of SARS-CoV RBD complexed with ACE2 revealed that an extended loop of the RBD, comprising residues 424–494, is in direct contact with ACE2 (Li et al. 2005). This receptor binding motif (RBM) is not glycosylated, but it is surrounded by two clusters of glycosylation sites (Han et al. 2007).

Various genetic factors influencing the susceptibility to or the outcome of SARS have been described (Gu and Korteweg 2007). The *ABO* gene stands out among the genes involved since O blood group individuals were shown to have very low odds of infection compared to non-O individuals in a hospital outbreak that occurred in March 2003 in Hong Kong (Cheng et al. 2005). Histo-blood group antigens are present not only on erythrocytes but also on many epithelial cells, which are their main site of expression (Marionneau et al. 2001). Since SARS-CoV replicates in epithelial cells of the respiratory and digestive tracts that have the ability to synthesize ABH carbohydrate epitopes, we hypothesized that the S protein of virions produced by either A or B individuals could be decorated with A or B carbohydrate epitopes, respectively. Natural anti-A or -B antibodies from blood group O, B, and A individuals could bind to the S protein and block its interaction with ACE2, thereby preventing infection in accordance with the rules of transfusion.

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In order to put this hypothesis to the test, we used a cell binding assay that reconstitutes the interaction between the S protein and ACE2 (Chou et al. 2005). We present data indicating that the S protein/ACE2-mediated adhesion between cells expressing ACE2 and cells coexpressing the S protein and the A histo-blood group antigen can be specifically blocked by anti-A antibodies. To further evaluate the potential effect of the ABO polymorphism on the epidemiology of SARS, we present a model of its transmission dynamics that takes into account the effect of the protection by anti-histo-blood group natural antibodies.

Results

Preparation of cells coexpressing the A antigen and SARS-CoV S protein for the study of the ACE2/S protein interaction in a cell adhesion assay

The interaction between the SARS-CoV spike protein and its cellular receptor ACE2 can be studied using a cell-based assay, as described previously (Chou et al. 2005). In this assay, the viral S protein expressed by transfection into Chinese hamster ovary (CHO) cells mediates adhesion to Vero E6 cells that possess ACE2. CHO cells do not express ABH antigens because of the lack of an α1,2-fucosyltransferase activity and of either the A or B histo-blood group enzymes. In order to obtain cells able to express the A antigen, parental CHO cells were stably transfected successively with the rat Fut2 cDNA and a rat A enzyme cDNA. Unlike mock-transfected cells, these double transfectants strongly express cell surface A antigen as detected by flow cytometry. Transfection of the S protein–EGFP fusion construction (S–EGFP) into these cells allowed the expression of the S protein together with the histo-blood group A antigen (Figure 1A). Observation of the triple transfectants by confocal microscopy revealed that, as expected, the A antigen and the S–EGFP fusion protein partially colocalized at the cell surface (Figure 1B). In addition, western blot analysis revealed that among various A antigen positive glycoproteins, a band at the expected size of the S–EGFP fusion protein, between 210—and 230 kDa (Chou et al. 2005), was present in the extract from the triple transfectant Fut2/A/SP but absent from the double Fut2/A transfectant cell extract. It indicated that the S-protein expressed by A-positive CHO cells carried A histo-blood group epitopes. Specificity of the anti-A labeling was ensured since no band was detected in extracts from CHO Fut2 only transfectants (Figure 1C). A stable A antigen and S-EGFP expressing clone showed significantly higher adhesion to Vero cells than either mock transfectants or the A expressing clone devoid of the S protein (Figure 2A and B). Similar results were obtained after transient transfection of the S-EGFP construct (not shown). The presence of the A and/or H antigens on the S protein expressing cells did not affect adhesion since CHO cells only transfected with the S-EGFP construct, as well as CHO cells transfected with both the S-EGFP and Fut2 cDNAs, adhered to Vero cells at a similar level as the A antigen S protein triple transfectants (Figure 2A). In order to control that the cell adhesion was dependent upon the ACE2/S protein interaction, blocking experiments with either an anti-ACE2 or an anti-S protein were performed. Both antibodies significantly inhibited adhesion, although the anti-ACE2 mAb proved more efficient (Figure 2C).

Inhibition of adhesion by anti-A antibodies

The effect of anti-A antibodies on the S protein/ACE2 interaction was first tested using a monoclonal anti-A. A clear-cut inhibition of the cell adhesion was observed using the monoclonal antibody 3-3A at 2 μg/mL. Specificity of the inhibition was confirmed since a control irrelevant antibody failed to inhibit and the adhesion to Vero cells of S protein-transfected CHO cells lacking the A antigen was not inhibited by the anti-A mAb (Figure 2D). Vero cells do not express the A histo-blood group antigen. Therefore, the inhibition of adhesion mediated by the anti-A mAb can only result from a binding to CHO S protein expressing cells and not to the glycans of ACE2. In order to assay the ability of natural human anti-A to inhibit cell adhesion, plasma samples from two O blood group individuals with high anti-A titers were selected. The samples were first adsorbed on silica beads conjugated to the A type 2 tetrasaccharide in order to specifically remove the anti-A natural plasma antibodies. Efficacy of the adsorption was controlled by ELISA which showed that the reactivity to the A type 2 tetrasaccharide was almost completely abolished following adsorption (Figure 3B). The A type 2 adsorbed and mock adsorbed plasma samples from the two individuals were then added in the cell-based assay. Both mock adsorbed samples, containing the anti-A as shown in Figure 3B, strongly inhibited the adhesion of A antigen-S protein expressing cells to Vero cells. In both cases, this inhibition was almost completely lost after A type 2 adsorption, showing that it was specifically mediated by anti-A plasma antibodies (Figure 3C). Moreover, the inhibition of adhesion by blood group O plasma was dose-dependent and still detected at a plasma dilution as low as 1/32 (Figure 3D).

