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An ELISA for the detection of rhinovirus specific antibody in serum and nasal secretion

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An enzyme-linked immunosorbent assay (ELISA) which detects rhinovirus specific antibody in human sera and nasal secretions, has been developed. This sandwich ELISA utilizes a rabbit antirhinovirus hyperimmune serum as the capture antibody and was found to be very sensitive, detecting rhinovirus specific antibody in the serum at dilutions of $1:10^6$ and $1:10^{3.5}$ for IgG and IgA immunoglobulins, respectively. Thus, this new assay is 10^2 – 10^4 times more sensitive than our standard neutralization test. Furthermore, this increase in sensitivity has enabled us to reliably detect rhinovirus specific immunoglobulins in unconcentrated nasal washings, which are thought to be particularly important for protection against rhinovirus reinfection. A preliminary study of the immune response in human volunteers challenged with rhinovirus using this new ELISA system is presented and further applications and potential of the method are also discussed.

Rhinovirus; ELISA; Nasal secretion; Immunoglobulin

Introduction

Specific antibodies produced after a rhinovirus infection have routinely been detected by their ability to neutralize virus in tissue culture. Consequently, the measurement of such antibodies has been dependent on virus isolation and is costly and time-consuming. In spite of this, the neutralization test has been used in both epidemiological studies (Fox et al., 1975) and for the laboratory diagnosis of a recent rhinovirus infection (Hamparian, 1979).

Epidemiological studies have revealed that a large proportion of infected individuals who shed virus do not show a serological response by neutralizing antibody (Fox et al., 1975). Furthermore, studies in our own laboratory indicate that many of our volunteers do not become infected following rhinovirus challenge even though they have no serum neutralizing antibody (W.S. Barclay, unpublished data).

Earlier studies have indicated that local antibody in nasal secretion measured by

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neutralization is a more reliable indicator of the rhinovirus immune status than is circulating antibody (Cate et al., 1966). However, the neutralization test lacks the sensitivity to reliably detect antibody in nasal washings. A haemagglutination-inhibition test has been developed which detected both serum and nasal secretion antibody to rhinoviruses, but prior concentration of nasal washings was still required to detect the local antibody (Reed and Hall, 1973). Furthermore, not all rhinovirus serotypes haemagglutinate, and so this latter test has not gained any widespread use.

We have therefore developed an enzyme-linked immunosorbent assay (ELISA) that detects rhinovirus class-specific antibody in both serum and nasal secretion. In this study, we describe the new assay and its application in the study of the immune response following inoculation with a rhinovirus.

Materials and Methods

Antigens and antisera

The rhinovirus used in this study was the untyped human rhinovirus-EL (HRV-EL). Although a member of the rhinovirus genus by physical and biochemical criteria, HRV-EL has not been assigned a serotype number in the collaborative scheme. However, cross neutralization tests in our laboratory suggest that HRV-EL is antigenically related to HRV-12 (C. Dearden, unpublished data).

HRV-EL was grown to a high titre by infecting confluent monolayers of Ohio Hela cells in 500 ml medical flats overlaid with 25 ml of Hela maintenance medium (BME containing 2% foetal calf serum, 0.13% tryptose phosphate broth, antibiotics, 0.088% NaH_2CO_3 and 30 mM MgCl_2). After the appearance of cytopathic effect, the cells were frozen and thawed rapidly 3 times and centrifuged at 2000 rpm for 10 min to remove cell debris. Crude tissue culture fluid containing the virus was harvested and stored at -70°C . Control antigen was prepared in the same way except that the cells were not infected.

Virus antigen for immunization was further purified by fluorocarbon extraction. Thus, 1 volume of Arklone (ICI) was added to 2 volumes of crude antigen, the mixture was shaken at room temperature and then centrifuged at 2000 rpm for 10 min. The aqueous layer was harvested and centrifuged at 30,000 rpm for 2 h. The virus pellet was then resuspended in a small volume of 0.3 M NaCl/0.1 M Tris buffer pH 7.5 and mixed with an equal volume of Freund's incomplete adjuvant. Rabbits were immunized following a short schedule; 0.5 ml emulsion (at least 10^7 PFU) intramuscularly in each hind leg followed by 1 ml virus intravenously 4 weeks later. Animals were bled 1 week after this and the hyperimmune serum was found to have a neutralization titre of 1:32,000 against 100 TCID₅₀ of the homologous virus.

