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The time course of the humoral immune response to rhinovirus infection

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SUMMARY

The specific humoral immune response of 17 volunteers to infection with human rhinovirus type 2 (HRV-2) has been measured both by neutralization and by ELISA. Six volunteers who had HRV-2-specific antibodies in either serum or nasal secretions before HRV-2 inoculation were resistant to infection and illness. Of the remaining 11 volunteers who had little pre-existing HRV-2-specific antibody, one was immune but 10 became infected and displayed increases in HRV-2-specific antibodies. These antibodies first increased 1–2 weeks after infection and reached a maximum at 5 weeks. All six resistant volunteers who had high pre-existing antibody and eight of the volunteers who became infected maintained their HRV-2-specific antibody for at least 1 year. At this time they were protected against reinfection. Two volunteers showed decreases in HRV-2-specific antibodies from either serum or nasal secretions. They became infected but not ill after HRV-2 inoculation 1 year later.

INTRODUCTION

Rhinoviruses are the major etiologic agents of the common cold (1). Following rhinovirus infection there is a specific humoral immune response which can be detected in both serum (2) and nasal secretions (3) by measuring rhinovirus type-specific neutralizing antibodies. Since neutralizing antibody is not detected until 14 days after infection (4), recovery from illness, which occurs within 7–10 days of infection, is probably mediated by other agents. On the other hand, resistance to illness and infection has been associated with high pre-existing neutralizing antibody titres (4–7), a finding which we have recently confirmed (8).

Serum-neutralizing antibody titres remain elevated for many years after infection (2, 9, 10). However, local specific antibody appears to be lost with time: that produced after intranasal rhinovirus vaccination was lost after 2 years (11). This rapid decrease would account for the high proportion of volunteers who

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present with serum specific antibody but who lack nasal secretion antibody (W. S. Barclay, unpublished data and (8)).

We have recently described an enzyme-linked immunosorbent assay (ELISA) which detected rhinovirus specific antibodies in both sera and nasal secretions of volunteers who had been infected with a rhinovirus (12). An evaluation of this ELISA has shown that it is more sensitive and reliable than the traditional neutralization test (8). Therefore we have used this new assay to document in detail the time course of the humoral immune response to rhinovirus infection. We describe here the response of volunteers who were infected with HRV-2 and identify the earliest and peak times that changes in specific antibodies in sera and nasal secretions were detected. In addition, the response of these volunteers to re-inoculation with the same virus after one year was documented and related to the persistence of the antibody acquired the previous year.

The high frequency of rhinovirus infections experienced by healthy adults has been attributed to infections with each of many distinct rhinovirus serotypes rather than repeated infections with the same serotype (see ref. (13)). In the present study we provide evidence that reinfection with the same rhinovirus occurred in some individuals but not in others, and we consider these differences in the light of differences in specific immune response.

MATERIALS AND METHODS

Human volunteers

Twenty-one adult volunteers were housed in isolation for 24 days according to standard procedures at the Common Cold Unit (14). Seventeen volunteers were inoculated intranasally with $10^{4.1}$ $TCID_{50}$ human rhinovirus type 2 (HRV-2) and the remaining four volunteers received an intranasal inoculation of saline. Volunteers were visited daily and assessed for the appearance of symptons and signs associated with a common cold. On this basis they were allotted a clinical score (see ref. (14)). Serum samples were collected 3 days and 1 day before inoculation, and 1, 3, 5, 8, 11, 14 and 20 days after inoculation. Nasal washing samples were collected each day for 3 days before inoculation and for 12 days after inoculation and thereafter on every other day until 20 days after inoculation. These were processed and stored as previously described (8) so that the same sample could be used for both virus isolation and local antibody measurement. For analysis of the immune response to rhinovirus infection those volunteers inoculated with HRV-2 were divided into three groups depending on their response to virus challenge assessed by virus isolation from nasal washings and by their clinical score. These groups were:

'colds' group – consisting of volunteers from whom virus was isolated and who showed symptoms and signs;
'virus shedders' – consisting of volunteers from whom virus was isolated but who showed no symptoms or signs;
'non-shedders' – consisting of volunteers from whom no virus was isolated and who showed no symptoms or signs.