Modeling the effect of protection by natural anti-A or -B antibodies on the virus transmission in populations

A total population $N_{TOT} = N_A + N_B + N_O + N_{AB}$ of 10^6 individuals including one blood group O-infected individual was simulated.

Three different patterns of transmission of the virus, with different probabilities of transmission, were assumed according to whether some protection by anti-histo-blood group natural antibodies was present (Figure 4). A strong or moderate group effect denotes a strong or moderate protection, respectively, whereas no group effect corresponds to an absence of protection. The impact of group effect was assessed on the number of infected individuals over time in four different populations with very different frequencies of ABO phenotypes (Figure 5). A strong group effect delayed the initiation of the epidemic as well as it decreased importantly the total number of infected individuals whatever the population considered as compared to no group effect and, to a lesser extent, a moderate group effect. Moreover, a strong group effect evidenced different starting times of the epidemic according to the different populations (Figure 5B). The impact of group effect was assessed on the number of infected individuals over time according to their blood groups in the Chinese (Hong Kong) population (Figure 6). The number of blood group O-infected individuals was always the most important one, closely followed by blood group B-, A-, and AB-infected individuals whatever the transmission pattern considered. A strong group effect also delayed the initiation of the epidemic as well as it markedly decreased the total number of infected individuals. Furthermore, a strong group effect also