Human specimens

Twenty-one volunteers inoculated intranasally with HRV-EL at the Common Cold Unit who took part in a separate trial (Zerial et al., 1985), provided samples for this study. Thus, serum samples were collected before virus challenge and 2–3 weeks later, and nasal washings were collected on days 3–7 after virus inoculation according to the standard procedure at the Common Cold Unit, i.e., 5 ml of Hank's balanced salt solution was instilled into each nostril and the expelled fluid was mixed with an equal volume of nutrient broth. Virus in nasal washings was isolated in Ohio Hela cells. All samples for antibody measurement were heated at 56°C for 30 min and stored at –20°C.

On the basis of clinical score (a measure of severity of symptoms assessed at the unit), virus isolation and seroconversion (as shown by a 4-fold or greater increase in neutralizing antibody titre between pre- and postinfection sera), the volunteers were divided into 3 groups. Thus, the first group of volunteers showed symptoms of the common cold (clinical score >10), and also all shed virus in nasal secretion. We have termed this group 'colds'. A second group of volunteers although infected showed no symptoms (clinical score <10) but shed virus and/or showed seroconversion. We have termed this group 'infected, no colds'. The rest of the volunteers despite challenge showed no clinical or laboratory evidence of infection, and we have termed this third group 'not infected'.

Conjugates

Antihuman IgG produced in goats and conjugated to alkaline phosphatase (Miles Laboratories) was used at a dilution of 1:1000 in phosphate buffered saline containing 0.05% Tween 20 (Sigma Chemical Co.) (PBS-T) and 0.1% bovine serum albumin (BSA). Similarly, antihuman IgA produced in goats and conjugated to alkaline phosphatase (Sigma Chemical Co.) was diluted 1:1000 in the same diluent, and antirabbit IgG produced in goats and conjugated to alkaline phosphatase (Miles Laboratories) was used at a dilution of 1:3000 also in PBS-T containing 0.1% BSA.

Substrate

The alkaline phosphatase substrate was *p*-nitrophenol phosphate (Sigma Chemical Co.). One mg/ml was dissolved in 10% diethanolamine buffer.

Sandwich ELISA

Polystyrene round-bottomed micro ELISA plates (Nunc Immunoplates U type II) were incubated overnight at 4°C with 100 µl per well of a 1:4000 dilution of rabbit hyperimmune antirhinovirus-EL serum in carbonate/bicarbonate buffer pH 9.6. The wells were drained and uncoated sites at which nonspecific binding might occur, were blocked with 1% BSA in PBS for 2 h at 37°C. Plates were washed 3

times with PBS-T. One hundred μl of a 1:10 dilution of crude virus antigen, which had an infectivity of about 10^7 TCID₅₀ per ml, or control antigen in PBS-T containing 0.1% BSA and 5% uninfected tissue culture fluid was added and plates were incubated overnight at 4°C. After washing as before, test samples at various serial dilutions in the same diluent were added and incubated for 2 h at 37°C. Plates were then washed and the appropriate conjugate added and incubated for 2 h at 37°C. Finally, plates were washed 5 times with PBS-T, and 100 μl of substrate added to each well. Plates were left uncovered at room temperature and the optical density at 405 nm was read at various time intervals in a Titertek Multiskan ELISA reader (Flow Laboratories).

Indirect ELISA

Wells of round-bottomed micro ELISA plates (Nunc Immunoplates U type II) were coated overnight with 100 μl of 1:10 dilution of virus antigen which had been partially purified by PEG 6000 precipitation and ultracentrifugation through a 15–45% sucrose density gradient. The starting material for this purification had an infectivity of about 10^7 TCID₅₀ per ml. Plates were drained and quenched with 1% BSA in PBS. The ELISA procedure from the point of adding the test samples was the same as for the sandwich ELISA.

