The time course of the immune response to experimental coronavirus infection of man

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SUMMARY

After preliminary trials, the detailed changes in the concentration of specific circulating and local antibodies were followed in 15 volunteers inoculated with coronavirus 229E. Ten of them, who had significantly lower concentrations of pre-existing antibody than the rest, became infected and eight of these developed colds. A limited investigation of circulating lymphocyte populations showed some lymphocytopenia in infected volunteers. In this group, antibody concentrations started to increase 1 week after inoculation and reached a maximum about 1 week later. Thereafter antibody titres slowly declined. Although concentrations were still slightly raised 1 year later, this did not always prevent reinfection when volunteers were then challenged with the homologous virus. However, the period of virus shedding was shorter than before and none developed a cold. All of the uninfected group were infected on re-challenge although they also appeared to show some resistance to disease and in the extent of infection. These results are discussed with reference to natural infections with coronavirus and with other infections, such as rhinovirus infections.

INTRODUCTION

Relatively little work has been done recently on the immune response to respiratory virus infection. Early studies on the immediate response to rhinovirus, coxsackievirus, influenza and parainfluenza infections gave some indication of when local and circulating antibodies first appear and when the maximum titre occurs [1–5]. The change in the concentration of total IgA and protein in nasal secretions after infection was also followed [2, 3, 6, 7].

While some studies have shown that the concentration of local specific antibody declines relatively quickly after infection [3, 8] in others it has been shown to be more long-lasting [9]. Serum antibody is usually more persistent, possibly lasting for life [8, 10]. More recently Barclay and colleagues [11] have shown that local and circulating rhinovirus antibodies are present at relatively high concentrations for at least 1 year after infection.

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Both local and circulating antibodies have been shown to be associated with protection from infection and disease due to coronavirus [12]. While Macnaughton [13] showed that circulating antibody may last no longer than 5 months, Reed [14] indicated that, in volunteers, immunity could last at least a year. However no more detailed studies on the immune response to coronavirus infection have been undertaken, particularly on the kinetics of antibody production after infection. In this study, volunteers at the Common Cold Unit were used to study short-term changes in specific antibody concentrations after infection with coronavirus 229E. This was in order to answer such questions as how soon local and circulating antibody concentrations start to increase and when the peak concentration is reached. Total blood lymphocytes and their subsets were also examined because it has been shown that lymphocytopenia, particularly of T-cells, occurs in the early stages of rhinovirus [15] and influenza infections [16–18]. Thereafter the long-term changes in antibody concentration were followed and resistance to intranasal virus challenge was estimated 1 year after first exposure. Finally, in order to compare these results with the longevity of immunity in natural coronavirus infections, sequential sera taken from subjects taking part in a field study of influenza prophylaxis [19] were examined. Some of these subjects had apparently been naturally infected with coronavirus 229E and others with the other major human coronavirus serotype, OC43.

METHODS

Volunteers

The isolation, inoculation and assessment of volunteers have been described elsewhere [20, 21]. In preliminary experiments, 16 volunteers stayed for a standard trial. After a 3-day quarantine period they were inoculated intranasally with 100 $TCID_{50}$ of coronavirus 229E contained in a filtered pool of nasal washings. They left the unit after a further 6 days but provided blood samples by post 2 and 11 weeks later. In addition, six volunteers returned for unrelated trials after about 1 year and thus provided further samples.

Extended trials were then organized in which volunteers stayed for 3 weeks after virus inoculation and frequent nasal washing and blood samples were taken. In addition, a blood sample was sent to the unit 8 weeks after inoculation and volunteers attended the unit at 11 weeks for further nasal washing and blood samples to be taken. At about 52 weeks, volunteers returned for a standard challenge trial in which coronavirus 229E was again inoculated. All these trials were approved by the Harrow District Ethical committee.

Nasal washings

Collection and treatment of nasal washings have been described elsewhere [22]. Briefly, 10 ml of phosphate buffered saline (PBS) were instilled into the nostrils in several aliquots and forcibly expelled. For antibody and protein measurement, washings were vortexed with glass beads, clarified by centrifugation, and stored at −20 °C. Nasal washings for virus isolation were mixed with nutrient broth and stored at −70 °C. Virus was detected by inoculation of roller tube cultures of the C-16 line of MRC-C cells [23].