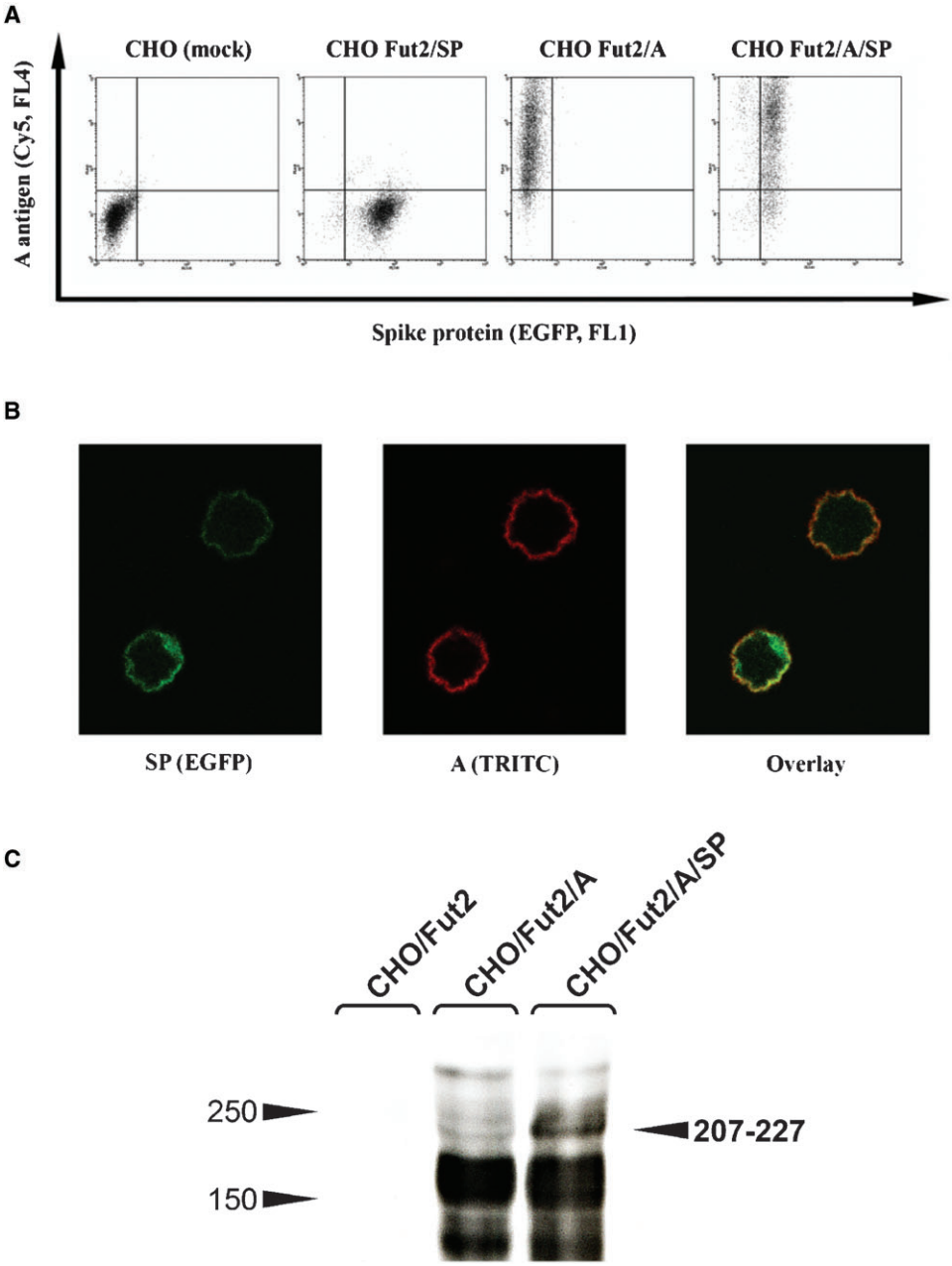

Fig. 1. CHO cells coexpressing the A histo-blood group antigen and the SARS-CoV S protein. (**A**) Flow cytometry analysis of the expression of A antigen and the S protein–EFGP construct on CHO mock-transfected cells (mock), double transfectants either with the Fut2 and S protein constructs (Fut2/SP) or the Fut2 and A enzyme cDNAs (Fut2/A), triple transfectants with the Fut2, A enzyme and S protein constructs (Fut2/A/SP). Fluorescence of the S–EFGP molecule was directly recorded on the FL1 channel. Detection of the A antigen was performed using an anti-A mAb followed by Cy5-labeled anti-mouse IgG and recorded on the FL4 channel. (**B**) Confocal microscopy analysis of the A antigen (A TRITC) and the S protein–EFGP construct coexpression on CHO cells triple transfectants. Detection of the A antigen was performed using an anti-A mAb followed by TRITC-labeled anti-mouse IgG. (**C**) Western blot analysis of transfected CHO cells glycoproteins. Total protein extracts from CHO Fut2 simple transfectants, CHO Fut2/A double transfectants, and CHO Fut2/A/SP triple transfectants were submitted to SDS–PAGE and Western blotting. Glycoproteins carrying A histo-blood group epitopes were detected with an anti-A mAb. The arrow shows the expected molecular size of the EGFP–SP fusion protein.

postponed the starting time of the epidemic in blood group O individuals as compared with the other blood groups. The blood group type of the index case in the Hong Kong outbreak was not provided (Cheng et al. 2005). The modeling was first performed using a blood group O index case, as described above. In order to assess whether the results could be affected by the blood group type of this first case, further simulations were performed with different index cases of either the A, B, or AB blood group. The main results were not modified since a strong group effect always delayed the initiation of the epidemic as compared to

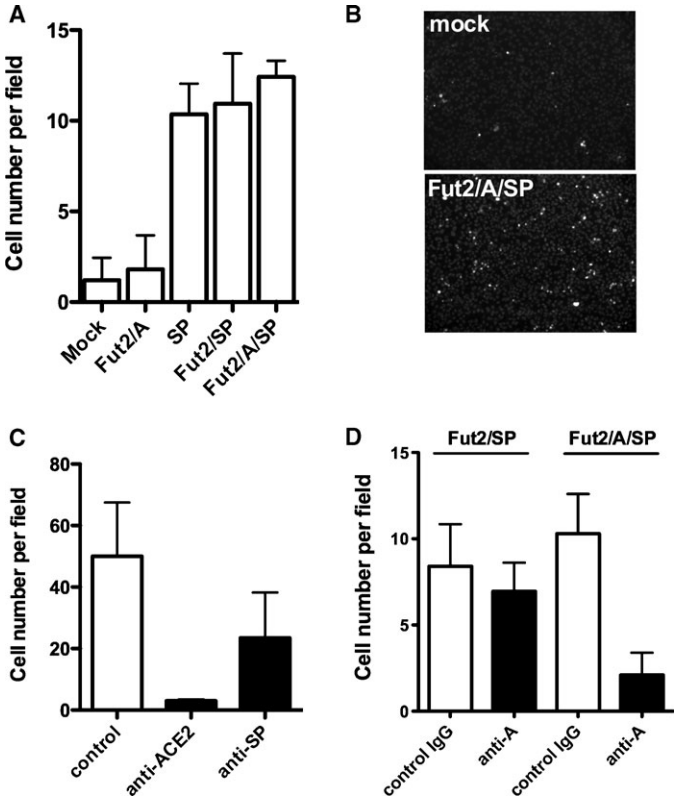

Fig. 2. S Protein/ACE2-dependent adhesion of CHO cells to Vero cells. The binding assay between CHO cells and Vero E6 cells was performed as described in *Material and methods*. Adherent cells were counted under a fluorescence microscope. Cells from a total of 36 fields from 6 wells were counted. (**A**) The results shown correspond to the mean ± SD of one representative experiment out of four obtained with CHO cells mock transfectants (mock), double transfectants with the Fut2 and A histo-blood group glycosyltransferases (Fut2/A), simple transfectants with the SARS-CoV S protein construct (SP), double transfectants with the Fut2 enzyme and the SARS-CoV S protein construct (Fut2/SP), and triple transfectants with the Fut2, A glycosyltransferases and the SARS-CoV S protein cDNAs (Fut2/A/SP). Adhesion of SP, Fut2/SP, and Fut2/A/SP cells is significantly higher than that of either mock or Fut2/A cells ($P < 0.001$, Student's *t*-test). (**B**) Representative fields illustrating the adhesion of either mock-transfected CHO cells or triple transfectants. (**C**) Inhibition of the adhesion of triple CHO transfectants to Vero cells by anti-ACE2 or anti-S protein mAbs. The mAbs were added to the CHO cells suspension at 20 and 25 μg/mL, respectively prior to incubation on the Vero cell layer. Adhesion in the presence of the anti-ACE2 and anti-SP are significantly lower than that of control cells ($P < 0.001$ and $P < 0.01$, respectively). (**D**) Inhibition of the adhesion to Vero cells of S protein-transfected CHO cells coexpressing either the H (Fut2/SP) or the A antigen (Fut2/A/SP) by an anti-A mAb or a control isotype matched antibody used at 4 μg/mL. Only the adhesion of the triple transfectants in the presence of the anti-A differs significantly from other conditions ($P < 0.01$).

no group effect and to a moderate group effect. Likewise, it markedly decreased the total number of infected individuals irrespective of the index case's blood group (data not shown).