Enzyme-linked immunosorbent assay (ELISA) for rhinovirus-specific immunoglobulins

Sera and nasal washing samples were tested for HRV-2-specific immunoglobulins by ELISA. This was essentially as previously described (8) except that the antigen was a purified fraction of HRV-2 which contained native virus particles rather than the mixture of full particles and empty capsids in crude tissue culture fluid harvests which was used previously. Use of purified full particles in the ELISA has been found to give a more serotype-specific response (W. S. Barclay, unpublished data). For the present study 10^9 p.f.u. HRV-2 particles diluted 1:500 in PBS containing 1% bovine serum albumin (BSA) were used as antigen. Sera and nasal washing samples were diluted $1:10^{2.5}$ and $1:10^{1.0}$ respectively in the same diluent. The antihuman IgG conjugate was the same as previously described (12) but the antihuman IgA was conjugated to horseradish peroxidase (Sigma) and used at 1:1000 dilution in the same diluent as above. The substrate was 3:3:5:5′-tetramethylbenzidine (TMB, Sigma) in 0·1 M sodium citrate buffer. This reaction was stopped after 5 min with 2·5 M-H_2SO_4 and the optical density of the product read at 450 nm with a reference filter of 550 nm. For each plate a standard curve was constructed from the titration of a known positive. The pool of positive sera was allotted 5·0 $\log_{10}$ antibody units, and the positive nasal washing pool 3·0 $\log_{10}$ antibody units. Thus, by reading off on the standard curve optical densities given by the samples, each was allotted a number of $\log_{10}$ antibody units. This method relies on the titration curves of the test samples being parallel to that of the positive sample, and this was confirmed for several serum samples chosen at random (data not shown).

Microneutralization test

Serum samples were tested for HRV-2 neutralizing antibodies as previously described (12). All samples from one volunteer were tested on the same microtitre plate, and in duplicate. A virus control and cell control were included on each plate. Any cytopathic effect was read microscopically after 5 days incubation at 33 °C and confirmed by fixing cell sheets in 10% citric acid and staining with crystal violet.

The volunteer experiments were undertaken with prior approval from the Harrow District Ethical Committee.

RESULTS

Response to HRV-2 inoculation

Of the 17 volunteers who received intranasal HRV-2 inoculation, 6 (35·3%) developed common cold symptoms, and virus was isolated from nasal washings of all of them. In addition, virus was isolated from nasal washings of 4 of the 11 asymptomatic volunteers. Thus these 4 volunteers (24·2%) were the 'shedders' whilst 7 (41·5%) were the 'non-shedders'.

The days on which virus was isolated from nasal washings of volunteers with colds are shown in Fig. 1. The majority of isolates were recovered between the first and the tenth day. However, two of these volunteers were still excreting infectious