Blood samples for enumeration of lymphocyte sub-sets

These were taken before, and 4 and 10 days after, virus inoculation. White cells were separated by standard methods, using lymphocyte separation medium (Flow Laboratories) and smears were prepared in a Cytospin 2 (Shandon). T-cells, B-cells and helper/suppressor cells were labelled by immuno-alkaline phosphatase staining as described previously [24]. Total lymphocytes were counted by standard methods.

Blood samples for serum were separated and sera were stored at −20 °C.

Neutralizing antibody titres were measured by a micro-neutralization test [21] in C-16 cells.

Protein assays

These were performed by a modification of the method of Lowry and colleagues [25] where, in the final step, samples were removed to ELISA plates and the optical densities (OD) read at 700 nm in a Dynatech MR700 ELISA reader.

Assay of specific IgA in nasal washings by ELISA

ELISA plates (Nunc Immunoplates U type II) were coated with coronavirus 229E or control antigen as described previously [12]. After washing three times with PBS containing 0·05 % Tween 20 (TPBS), the plates were blocked with 0·5 % bovine serum albumen (BSA) for 1 h at 37 °C. After washing the plates nasal washings, diluted 1:10 in TPBS containing 10 % control antigen and 0·1 % BSA, were added and incubated for 5 h at 4 °C. Goat anti-human IgA (α chain) conjugated to alkaline phosphatase (Sigma Chemical Co.), diluted 1:1000, was added and incubated at 4 °C overnight. The substrate, p-nitrophenol phosphate, was added as described previously [12]. A standard pool of nasal washings given an arbitrary concentration in units was titrated on each assay plate [22].

Assay of specific antibodies in sera

Sera were diluted 1:80 for specific IgA and 1:240 for IgG assay, using the same diluent as for nasal washings. Plates were coated and blocked as before except that, for specific IgG measurement, control antigen-coated wells were not used as these always gave very low readings. Sera were incubated for 2 h at 37 °C. Anti-IgA conjugate, or anti-IgG conjugate (Miles Laboratories), diluted 1:1000 was added and incubated for 2 h at 37 °C and substrate was added as before. A pool of standard serum, given an arbitrary concentration in units, was titrated on each plate, except in some experiments where results are quoted as OD.

Reading the plates and calculation of results

Plates were read on a Titertek Multiskan ELISA reader (Flow Laboratories) at 405 nm. For specific IgG assays, plates were read at 20 and 30 min, and for specific IgA at 60 and 75 min. The ELISA reader was linked to a Sirius 1 microcomputer, which ran a program for collection of data, calibration of standards and calculation of the results. All the following calculations were performed by the program which was written by Clive Thomas of the Clinical Research Centre, Harrow, Middlesex. Except in the case of the specific IgG assay, the OD in control

antigen-coated wells were subtracted from those in the virus-coated wells. All samples and standards were run in duplicate and means were calculated. The concentration in units of the samples was calculated from the calibration curve of the standards. The final concentration of antibody was calculated from the mean of readings taken at two different times.

Specific IgA in nasal washings was standardized against protein concentration [1]. Concentrations of protein in any one individual remain fairly constant from day to day [4; Callow, unpublished observations].

The method used to establish a significant rise in serum specific IgG has been described previously [12, 22]. Paired sera from 14 volunteers who received intranasal saline instead of virus were tested for specific IgG as described above. A significant rise was set as the mean difference between these paired sera plus two standard deviations, i.e. $0.14 \log_{10}$ units in this study.

Statistical methods

The analysis was performed on an IBMXT computer using the program, Statistical Package for Personal Computers (SPP) written by Patrick Royston of the Clinical Research Centre. To examine the difference in antibody concentration on different days, in particular on any day compared with the pre-challenge value, and to compare antibody concentrations in infected and uninfected volunteers, t-tests were used.

Treatment of missing values

There were a few instances when samples were not available for investigation. In these cases, for the graphical representation of results, an estimate of the value of single missing samples was obtained by interpolation from the value of the samples taken just before and after the missing ones. This estimate was not used for statistical analysis.

Field study

Serological response to natural coronavirus infection was investigated by testing sera obtained in a field study of influenza prophylaxis described elsewhere [19]. Briefly, this involved military personnel volunteers who were given either influenza vaccine, amantadine (during influenza epidemics) or placebo prophylactically. Respiratory tract illnesses were recorded over a 3-year period, and infections with common respiratory viruses diagnosed by isolation from nose or throat swabs, and/or by seroconversion. Serum samples were obtained at the beginning of the study, at 4–6 week intervals thereafter, and in the acute and convalescent stages of any respiratory illness.