Calculation of corrected optical density and ELISA titre

Sera were tested in duplicate 10-fold serial dilutions and nasal washings in duplicate 2-fold serial dilutions. The means from the duplicates were corrected for each dilution by subtracting the mean optical density (OD) in the absence of sample. From this value, the OD with control antigen coated wells (corrected in the same way) was then subtracted. The corrected OD was plotted against the log of the sample dilution and this generated a sigmoid curve. An assay line was chosen at an OD level which intersected this curve in the linear portion for the majority of samples. The ELISA titre was taken as the reciprocal of the sample dilution corresponding to that OD.

Microneutralization test

Neutralizing antibodies were detected by the standard microneutralization test used at the Common Cold Unit. Briefly, 2-fold serial dilutions of each serum were incubated with 100 TCID₅₀ virus per well for 1 h at room temperature. 3×10^4 Ohio Hela cells in Hela maintenance medium containing 5% foetal calf serum were then added. Plates were incubated at 33°C for 5 days and read microscopically for CPE. The neutralization titre was the reciprocal of the lowest serum dilution which neutralized 100 TCID₅₀ of virus. A virus titration and cell controls were included in every test.

Results

Comparison of sandwich and indirect ELISA procedures

Fig. 1a shows the measurement of HRV-EL specific IgG in rabbit pre- and post-immunization sera by the indirect ELISA, compared with the measurement of HRV-EL specific IgG and IgA in pre- and postinfection human sera by the same method (Fig. 1b,c). As illustrated, the indirect ELISA detected a high titre of HRV-EL specific antibody in both pre- and postinfection human sera and hence failed to show any specific antibody response following HRV-EL infection although this individual was found to excrete virus, showed serological evidence of infection by neutralization test, and had symptoms of the common cold. In contrast, the sandwich ELISA (Fig. 1d,e) demonstrated an increase in HRV-EL specific antibody after infection of both the IgG and IgA classes of immunoglobulins in the sera of the same individual. However, it is noteworthy that the titre of HRV-EL specific IgG in the preinfection serum was high and did not rise as much following infection as that of the IgA class.

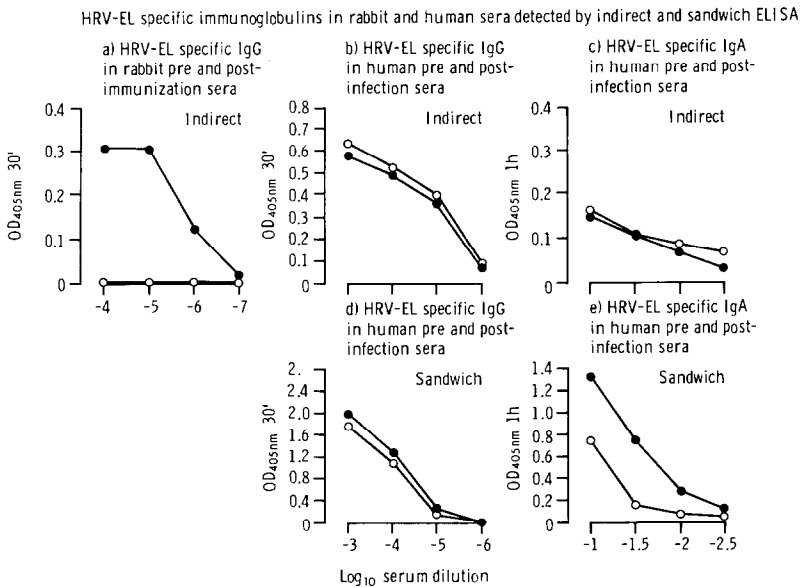


Fig. 1. ELISA titration curves of HRV-EL specific immunoglobulins (IgG) in rabbit pre- (○) and postimmunization (●) sera (a) and of rhinovirus specific IgG (b) and (d) and IgA (c) and (e) in human pre- (○) and postinfection (●) sera as measured by the indirect (a-c) and the sandwich (d-e) ELISA systems