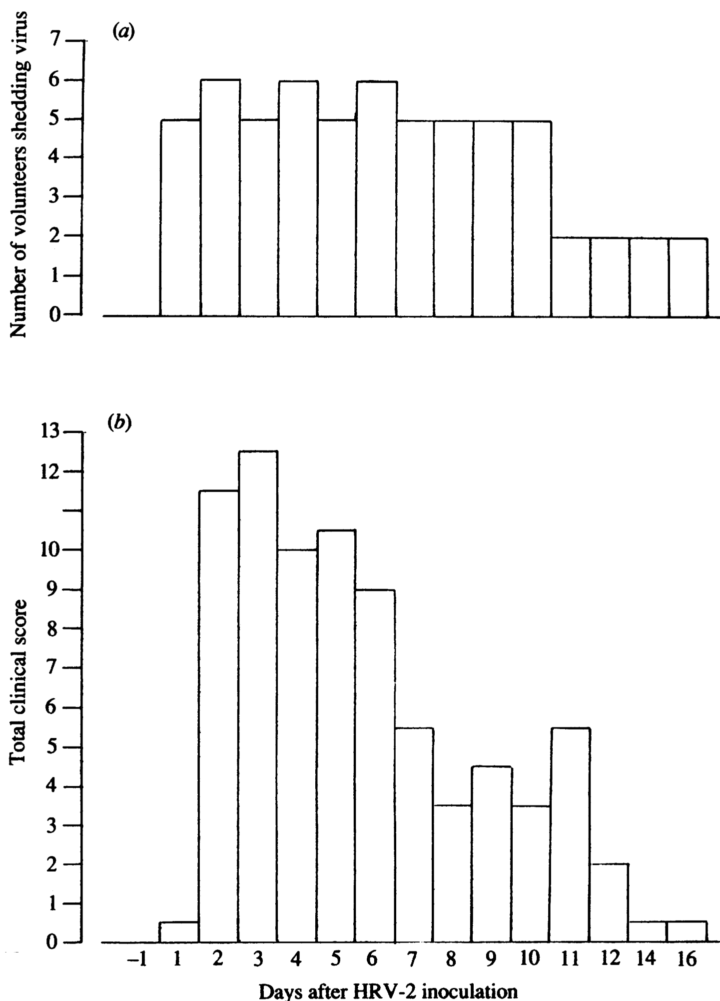

Fig. 1. (*a*) The number of volunteers with colds from whose nasal washings virus was isolated on each day after HRV-2 inoculation. The total number of volunteers with colds was six. (*b*) The total clinical score of volunteers with colds on each day after HRV-2 inoculation.

virus 16 days after inoculation even though their symptoms had ceased several days before.

All the viruses isolated from volunteer nasal washings were typed as HRV-2 by neutralization with a rabbit anti-HRV-2 hyperimmune serum.

The appearance of HRV-2-specific antibodies in sera

The geometric mean titres of HRV-2-specific IgG and IgA in consecutive serum samples from the three groups of volunteers who received intranasal inoculation are shown in Figs 2 and 3. Also shown are mean specific antibody titres of the group of four volunteers who received a saline inoculum. The time course of the response to virus inoculation was very similar for the two classes of immunoglobulins.

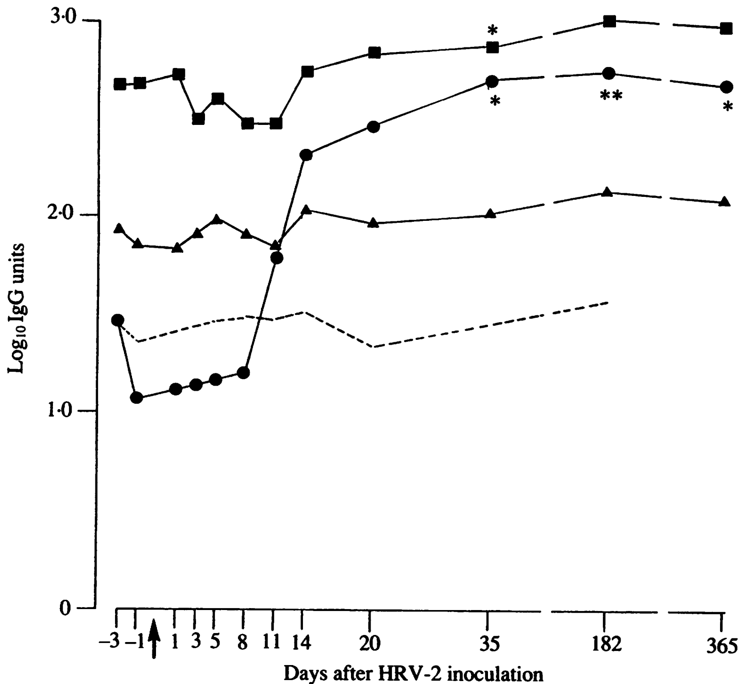

Fig. 2. HRV-2-specific IgG in sera after HRV-2 or saline inoculation (↑). Geometric mean titres are shown for volunteers with colds (●) $n = 6$; volunteers who were 'shedders' (■) $n = 4$, volunteers who were 'non-shedders' (▲) $n = 7$ and volunteers who received saline inoculum (---) $n = 4$. Significant rises over pre-inoculation titres are indicated as $*P < 0{\cdot}05$ and $**P < 0{\cdot}001$.