RESULTS

Preliminary trials

Neutralizing antibodies and specific IgG and IgA in sera were monitored during and after infection with coronavirus 229E in 16 volunteers on standard 10-day trials. Although concentrations of all three types of antibody were significantly raised in sera taken about 3 weeks after inoculation, by 12 weeks the concentrations had fallen considerably. Fig. 1 shows these effects in six volunteers
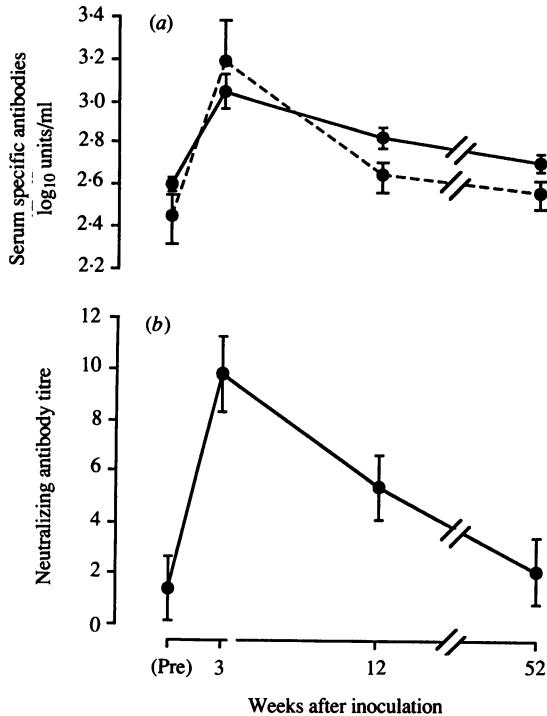
Fig. 1. Changes in concentration of serum antibodies (geometric mean) of six volunteers infected with coronavirus 229E in preliminary trials. Bars represent the standard error of the mean. (a) Specific IgG ●——●; Specific IgA ●----●. (b) Neutralizing antibody titres.

from whom serum samples were also available 1 year later. By this time mean antibody concentrations were only slightly higher than those measured before the trial. There were considerable individual differences however. Two volunteers had retained high concentrations of specific IgG, the others had returned to their baseline values or just above.

With this preliminary information extended trials were planned to obtain details of changes in both serum and nasal antibody in the first 3 weeks after virus inoculation as well as long-term changes.

Extended trials

(a) Infection and illness resulting from coronavirus inoculation

Of 15 volunteers inoculated with coronavirus, 10 became infected as indicated by virus shedding on at least 1 day. All of these also had a significant rise in serum specific IgG. Eight of the 10 infected volunteers had clinical colds of varying severity. For the purposes of this study the volunteers were divided into two groups, an infected and an uninfected group.

(b) Changes in total blood lymphocytes

The mean total lymphocyte count before challenge was higher in the infected group than the uninfected group, although this difference was not statistically significant (Table 1). In the infected group there was a significant decrease in

Table 1. *Mean concentration ($\times 10^{-9}/l$) of total blood lymphocytes in volunteers inoculated with coronavirus 229E*

	Days after challenge		
	Pre	4	10
Infected volunteers	2·41	2·13*	2·55*
Uninfected	2·02	2·10	2·17

* Significantly different ($P < 0.05$) from previous value.

counts 4 days after virus inoculation ($P < 0.05$), 8 out of the 10 showing lymphocytopenia of up to 25%. There was a significant increase again 1 week later. There appeared to be no relationship between these changes and the severity of symptoms. No other parameters, i.e. %T or B cells or helper/suppressor ratios were significantly changed in either group (data not shown).

(c) Changes in nasal-washing IgA

Mean concentrations of specific IgA, standardized relative to protein concentrations, in the two groups are shown in Fig. 2a. Similar curves were obtained if concentrations were not standardized, or standardized relative to total IgA concentrations. The infected volunteers entered the trials with a significantly lower concentration of nasal antibody, 74 units/mg protein compared with the uninfected volunteers, 398 units/mg ($P < 0.05$). The day after inoculation there was an immediate increase in the amounts of antibody in both groups compared with those existing before the trial. In the case of the uninfected volunteers this increase was significant at $P < 0.05$. After some fluctuations in this group, the concentrations slowly declined and by 11 weeks they were slightly below those existing at the start of the trial. In contrast the infected group showed an initial decrease of antibody concentration, in some subjects becoming undetectable. This was followed by a sharp increase starting about 1 week after challenge, which coincided with the end of virus shedding in some subjects, while in others it occurred after virus shedding stopped (see legend to Fig. 2). After reaching a maximum about 10 days later the concentration slowly declined, and although from 12 days to 11 weeks after inoculation concentrations were significantly higher than before the trial, by 11 weeks the mean concentration was less than in the uninfected group.