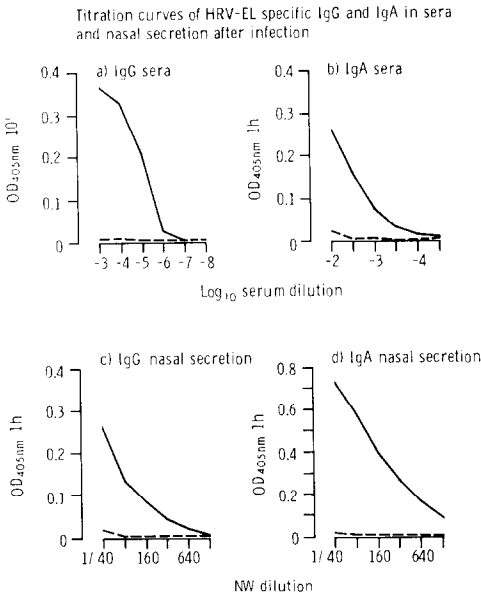


Fig. 2. ELISA titration curves of HRV-EL specific IgG and IgA in postinfection serum (a-b) and in postinfection (7 days) nasal secretion (c-d) as measured by the sandwich ELISA. Dotted lines represent OD values with control antigen coated wells.

Specificity and sensitivity of the sandwich ELISA for rhinovirus specific antibody measurement in both the serum and nasal secretions

Fig. 2 shows titration curves of IgG (Fig. 2a,c) and IgA (Fig. 2b,d) immunoglobulins specific to HRV-EL in postinfection sera (Fig. 2a,b) and nasal secretion (Fig. 2c,d) of an infected volunteer who was symptomatic, as measured by the sandwich ELISA. The OD values obtained with control antigen coated wells were low and independent of sample dilution, indicating that the test specifically detected antibody to the virus.

The highest dilutions at which HRV-EL specific serum antibody was detected, were 1:10⁶ and 1:10^{3.5} for IgG and IgA, respectively. The neutralizing antibody titre for this serum sample was 1:128. In addition, the neutralization test failed to detect HRV-EL specific antibody in this postinfection nasal washing sample, and yet the sandwich ELISA detected HRV-EL specific IgG and IgA in this sample at dilutions of up to 1:10³.

Measurement of the immune response following HRV-EL challenge by the sandwich ELISA

Of the 21 volunteers challenged with HRV-EL, 4 became infected and symptomatic ('colds' group), 9 were infected but asymptomatic ('infected, no colds'

Geometric mean titre of HRV-EL specific antibody measured by ELISA and by microneutralization of paired sera from 3 groups of volunteers

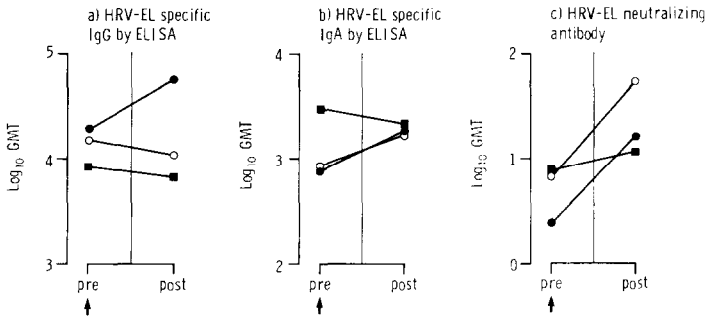


Fig. 3. The geometric mean titres of HRV-EL specific IgG and IgA in pre- and postinfection serum as detected by ELISA, and of HRV-EL neutralizing antibody. The means are for samples from 4 volunteers in each of the response groups, 'colds' (●), 'infected, no colds' (○) and 'not infected' (■). ELISA titres were calculated from titration curves at 30 min after substrate addition using an assay line of OD = 0.1 and OD = 0.02 for IgG and IgA, respectively.