In agreement with previous findings (8), the volunteers who developed colds had very little HRV-2-specific serum antibody before inoculation. The mean IgG titre was 1·1 $\log_{10}$ units and for IgA this figure was 0.7 $\log_{10}$ units. By 35 days after infection these titres had increased to 2·7 and 2·2 $\log_{10}$ units for IgG and IgA respectively. Although inspection of Figs. 2, 3 and 4 would suggest that these increases began as early as day 8, in fact they did not become statistically significant until later due to the spread of data and the low number of volunteers. One year after inoculation the mean HRV-2-specific serum IgG titre was the same as that at day 35. The mean IgA titre had begun to decline after 35 days to 2·0 $\log_{10}$ units at 6 months and by 1 year after infection was 1.9 $\log_{10}$ units.

The mean serum antibody titres of volunteers who shed virus but were asymptomatic were relatively high before inoculation: the IgG titre was 2·7 $\log_{10}$ units and the IgA titre was 1·7 $\log_{10}$ units. After asymptomatic infection antibody titres increased and by 21 days the titres of both HRV-2-specific IgG and specific IgA were very similar in sera from the two infected groups. As in the 'colds' group, the specific IgA titres in sera of subclinically infected volunteers began to decline by 6 months after infection, whereas the specific IgG titre remained high.

Neither of the groups of volunteers who did not shed virus, whether they had received virus or saline inoculum, showed any change in HRV-2-specific serum IgG or IgA throughout the length of the study (Figs 2 and 3). For the 'non-shedders' group the specific IgG titre was around 2·0 $\log_{10}$ units and the specific

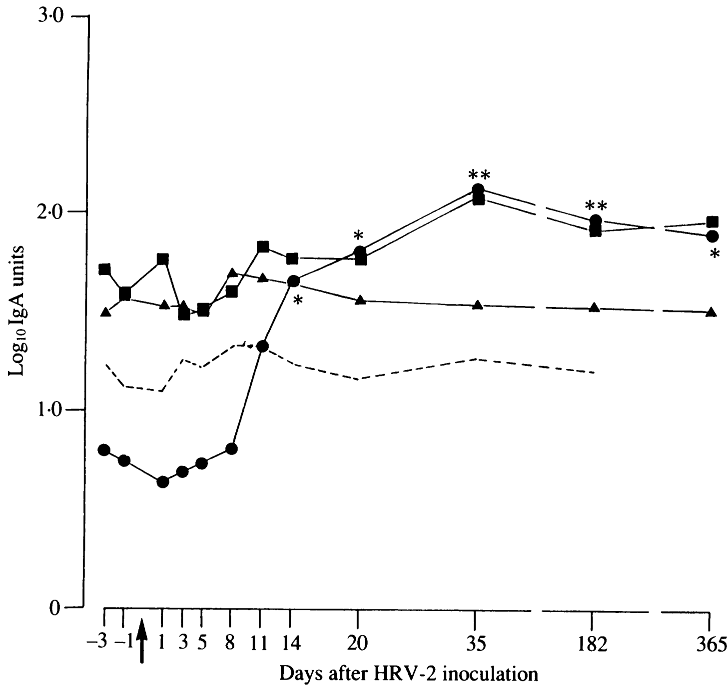

Fig. 3. HRV-2-specific IgG in sera after HRV-2 or saline inoculation (↑). Geometric mean titres are shown for volunteers with colds (●) $n = 6$; volunteers who were 'shedders' (■) $n = 4$, volunteers who were 'non-shedders' (▲) $n = 7$ and volunteers who received saline inoculum (---) $n = 4$. Significant rises over pre-inoculation titres are indicated as $*P < 0{\cdot}05$ and $**P < 0{\cdot}001$.

IgA titre remained at about 1·7 $\log_{10}$ units. In addition, the mean neutralizing antibody titre appeared approximately the same in these groups although statistical analysis did reveal some significant changes which we find hard to explain (Fig. 4). In contrast, increases in neutralizing antibody titres in the sera of the 'colds' group did not become statistically significant until 20 days after inoculation. Thereafter, the increase in serum HRV-2 neutralizing antibody titres for volunteers with colds coincided with the changes in HRV-2-specific IgG and IgA antibodies measured by ELISA, reaching mean peak titres of 2·4 $\log_{10}$ units 35 days after infection and declining to 1·9 $\log_{10}$ units by 1 year.