Discussion

Probably due to mimicry of A or B antigens by flora or infectious bacteria, individuals acquire anti-A or -B blood group antibodies to the antigen that they do not synthesize. These so-called natural anti-histo-blood group antibodies have long been suspected to play a role in anti-viral immunity since viruses may carry ABH structures as terminal carbohydrate motifs of their envelope glycoproteins or possibly as inserted glycolipids (Greenwell 1997). In line with this concept, a monoclonal anti-A was shown to neutralize HIV produced by lymphocytes from blood group A donors only (Arendrup et al. 1991). More recently, anti-A or -B from human serum were shown to sensitize HIV to complement-mediated inactivation (Neil et al. 2005). Likewise, measles virus produced by cells expressing either A or B histo-blood group epitopes was neutralized by natural anti-HBGAs in a complement-dependent manner (Preece et al. 2002). Though these in vitro data suggest that natural anti-HBGAs may provide protection against some viruses, they have not been substantiated by epidemiological observations so far. If natural anti-A or -B serum antibodies provide protection, it is expected that during an outbreak, blood group O individuals should experience a lower risk of infection than non-blood group O individuals. This has not been observed for either HIV or measles virus at present. Yet, it is precisely what was observed in the case of a hospital outbreak of SARS in Hong Kong, where O blood group individuals appeared at a much lower risk of being infected by SARS-CoV than subjects of other blood types (Cheng et al. 2005). Interestingly, SARS-CoV infects cells that express ABH antigens according to the individual's ABO phenotype. Indeed, SARS-CoV infection has been documented in pneumocytes, enterocytes of the small intestine as well as in cells of the kidney distal tubular epithelium, all cell types known to be able to synthesize ABH antigens (Chen and Subbarao 2007; Gu and Korteweg 2007). Since the glycosylation of viral glycoproteins necessitates the glycosylation machinery of the infected cell, viral particles synthesized in cells that may express that histo-blood group antigens are expected to be tagged with these antigenic motifs, and therefore natural antibodies directed against these carbohydrate tags may have a protective role (Seymour et al. 2004).

In order to determine if anti-HBGAs could block SARS-CoV entry into target cells, we used an experimental model of cell adhesion that has been developed with the aim of screening molecules that block the virus entry without using infectious particles. The model allowed us to show that either a monoclonal anti-A antibody or natural plasma anti-A specifically inhibited the SARS-CoV S protein/ACE2-dependent adhesion. This is in accordance with the hypothesis of the protective role of natural anti-HBGAs and strongly suggests that the low risk of infection of blood group O individuals during the Hong Kong hospital outbreak was due to the presence of these antibodies prior to the outbreak.

Although the RBM of the SARS-CoV S protein does not involve any glycan chain, clusters of glycosylation sites are located in its vicinity (Li et al. 2005; Han et al. 2007). Large molecules such as lectins or antibodies binding to these glycans are thus expected to interfere with the S protein/ACE2 interaction. This has already been observed with mannose-specific lectins which have shown anti-viral activity against SARS-CoV by blocking virus attachment to its receptor (Keyaerts et al. 2007). Our data indicate that natural anticarbohydrate antibodies could have a similar effect. In addition to the blocking of virus attachment to its receptor, natural antibodies could block entry or opsonize viral particles leading to complement-mediated neutralization (Neil et al. 2005). Moreover, it was recently shown that natural antibodies can contribute to help the generation