group), and 8 showed no clinical or laboratory evidence of infection ('not infected' group). From these 21 volunteers, 12 were chosen for further study such that a wide range of responses to HRV-EL challenge was represented (4 from each group). The titres of the various immunoglobulins specific for HRV-EL were measured by the sandwich ELISA. Fig. 3 shows the geometric mean titre of rhinovirus specific antibody measured by both the ELISA and the microneutralization test for the paired sera of the 4 volunteers in each group. The figure suggests that detection of HRV-EL specific IgA measured by ELISA (Fig. 3b) was a better indicator of the group susceptibility to infection than the detection of specific IgG (Fig. 3a) or neutralizing antibody (Fig. 3c). Thus, the GMT of the preinfection HRV-EL specific serum IgA was much higher among volunteers who despite challenge did not become infected than among volunteers who did become infected. Furthermore, after infection the GMT of HRV-EL specific IgA and of neutralizing antibody showed substantial rises in the sera of all the volunteers who became infected, whereas the GMT of HRV-EL specific IgG showed only very small rises following challenge among the symptomatic volunteers. Indeed, the level of preexisting HRV-EL specific IgG was generally high and it neither correlated with serum neutralizing antibody rises nor appeared to be relevant for protection against virus challenge.

Fig. 4 shows the mean ODs obtained when the nasal washings from the 4 volunteers in each group collected on days 3, 5 and 7 after inoculation were tested at a 1:40 dilution in the sandwich ELISA, for both HRV-EL specific IgG and IgA. The mean OD values for each group showed a distinct pattern. Thus, volunteers who were infected and became symptomatic (Fig. 4a,d), showed HRV-EL specific increases in both the IgG and IgA immunoglobulin classes after infection. In contrast, volunteers who did not become infected (Fig. 4c,f), had higher levels of local rhinovirus specific IgA by day 3 after challenge which then fell off during the fol-

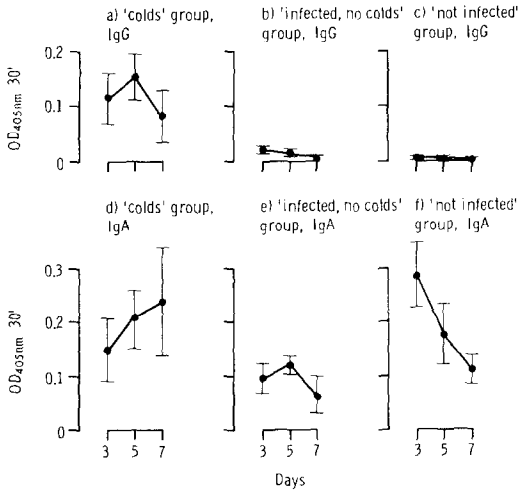


Fig. 4. Time course of appearance of HRV-EL specific IgG (a-c) and IgA (d-f) immunoglobulins in nasal secretion 3-7 days after HRV-EL inoculation. Mean ODs obtained with a nasal washing dilution of 1:40 read at 30 min after substrate addition are shown for 4 volunteers in each of the response groups 'colds' (a-d), 'infected, no colds' (b-e) and 'not infected' (c-f), with bars representing standard errors of the means.

lowing days. On the other hand, volunteers who were subclinically infected (Fig. 4b,e), showed only intermediate changes in specific IgA levels and very little HRV-EL specific IgG.

Agreement between the sandwich ELISA and neutralization test data

Table 1 shows the HRV-EL antibody titres of paired sera of the 12 individual volunteers as measured by the ELISA and by the neutralization test. When the differences in titre after infection measured by the ELISA for IgG and by the neutralization test were compared, the rank coefficient of correlation was 0.17 indicating no agreement between the two measurements. A similar analysis of the ELISA IgA measurements and neutralizing antibody titre differences gave an r value of 0.53, which illustrated that, although the agreement was better than for the ELISA IgG measurements, it was nonetheless incomplete.