Individual volunteers within the groups showed differences in the class of immunoglobulins they produced in response to HRV-2 infection. For example, one of the volunteers with a cold showed a maximum IgG increase of only 0·78 $\log_{10}$ antibody unit 182 days after infection whereas his IgA increase was 1·82 $\log_{10}$ antibody units by 35 days after infection. In contrast, another symptomatic volunteer, had maximum rises of 2·54 $\log_{10}$ IgG units at 21 days but only 1·2 $\log_{10}$ IgA units at 11 days.

The appearance of HRV-2-specific antibodies in nasal secretions

HRV-2-specific IgG in nasal washings was low and did not change during the course of the study (data not shown). Therefore the local specific immune response described below refers only to the IgA class of immunoglobulins.

The pre-inoculation titres of specific IgA antibody in nasal washings were lowest

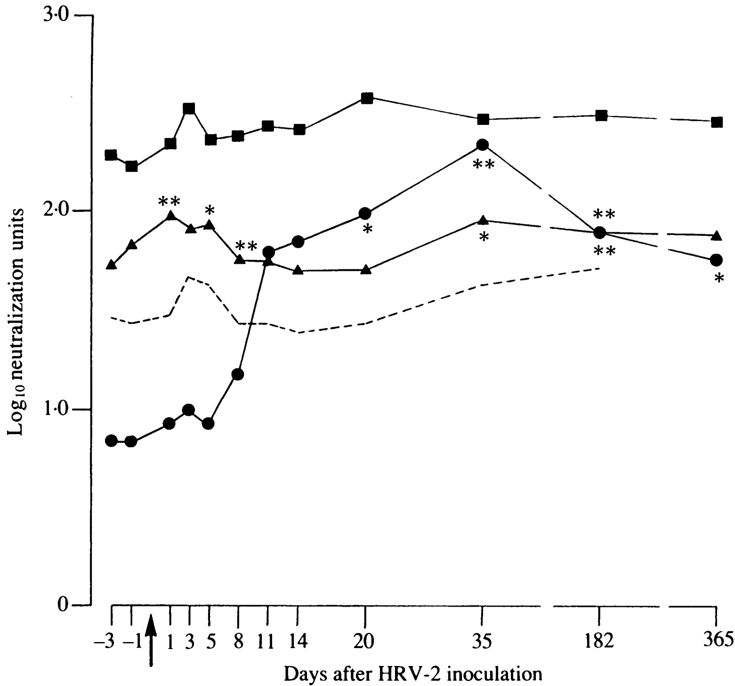

Fig. 4. HRV-2 neutralizing antibody in sera after HRV-2 or saline inoculation (↑). Geometric mean titres are shown for volunteers with colds (●) $n = 6$; volunteers who were 'shedders' (■) $n = 4$, volunteers who were 'non-shedders' (▲) $n = 7$ and volunteers who received saline inoculum (---) $n = 4$. Significant rises over pre-inoculation titres are indicated as $*P < 0{\cdot}05$ and $**P < 0{\cdot}001$.

(0·9 $\log_{10}$ unit) for the volunteers who went on to develop colds. Volunteers who were shedders also had low nasal washing antibody before inoculation (1·1 $\log_{10}$ units), whereas volunteers who did not become infected (non-shedders) had higher specific IgA in nasal secretions before inoculation (1·8 $\log_{10}$ units). These levels were similar to those found in three corresponding groups of volunteers in a previous study (8). Thus for the purposes of the analysis of HRV-2-specific antibodies in nasal secretions, both groups of infected volunteers, those with and those without colds, were considered together as an 'infected' group.

The amounts of local specific antibody detected by ELISA after HRV-2 inoculation fluctuated much more than those of serum antibody (Fig. 5). Attempts to standardize antibody content of nasal washings in terms of total IgA or total protein did not make the results any easier to interpret (data not shown). None of the post-inoculation mean antibody levels were significantly different from the pre-inoculation level (by Student's paired t test). However it is worth noting that over the first week after inoculation there was a consistent decrease in the specific IgA detected in nasal washings of those volunteers who possessed pre-inoculation antibody (data not shown). In addition there was a specific IgA increase in nasal washings of infected volunteers which began on day 10 and continued to rise for at least 6 days after that. In fact one year after inoculation, the mean titres of local specific IgA for volunteers who had been infected and volunteers who had not were the same (2·38 and 2·40 units/ml, respectively) (Fig. 5).