No significant changes in coronavirus specific IgA concentrations were seen in nasal-washings of four volunteers inoculated with saline or of five inoculated with rhinovirus (data not shown).

(d) Changes in serum specific IgA

The uninfected group had more serum specific IgA (120 units/ml) before inoculation than the infected group (38 units/ml) ($P < 0.05$, Fig. 2b). Although there were minor fluctuations, the concentrations remained fairly constant in the former group throughout. In contrast the infected group showed a sharp increase about 8 days after inoculation. From 10–20 days after inoculation concentrations were significantly higher than before but after a peak at 14 days, specific IgA declined and by 8 weeks was slightly less than in the uninfected group. There were
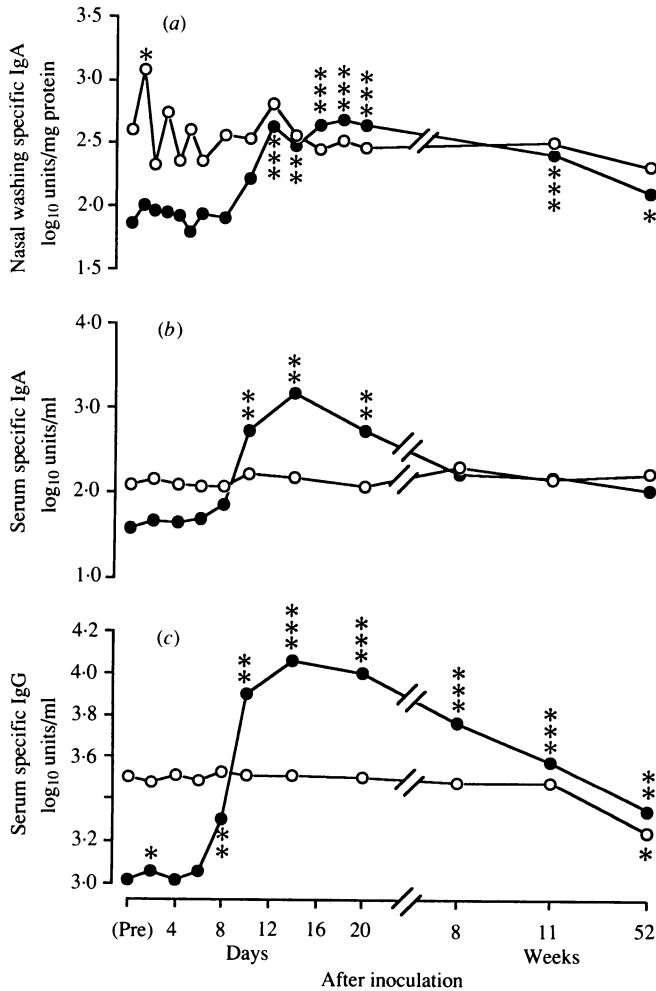
Fig. 2. Changes in specific antibodies up to 1 year after coronavirus 229E inoculation. Geometric mean concentration in uninfected (○) and infected (●) volunteers. Significance of difference from pre-inoculation values: $***P < 0.001$, $**P < 0.01$, $*P < 0.05$. All these significant differences represent increases except for IgG 1 year after inoculation in uninfected volunteers where there was a significant decrease. (a) IgA in nasal washings. (The percentage of infected volunteers shedding virus was: 1 day after inoculation, 10%; 2 days, 80%; 3 days, 90%; 4–6 days, 100%; 8 days, 50%; 10 days, 20%; 12 days, 0%. (b) IgA in sera. (c) IgG in sera.

considerable individual differences in both groups. For example, two uninfected volunteers maintained high concentrations throughout the period, while two infected volunteers showed no significant rise in serum specific IgA. In 4 of the remaining 8 infected volunteers, concentrations had returned to pre-inoculation values by 8 or 11 weeks.