Discussion

The ELISA described in this study is a simple, rapid assay which detects rhinovirus class-specific immunoglobulins in both the serum and nasal secretion. It is an extremely sensitive technique, detecting HRV-EL specific IgG and IgA antibody in postinfection serum at dilutions 10^4 - and 10^2 -fold greater respectively than the end-point dilution for the neutralization test. In unconcentrated nasal wash-

TABLE 1

HRV-EL specific antibody titres in paired sera of 12 volunteers challenged with homologous virus, as measured by ELISA and microneutralization test.

Volunteer	Titre (log)			ELISA IgG			ELISA IgA			Neutralization		
	Difference (post-pre)			Pre Post			Pre Post			Pre Post		
	Pre	Post	Difference (post-pre)	Pre	Post	Difference (post-pre)	Pre	Post	Difference (post-pre)	Pre	Post	Difference (post-pre)
1	4.00	4.40	0.40	2.00	3.62	1.62	0.90	2.41	1.51	0.30	0.90	0.60
2	4.40	4.82	0.40	3.15	3.55	0.40	0.00	0.90	0.90	0.00	0.90	0.90
3	4.20	4.50	0.30	3.40	3.40	0.00	0.30	0.60	0.30	0.30	0.60	0.30
4	4.50	5.35	0.85	3.00	2.50	0.50	0.60	2.41	1.81	0.60	2.41	1.81
5	4.40	4.20	-0.20	3.30	3.57	0.27	0.90	1.51	0.61	0.90	1.51	0.61
6	4.00	4.00	0.00	3.30	3.50	0.20	1.81	2.41	0.60	1.81	2.41	0.60
7	4.20	4.00	-0.20	2.52	3.50	0.98	0.00	0.60	0.60	0.00	0.60	0.60
8	4.10	4.00	-0.10	2.65	2.42	-0.23	0.48	0.90	0.42	1.10	1.38	0.18
9	3.70	3.80	0.10	3.62	3.72	0.10	0.60	2.60	-0.30	0.60	2.60	-0.30
10	4.00	3.80	-0.20	3.50	3.30	-0.20	1.20	1.38	0.18	1.20	1.38	0.18
11	3.80	3.80	0.00	2.90	2.60	-0.30	0.60	0.60	0.00	0.60	0.60	0.00
12	4.20	3.90	-0.30	3.85	3.75	-0.10	1.20	1.38	0.18	1.20	1.38	0.18

ings, it consistently detected HRV-EL specific antibody of both the IgG and IgA classes whereas the neutralization test lacks the sensitivity to reliably detect this local specific antibody. This, we feel, is an important feature of our rhinovirus ELISA system since it is now possible to look more closely at the immune response following a rhinovirus infection and perhaps be able to identify the feature(s) of the humoral immune response which may be relevant for protection against reinfection with homologous or heterologous virus. Indeed, preliminary data presented in this study suggest that the presence of rhinovirus specific IgA in both serum and nasal secretion appears to be more important for protection against reinfection than does the presence of specific IgG. Thus, the group of volunteers who showed no evidence of infection despite challenge with HRV-EL ('not infected' group), had high levels of HRV-EL specific IgA in both their sera and nasal secretions. Furthermore, they did not show rises in specific serum IgG, IgA or neutralizing antibody. In contrast, volunteers who became subclinically infected ('infected, no colds' group), had low levels of HRV-EL specific IgA in their serum and nasal secretions before infection, and showed rises in both specific serum IgA and neutralizing antibody after infection. In addition, those volunteers who became infected and who were symptomatic ('colds' group) also had low levels of preinfection specific IgA and showed rises in specific serum IgG, IgA and neutralizing antibody as well as specific IgG and IgA in nasal secretion after infection. These data demonstrate the potential of our ELISA in detecting different components of the humoral immune response following a rhinovirus infection. This may be important should vaccination against rhinovirus infection be contemplated. However, in view of the fact that the number of volunteers in this study was small (4 per group), confirmation and elaboration of these findings will have to await a much larger study that needs to involve many more volunteers.