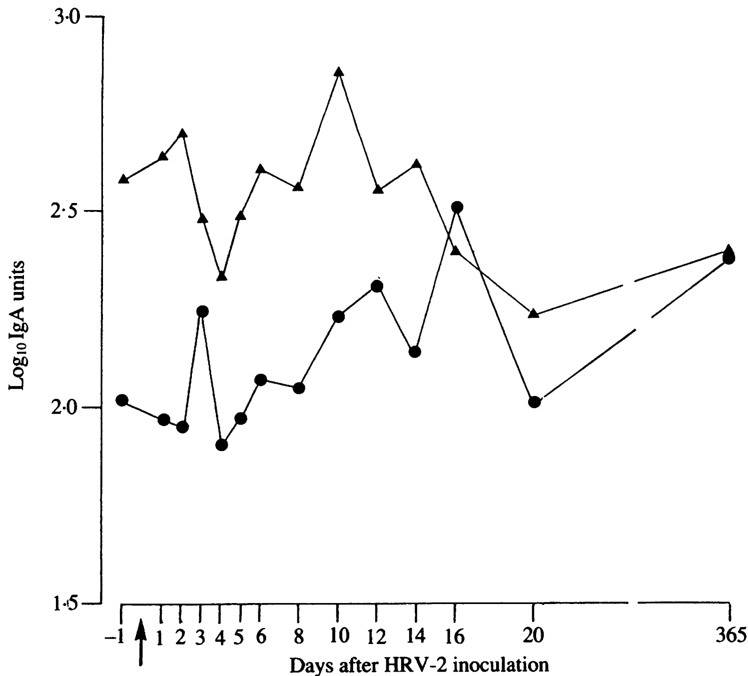

Fig. 5. HRV-2-specific IgA in nasal secretions after HRV-2 inoculation (↑). Geometric mean titres are shown for volunteers who were infected (●) $n = 10$ and for volunteers who were 'non-shedders' (▲) $n = 7$.

Response of volunteers to reinoculation with HRV-2

Sixteen of the 17 volunteers who had received intranasal HRV-2 inoculation agreed to return after approximately 1 year for reinoculation with the homologous virus.

Seven of the nine volunteers who had been infected and had mounted an antibody response to HRV-2 had maintained that concentration of antibody for at least 1 year. Indeed, all these volunteers also had higher titres of local HRV-2-specific IgA after one year than they had before the first inoculation. None of these seven volunteers developed colds or became infected after the second HRV-2 inoculation. However, the remaining two volunteers, both of whom had developed colds after the first inoculation, did become subclinically infected after the second HRV-2 challenge as indicated by virus shedding. One of these volunteers had maintained high titres of neutralizing antibody, but the HRV-2-specific IgA titres in serum measured by ELISA were lower than those for the rest of the group. After the second infection with HRV-2 this volunteer showed greatly enhanced HRV-2-specific antibody responses (Fig. 6*a*). The second volunteer who became reinfected with HRV-2 had mounted good serum antibody responses to the first infection but had not maintained these well (Fig. 6*b*). In addition, there had been very little local antibody response by this volunteer to the first infection so that the HRV-2-specific IgA in nasal secretion was lower before the second inoculation than it had been before the first (data not shown). After the second infection, the HRV-2-specific antibody responses in serum and nasal secretions of this volunteer were no larger than they had been after the first infection (Fig. 6*b*).