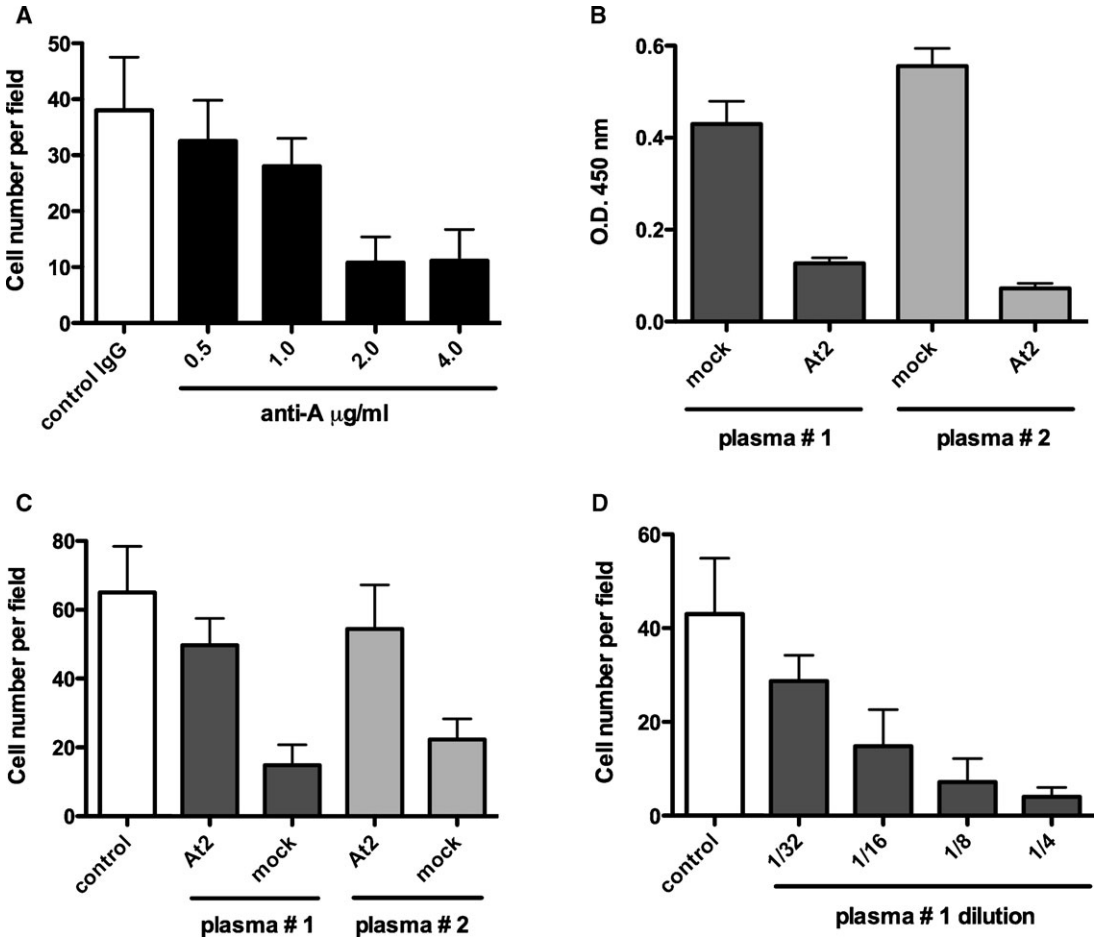

Fig. 3. Effect of anti-A antibodies on the interaction between the SARS-CoV S protein and ACE2. (**A**) The anti-A monoclonal antibody 3-3A was added at the indicated concentrations to the triple transfected CHO cells suspension prior to incubation on the Vero cell layer. An irrelevant IgG1 was used as control at 4 μg/mL. The results are presented as mean cell number per field ±SD of one representative experiment out of two. From 1.0 μg/mL to 4.0 μg/mL anti-A, values are significantly different from those for the control IgG ($P < 0.05$ and 0.001, respectively). (**B**) Adsorption of the anti-A natural antibody from human O plasmas. Plasma samples from two individuals were adsorbed on either control silica beads (mock) or A type 2 tetrasaccharide conjugated to silica beads (At2). The postadsorption plasma reactivity on A type 2 conjugated to polyacrylamide was tested by ELISA. Results are shown as O.D. 450 nm values of duplicate wells ±SD for each plasma sample diluted at 1/4. In the absence of A type 2 conjugate, mean O.D. values were 0.13. (**C**) Inhibition of the adhesion of CHO triple transfected cells to Vero cells by mock adsorbed (mock) or A type 2 (At2) adsorbed human blood group O plasma samples from individuals 1 and 2. Plasma samples were diluted at 1/8 in PBS. Control values were obtained in the absence of plasma. Values for the mock adsorbed plasma were significantly different from the control values ($P < 0.001$). (**D**) Inhibition of the adhesion in the cell-based assay as in **C** by serial dilutions of unadsorbed plasma from individual 1. All values obtained in the presence of plasma were significantly different from the control value (from $P < 0.05$ to $P < 0.0001$).

of cytotoxic T cells against the pathogen (Stäger et al. 2003; Dürrbach et al. 2007). These additional mechanisms of protection may also have participated to the protection of blood group O individual during the SARS outbreak.

Since the epidemiology of SARS has been well documented (Anderson et al. 2004; He and Chinese 2004), it was possible to develop a model of the virus transmission that takes into account the effect of the ABO polymorphism. Critical transmission parameters were deduced from those of the global SARS outbreak and from those of the Hong Kong hospital outbreak where the ABO effect was observed. The model considers that no prophylatic measures were taken; although this is clearly unrealistic, it allows us to fully appreciate the impact of the blood group polymorphism itself. Of note, the expression of ABH antigens in epithelial cells where SARS-CoV replicates is also controlled by polymorphisms of the *FUT2* gene. Thus, individuals with two *FUT2* null alleles, the so-called nonsecretors, are unable to synthesize H antigen and hence A or B antigens in these cells (Marionneau et al. 2001). For simplicity, the model did not consider such individuals since with regard to the virus transmission, they would behave as O blood group donors. Including them in the analysis is therefore similar to slightly increase the pool of O individuals. Since anti-A and anti-B titer interindividual variability is quite high, and since we observed that plasmas from O blood group individuals with low anti-A titers were not inhibitory in the cell adhesion assay (not shown), we considered transmission parameters allowing for a moderate ABO effect only. In this case, virus transmission in incompatible ABO situations remains possible but with a lower probability of occurrence than in compatible situations. That is certainly more realistic than the case of the strong ABO effect where ABO incompatibility completely impairs virus transmission. The latter case was analyzed in order to evaluate the maximal potential of the ABO polymorphism. The model indicated that both in

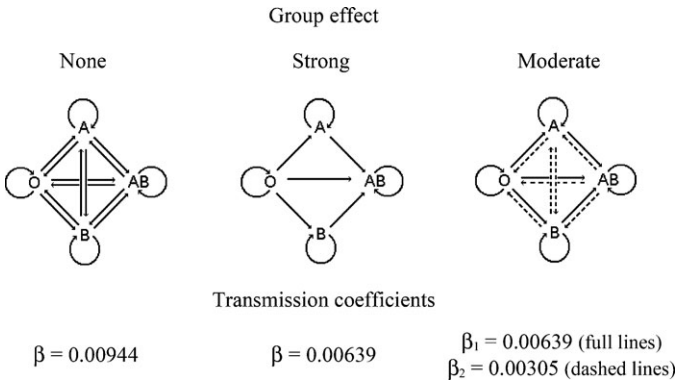

Fig. 4. Transmission patterns used to model the effect of the ABO polymorphism. In the absence of ABO effect, transmission can occur irrespective of the ABO type (full arrows in all directions). In the presence of a strong ABO effect, transmission occurs strictly according to the rules of transfusion, whereas in the case of a moderate ABO effect, some incompatible transmission can occur (dashed arrows). Determination of the values of the transmission coefficients β, β_1, β_2 has been done based on the Hong Kong hospital outbreak data, as described in the supplemental material. Transmission coefficients correspond to the transmission rates of the disease for each contact.