(e) *Changes in serum specific IgG*

The uninfected group had more specific IgG in pre-inoculation sera (3162 units/ml) than the infected group (1047 units/ml) ($P = 0.05$), and in the former group the mean concentration remained remarkably constant throughout the trial

(Fig. 2c). The infected group had an immediate slight increase ($P < 0.05$) in antibody titres followed by a decrease. A sustained rise began about 8 days after inoculation, reaching a peak at 14 days. Thereafter there was a slow decline, and although between 8 days and 11 weeks after inoculation concentrations were significantly higher than at the start of the trial, at 8 and 11 weeks they were not significantly higher than in the uninfected group.

(f) Antibody concentration 1 year after challenge

All of the volunteers, except for one infected individual were able to return to the unit approximately 1 year later.

Between 11 weeks and 1 year after challenge, nasal washing specific IgA appeared to decline in both groups (Fig. 2a). However the uninfected group showed no significant difference from before the trial, and, while in the infected group concentrations were still significantly raised ($P < 0.05$) compared with before the trial, they were now lower than in the uninfected group. Serum specific IgA showed no significant changes (Fig. 2b). In the infected group the mean concentration was still slightly raised compared with before the trial, but concentrations had become slightly lower than in the uninfected group.

Serum specific IgG declined in both groups (Fig. 2c). However, while concentrations in the uninfected group were significantly lower than before the trial ($P < 0.05$), in the infected group concentrations were still significantly higher than before the trial ($P = 0.01$). However in one infected volunteer the concentration of this antibody was lower than before the trial, and in another had almost returned to its original value.

(g) Results of re-challenge

Volunteers were re-challenged with the same dose of coronavirus. Of the original uninfected group of five, all became infected as judged by virus shedding on at least one day and/or a significant rise in serum specific IgG and one had a cold. Of the original infected group, 6 out of 9 became reinfected, using the same criteria, but none developed a cold.

One distinguishing feature of the two sets of trials was that virus shedding was of a much shorter duration, in both groups, on re-challenge. In those that shed virus at all, the mean duration was 5.6 days in the first trial, and 2.0 days in the second.

Field study – changes in serum specific IgG in natural infections

Serial serum samples had been taken over 2 years from 11 subjects in a field study of respiratory disease in servicemen in Canada. Infections with either coronavirus 229E or OC43 were detected by rises in serum specific IgG against one or the other serotype. Three subjects showed a decrease in antibody to 229E, as measured by OD in the ELISA test, similar to that seen in volunteer trials (Fig. 3a). Eight subjects infected with OC43 were also followed up. Despite individual variations, all showed a distinct decline in antibody after convalescence (Fig. 3b, c). In two subjects from whom later samples were available, antibody returned to pre-infection values by 33 and 50 weeks respectively (Fig. 3c).
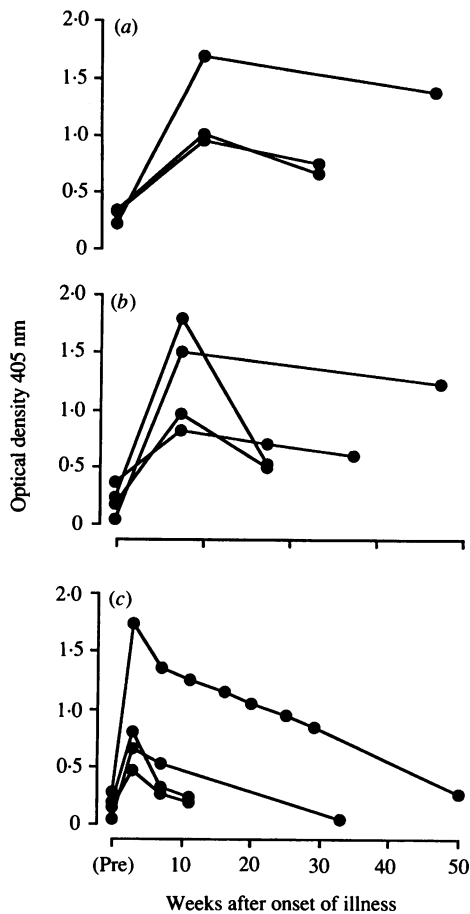
Fig. 3. Changes in concentration of serum specific IgG in individual subjects naturally infected with coronavirus, as measured by ELISA optical density. (a) Infected with the serotype 229E. (b, c) Infected with the serotype OC43.

DISCUSSION

This extended trial represents the most detailed study yet reported of immune responses to human coronavirus infections. It shows that the immunological changes during and after coronavirus infection, are in some respects like those which follow other respiratory infections such as rhinovirus infections, while in other respects they are different.