The present ELISA data were obtained with serum specimens ideally collected for the measurement of neutralizing antibody titres, i.e., a prechallenge serum specimen and another 2-3 weeks later. Hence, measurements made with these samples may not be optimal for detecting antibody rises by ELISA. Also, the nasal washings used in this study were collected primarily for virus isolation and not for antibody measurement. Therefore, these specimens were also probably not optimal for measuring the sequential appearance and decline of the various rhinovirus specific immunoglobulins. Furthermore, a series of preinfection nasal washes would ideally be required to establish the presence and day-to-day variation of any preexisting local specific antibody. Since rhinovirus infection is localized and short-lived it presents a relatively light antigenic load, and so it is expected that a sensitive assay such as the ELISA would detect rapid immunoglobulin responses very soon after inoculation rather than 3 weeks later. Indeed, the use of ELISA systems to detect class-specific responses to other respiratory viruses such as parainfluenza virus and influenza A virus indicated that such responses appear within 3-6 days of onset of symptoms (Van der Logt et al., 1985; Leinikki and Passila, 1976). Therefore, it is possible that in this study we have missed the peak of the rhinovirus specific immunoglobulin responses at least in the serum. We are now planning a more extensive and detailed study in human volunteers whereby closely

spaced serum and nasal secretion specimens will be collected so that we can define more precisely the time course of appearance of specific immunoglobulins after a rhinovirus challenge.

Antibody to several other respiratory viruses, such as influenza A and coronavirus, has been detected using indirect ELISA systems in which the virus is directly bound to the solid phase (Murphy et al., 1980; Kraaijeveld et al., 1980). However, picornaviruses may be prone to changes in their surface structure after direct electrostatic binding which reveal internal antigenic regions (McCullough et al., 1985). Indeed, using an indirect ELISA in which rhinovirus antigen was immobilised on the plate, we have found high levels of HRV-EL antibody in both pre- and postinfection human sera, and hence no differences in antibody levels between the paired sera could be demonstrated. Such antibody probably persists from previous infections by heterologous serotypes and may cross-react with our EL-rhinovirus after interaction with the solid phase has exposed common internal regions. On the other hand, in the same indirect ELISA the rabbit sera showed clear rises of HRV-EL specific antibody after immunization. This may be explained by the fact that the rabbit immune system has not encountered any previous rhinovirus other than that used for hyperimmunization, and so the indirect ELISA detected no cross-reacting antibody in the preimmunization serum. In the sandwich ELISA described here, the solid phase was coated with a rhinovirus specific antibody which captured the rhinovirus antigen and hence the surface structure was less likely to have been distorted. Thus, we were able to detect an immune response to the homologous rhinovirus infection in both human sera and nasal secretions.

We feel that there is still considerable scope for improving the present ELISA system. For example, preliminary results suggest that the use of purified virus as antigen instead of crude tissue culture fluids containing virus will result in increased sensitivity (W.S. Barclay, unpublished data). Also, if D-type rhinovirus particles were purified and separated from C-type particles, and used as antigen, it is likely that an immunoglobulin response more closely associated with the infecting homologous virus would be detected rather than any heterologous reaction (Lonberg-Holm and Yin, 1973). The existence of such heterologous reactions is expected in view of the fact that most human adults have experienced many rhinovirus infections. This may explain to some extent the detection of consistently high levels of rhinovirus specific IgG in preinfection sera, and our failure to show a good correlation with antibody rises detected by our neutralization tests since these latter assays detect rises in antibody to the infecting homologous virus.

Conversely, the use of purified C-type or denatured rhinovirus particles as antigen may allow the detection of broader antibody reactivity in the immune response following rhinovirus infection. However, the relevance of these measurements in terms of protection against reinfection by the homologous or a heterologous rhinovirus, or whether such measurements will consistently detect diagnostic rises following a recent rhinovirus infection will have to await future experiments in human volunteers designed to provide answers to these questions.

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