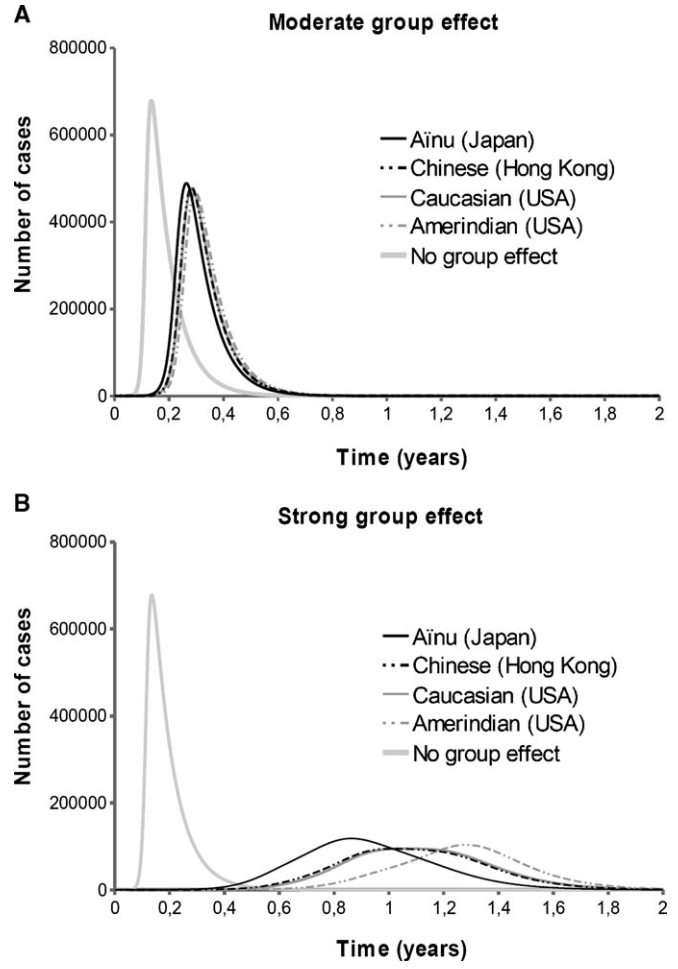

Fig. 5. Influence of the ABO polymorphism, with either a moderate group effect (**A**) or a strong group effect (**B**) as compared with no group effect, on the number of infected individuals over time in four different populations presenting large differences in the frequencies of ABO phenotypes.

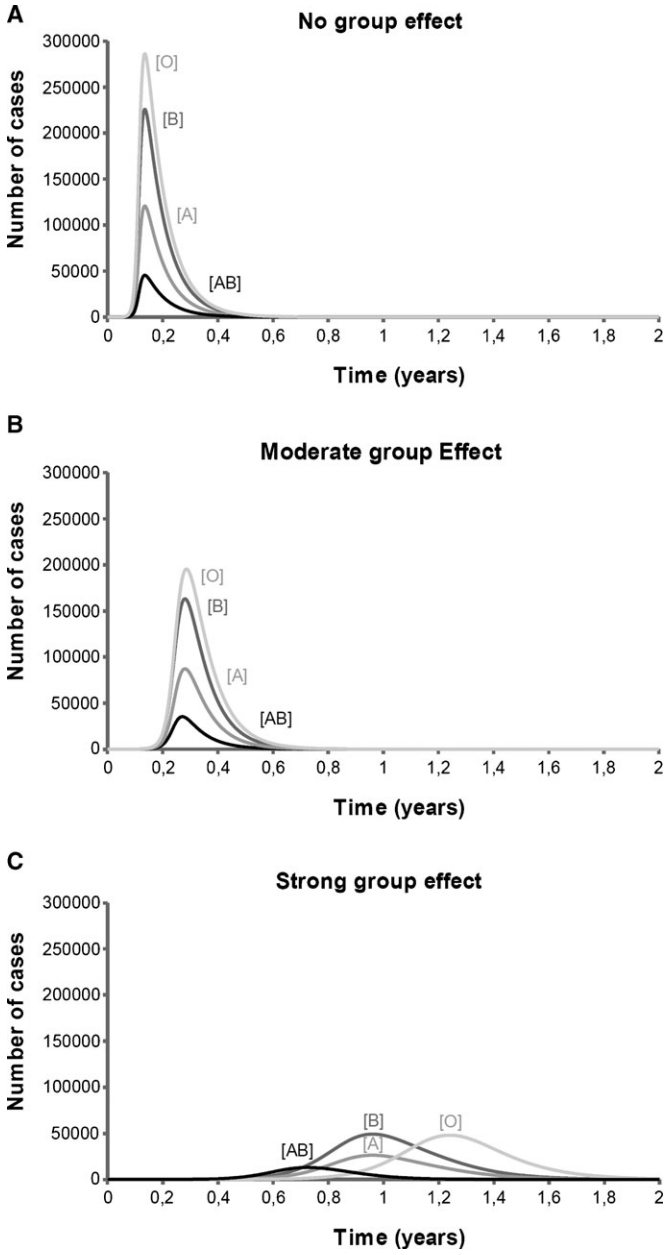

Fig. 6. Influence of different transmission patterns, no group effect (**A**), moderate group effect (**B**) or strong group effect (**C**), on the number of infected individuals over time according to their blood groups in the Chinese (Hong Kong) population.