As in infections with rhinovirus [15] and influenza [16–18], lymphocytopenia occurred in the early stages of infection. It is thought that this reduction may reflect migration of lymphocytes to the nasopharyngeal site of infection [15, 17]. However in contrast to these other studies the reduction was transient and slight; a mean decrease of 11·6% compared with 28% in rhinovirus infection [15]. Furthermore there was no relationship between the amount of decrease and symptom scores, and no significant changes in the proportions of T-cells, B-cells or T-cell subsets, as was shown in other infections. If we had been able to take

more frequent samples we might have detected a greater decrease on another day, together with changes in T- or B-cells. Further work should determine whether this is the case.

The initial sharp, transient increase in local antibody in the uninfected group has been observed previously in coronavirus infections (Callow, unpublished observations). One can discern a similar, but slightly later, peak in the relevant figure in the study of Barclay and colleagues [11] of rhinovirus infections. Butler and co-workers [2] recorded an increase in total IgA in nasal secretions 24 h after rhinovirus inoculation. Until now, sufficiently sensitive methods for recording such changes in specific antibody have not been available. The increase may represent an anamnestic response resulting from previous infections, being a specific discharge from loaded cells, or it may have resulted from an as yet unrecognized mechanism. The later steep rise in concentration of nasal antibody in the infected group coincided with, and may have been the reason for, the cessation of virus shedding.

Maximum concentrations of both circulating and local antibody occurred at 12–14 days. In rhinovirus infections [11] the rise and particularly the maximum (35 days) are later. After this maximum there was quite a steep decline, whereas in rhinovirus infection there is no decline. Barclay and colleagues [11] suggest that this may be due to repeated stimulation by related cold viruses. Since there are only two sub-types of coronaviruses, such stimulation would be less likely to occur.

Although after 1 year concentrations of specific serum IgG and nasal IgA were still significantly raised in the infected group, they were much lower than at the maximum. This latter observation confirmed that seen in preliminary trials.

When challenged after 1 year all of the original uninfected group were infected and 6/9 of the infected group were reinfected. These data do not fit any simple model. It may be that the small amounts of antibody remaining in the original infected group contributed to resistance to reinfection in some volunteers. It may also have prevented colds and shortened the duration of virus shedding. The original uninfected group also shed virus for only a short time and only one volunteer got a cold. Their mean antibody concentrations were still higher than in the infected group before the first trial. They could have had an intervening coronavirus infection or perhaps tend to have higher concentrations of specific antibody at any time. This could be because they produce more antibody as a result of infection or, as appears more likely from this study, they retain antibody for longer. Further study of peak concentrations and rates of decline is necessary to ascertain which is the case. In the infected group there were considerable individual differences. Some lost their antibody, particularly serum antibody, completely by 1 year, some did not. Such differences are also seen in rhinovirus infections [11]. They indicate that resistance may be a more inherent characteristic than is generally supposed. It is known that extroversion, obsessionality and other essentially permanent psychological characteristics seem to be related to infection rates [26, 27]. We are now studying links between such characteristics and antibody concentrations. Other factors must be analysed as well; for example, the cellular arm of the immune system may play a role.

The incidence of reinfection after 1 year was greater than that observed with

rhinovirus infection [11] and did not support Reed's study [14] in which reinfection with a homologous coronavirus strain after 1 year did not occur. Also, antibody was still declining, albeit slowly. Coronaviruses have been shown to cause almost 20 % of all colds [28] and since the two main serotypes are roughly equally prevalent (Callow, unpublished observation), any one serotype would cause about 10 % of all colds. Since adults have about two colds per year on average [29, 30] and about half of all coronavirus infections are subclinical [31, 32], each adult should have a coronavirus infection every 2–3 years. This agrees with Monto [33] who suggested a 2–3 year cyclic pattern. It indicates that protective amounts of antibody may have disappeared by 2 years, and that if we had been able to reinoculate the volunteers after a further year, the reinfection rate would have been even higher.

In the few natural infections studied a marked decline in antibody was also observed, but a strict comparison with the size and speed of decline in volunteers is impossible because the results were obtained using OD rather than antibody concentrations. Furthermore, data for an extended period were only obtained for the strain OC43 and only on a few individuals. Again large individual differences were observed.

The observed differences from rhinovirus infection may simply reflect differences in the number of serotypes. However they may also be due to differences in the structure of the viral epitopes and the frequency and nature of their interaction with the immune system.

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