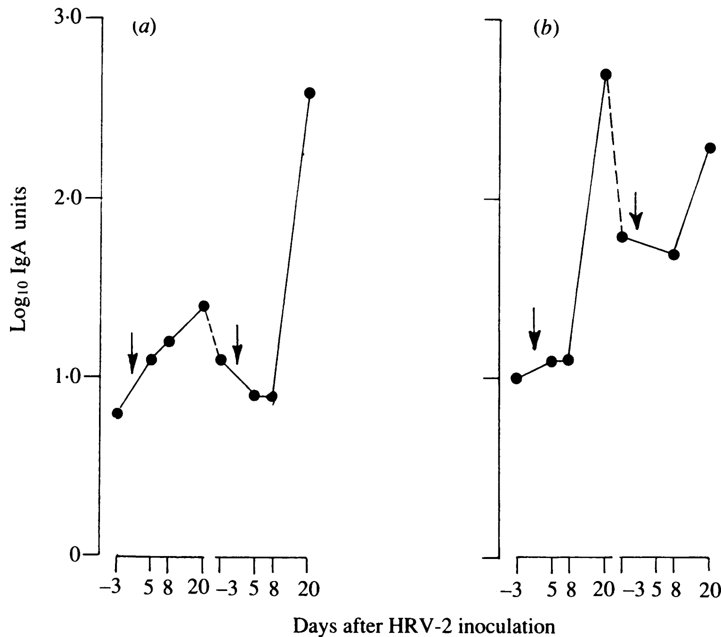

Fig. 6. HRV-2-specific IgA in serum of two volunteers (*a*) and (*b*) after two HRV-2 inoculations (↓) separated by an interval of 1 year (---). Both volunteers developed a cold after the first HRV-2 inoculation and became infected but not ill after the second.

Volunteers who had not become infected after the first HRV-2 inoculation also did not become infected after the second with the exception of one individual who developed a cold. This individual had not displayed high antibody titres the previous year and it is possible that his resistance to the first HRV-2 inoculation was non-specific for HRV-2 and thus short lived (15). His antibody titres were as low before the second HRV-2 inoculation as the preinoculation titres of the six volunteers who had developed colds the previous year.

DISCUSSION

In the present study rhinovirus specific antibodies did not increase in sera or nasal secretions until 1–2 weeks after infection, by which time common cold symptoms had declined. Furthermore, most infected volunteers had ceased to shed virus in nasal washings by day 10. Thus it seems that clearance of virus and recovery from illness were probably mediated by factors other than the humoral immune system. In addition, two volunteers who had high titres of HRV-2-specific IgA in nasal secretions by 16 days after infection were still excreting virus. This demonstrates that possession of antibody was not sufficient to eliminate the infection. However it is possible that these late virus isolates were a selected population resistant to the local neutralizing antibodies. It is known that such escape mutants are rapidly selected in the presence of monoclonal antibodies (16) and even after replication in the presence of polyclonal sera (17). We are currently investigating the antigenic characteristics of these late HRV-2 isolates.

Rhinovirus specific antibodies reached maximum titres in the sera at 35 days

after infection. Therefore for diagnostic and epidemiologic purposes this would be the optimum time for collection of samples. Thereafter the titres of specific antibodies were well maintained even though the antigenic stimulus was presumably no longer present. It is likely that affinity maturation of the antibodies contributes to the apparent persistence of high titres since high affinity antibody would have greater potency in both the neutralization test and ELISA. However, such maturation would also require the presence of antigenic stimulus. This may be provided either by retention of processed antigen in lymphatic organs, or by repeated infections with related antigens once volunteers are in the open community. In this respect it is noteworthy that British Antarctic Survey Personnel who had lived as an isolated community for at least 1 year had lower HRV-2-specific antibody, particularly in nasal secretions, than volunteers taken from the open community (18). Furthermore, specific antibodies produced after infection with human coronavirus 229E were lost rapidly (Callow, in preparation). Since only a few serotypes of coronavirus exist and these are antigenically unrelated, and since coronavirus infections are half as frequent as rhinovirus infections, this observation suggests that rhinovirus specific antibodies may be maintained by repeated stimulation from infections with related antigens.

We have demonstrated that some volunteers who have been infected with HRV-2 can be reinfected with the same virus one year later whereas others cannot. This difference was related to the ability of individual volunteers to mount and maintain a specific immune response, especially of the IgA class of immunoglobulin, in both sera and nasal secretions. The ability to do this may depend on factors internal to the host, for example the HLA haplotype of an individual may influence the magnitude of a specific response to HRV-2 epitopes. Alternatively the frequency of exposure to related rhinovirus serotypes may influence the time for which the HRV-2-specific response is maintained as discussed above.

In previous studies we have noted a large proportion of volunteers who have serum specific antibody but lack local specific antibody and who become subclinically infected after rhinovirus inoculation (8). It now seems likely that these represent individuals who have encountered the same rhinovirus serotype before but who have not maintained a good IgA response in either serum or in nasal secretions. The observation that the specific antibodies of these individuals may be boosted by the subclinical infections that they experience bodes well for rhinovirus vaccine development.

ACKNOWLEDGEMENTS

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