the presence of a moderate or a strong group effect, virus transmission was decreased, supporting the hypothesis that natural anti-A and -B antibodies can contribute to protect against selected viral diseases at the population level. Less intuitively, the model shows that the main effect of the natural anti-histo-blood group antibodies is to delay and slow down the epidemic. In the case of a full protection (strong effect), the delay between the occurrence of the first cases and the full development of the outbreak can be very large. It is linked to the frequency of O individuals in the population, but remains very significant even in a population with an unusually low blood group O frequency such as the Aïnous of Japan. This delay, already clearly visible

when taking into account a moderate ABO effect only, might have had an adaptative value, in past epidemics of other viruses with transmission characteristics similar to those of SARS, since it allows for modifications of behavior limiting the spread of epidemics. This could have contributed to the maintenance of the ABO polymorphism throughout human evolution and history. Mean natural anti-A or -B titers tend to decrease over the years in developed countries, possibly due to improved hygiene (Dr. A. Blancher, personal communication). It is thus possible that in the past their protective effect was higher than in most contemporary populations. It could thus be of interest to raise the levels of anti-A or -B in all populations so as to slow and limit the spread of some emergent pathogens. This could prove a valuable prevention strategy against SARS but also against other coronaviruses which are responsible for a significant proportion of common colds and can contribute to more severe respiratory tract infections (van der Hoek 2005).

Material and methods

Cell culture and transfections

Cell lines CHO-K1 and Vero E6 were purchased from American Type Cell Collection (Manassas, VA). Vero-E6 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% decomplemented fetal-calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Paisley, UK).

CHO cells are devoid of α1,2-fucosyltransferase activity and therefore of ABH antigens. To obtain the expression of the A antigen, they were transfected first with the rat Fut2 cDNA, and then with an A enzyme cDNA (Bureau et al. 2001; Cailleau-Thomas et al. 2002). The rat Fut2 cDNA was inserted into the pDR2 eukaryotic expression vector (Clontech, St Germain en Laye, France) deleted of the sequences lying between the EcoRV and ClaI sites. This vector possesses a hygromycin selection marker. The rat A enzyme cDNA was inserted into the pcDNA3.1 vector (Invitrogen, Paisley, UK) with a zeocin selection marker. Cells were transfected with the rat Fut2 cDNA using Lipofectamin 2000™ according to the manufacturer's instructions (Invitrogen). After selection in hygromycin containing medium (0.4 mg/mL), cells were cloned by limiting dilution, and a clone strongly expressing cell surface H antigen, as detected using the FITC-labeled UEA-I lectin, was selected. This clone was further transfected with the rat A enzyme cDNA. Transfectants were then selected in a zeocin-containing medium (0.6 mg/mL). After cloning by limiting dilutions, a clone strongly expressing the A histo-blood group antigen was selected. Control transfected cells were prepared by transfection with the empty vectors. These stable transfectants were cultured in RPMI 1640 supplemented with 10% decomplemented fetal-calf serum, 2 mM L-glutamine, 10 μg/mL free nucleotides, 100 U/mL penicillin, and 100 μg/mL streptomycin, 0.4 mg/mL hygromycin, and 0.6 mg/mL zeocin. To obtain the expression of the SARS-CoV S protein, control CHO cells, transfected CHO cells expressing the H antigen, and double transfected CHO cells expressing the A antigen were transfected with the previously described pEGFP-N1 vector containing an S protein–EGFP construction (CMV-SG) (Chou et al. 2005). Stable transfectants were obtained after selection with 1 mg/mL G418 (Gibco). Since the expression of the S protein was progressively lost, even when cells were continuously cultured in the presence of G418, part of the experiments were performed 48 h after transient transfection with the CMV-SG vector.

Cells were passaged at confluence after dispersal with 0.025% trypsin in 0.02% EDTA and routinely checked for mycoplasma contamination by Hoechst 33258 (Sigma, St Louis, MO) labeling.

Flow cytometry, immunofluorescence, and Western blot analyses

Cells at near confluence were detached by a brief 0.025% trypsin/0.02% EDTA treatment. Viable cells, 2×10^5 per well of 96 culture microtiter plates, were incubated with primary anti-H type 2 or broad reactive anti-A monoclonal antibodies 19-0LE and 3-3A respectively, in PBS containing 0.1% gelatin for 30 min at 4°C (Bara et al. 1988; Mollicone et al. 1996). After three washes with this same buffer, a 30-min incubation with the secondary anti-mouse IgG FITC-labeled antibody (Sigma) was performed at 4°C. After washing, fluorescence analysis was performed on a FACSCalibur (Becton-Dikinson, Heidelberg, Germany). The expression of the EGFP-S protein construct was detected by its autofluorescence on the FL1 channel.

CHO cells transfectants, cultivated on glass lamellae, were fixed by the addition of 2% formaldehyde in a culture medium for 10 min. After washing in PBS, the cell monolayer was incubated with the anti-A mAb 3-3A at 0.5 μg/mL in PBS for 1 h and washed thrice in PBS before incubation for 30 min with TRITC-labeled anti-mouse IgG (Sigma) diluted at 1/400. After three final washings in PBS, slides were mounted in Mowiol and observed under a Leica TCS SP (Heidelberg, Germany) confocal fluorescence microscope. Negative controls were incubated with the secondary antibody alone.

Confluent cells (CHO Fut2 simple transfectants, CHO Fut2/A double transfectants, and CHO Fut2/A/PS triple transfectants) were rinsed with ice-cold PBS, pH 7.2, and then recovered by scraping. After washing with ice-cold PBS, cells were solubilized in 50 mM potassium phosphate, pH 6.0, containing 2% (v/v) triton X-100 on ice for 30 min. Following a centrifugation at 13,000 × *g* for 10 min, the supernatant was collected and the protein concentration was determined using the BC assay protein quantification kit (Uptima, Montluçon, France). Thirty micrograms of total proteins of each extract were separated on 8% SDS–PAGE under reducing conditions and electrotransferred to immobilon P sheets (Millipore, Bedford, MA). Immunoblots were saturated for 1 h at room temperature with Western blocking reagent (Roche Diagnostics GmBH, Mannheim, Germany) and strips were cut and incubated overnight at 4°C with the anti-A 3-3A mAb at 2 μg/mL in antibody Western blocking reagent. Following three washes for 15 min with TBS containing 0.05% Tween 20, strips were incubated with horseradish peroxidase-labeled anti-mouse IgG (H + L) (Beckman Coulter, Fullerton, CA) for 1 h at room temperature. After three final washes, detection was performed with a chemiluminescence kit (Roche Diagnostics).

Human plasma preparation

Plasma samples from two blood group O healthy donors with anti-A titers 1/256 by classical hemagglutination were used. The presence of anti-A natural antibodies was confirmed by ELISA on synthetic A type 2 tetrasaccharide conjugated to

polyacrylamide (Lectinity, Moscow, Russia). The polyacrylamide conjugate at 10 μg/mL in a carbonate–bicarbonate buffer, 100 mM, pH 9.6, was coated onto Maxisorp ELISA plates (NUNC, Roskilde, Denmark) by overnight incubation at 37°C. After three washes with PBS containing 0.05% Tween 20 (TPBS), wells were incubated with 5% defatted milk in PBS for 1 h at 37°C. PBS 2-fold serially diluted plasma samples were then incubated for 1 h at 37°C. After washing with TPBS, peroxidase-labeled anti-human IgG (H + L) (Uptima, Montluçon, France) diluted at 1/10,000 were incubated for 1 h at 37°C. After final washes with TPBS, reactivities were detected using TMB (5-tetramethylbenzidine) as a substrate (BD Bioscience, San Jose, CA) and read at A_{450} nm. To adsorb the anti-A natural antibodies, 1600 μL of plasma diluted 1/4 in PBS were incubated onto 120 mg of silica beads conjugated with either synthetic A type 2 tetrasaccharide or a methyl group (kind gift from the late Pr. R.U. Lemieux, Edmonton, Canada). The latter being used as mock adsorbed controls. After a 1-h incubation under gentle agitation and centrifugation at 13,000 × *g* for 10 min, the supernatant was collected and kept at 4°C until used.

Cell-based binding assay

Vero E6 cells were grown to a confluent layer in wells of 48-well plate (NUNC). The CHO transfected cells were labeled with Hoechst 33258 at 2 μM for 5 min, rinsed three times with PBS, and then suspended after incubation with 0.025% trypsin in 0.02% EDTA. 1×10^5 cells suspended in 500 μL were gently laid onto the Vero cell layer in at least triplicate wells. After 2-h incubation at 37°C, wells were gently rinsed with a culture medium three times, and cells were fixed by a 10-min incubation with 2% formaldehyde. Adherent CHO cells were counted under an epifluorescence microscope (Zeiss, Jena, Germany) with a 10× objective. Three to six fields/well were counted and results expressed as the mean number of cells per field. In the antibody blocking experiments, monoclonal antibodies or human plasma samples were added to the transfected CHO cells suspension before seeding on the Vero cell layer.

Modeling the effect of protection by natural anti-A or -B antibodies on the virus transmission in populations

A deterministic SIR (susceptible, infectious, recovered) model of the transmission dynamics of SARS that takes into account the effect of the protection by anti-histo-blood group natural antibodies was developed. In this model, the whole population was divided according to the different blood groups (A, B, O, or AB) into four interacting subpopulations N_A, N_B, N_O and N_{AB}. For each subpopulation $N = S(t) + I(t) + R(t)$ where $S(t)$, $I(t)$, and $R(t)$ represent, for each blood group, the number of individuals susceptible, infected, or recovered at time t, respectively. Four different populations reflecting the variability in blood groups distributions were studied (Table I): Chinese from Hong Kong, Aïnu from Japan, Amerindians and white Americans from the United States.

Mathematical details are given in the appendix available as supplementary data; the program was written in C++, a 1-day step was used for all the simulations. In each simulation, changes in the number of infected individuals (cases) over time were determined.

Table I. Distribution of ABO blood groups in four different populations used to model the impact of the ABO effect on SARS transmission.

	[O]	[A]	[B]	[AB]
Aïnu (Japan)	0.17	0.32	0.337	0.173
Chinese (Hong Kong)	0.422	0.178	0.333	0.067
Caucasians (USA)	0.45	0.40	0.111	0.039
Amerindians (USA)	0.79	0.16	0.046	0.004

The frequencies of each phenotype were obtained from Mourant (1983).

Supplementary Data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest statement

None declared.

Abbreviations

ACE2, angiotensin-converting enzyme 2; CHO, chinese hamster ovary; CoV, coronavirus; EGFP, enhanced green fluorescent protein; HBGA, histo-blood group antigen; mAb, monoclonal antibody; PBS, phosphate buffered saline; RBD, receptor binding domain; RBM, receptor binding motif; S protein, spike protein; SARS-CoV, severe acute respiratory syndrome coronavirus; S-EGFP, S protein-EGFP fusion construction.

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