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Evaluation of an Enzyme-Linked Immunosorbent Assay That Measures Rhinovirus-Specific Antibodies in Human Sera and Nasal Secretions

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Rhinovirus-specific antibodies have traditionally been detected by their ability to neutralise the homologous rhinovirus serotype in tissue culture. Recently, however, we have described an enzyme-linked immunosorbent assay that detects rhinovirus-specific antibodies in sera and nasal secretions [Barclay and Al-Nakib, 1987]. Here we describe an evaluation of the ELISA in a study involving 71 adult volunteers inoculated intranasally with human rhinovirus type 2 (HRV-2). Pre- and post-inoculation serum samples and pre-inoculation nasal washings were tested for the presence of HRV-2-specific antibodies by ELISA. Such antibodies were associated with protection against infection when present locally in nasal secretions, but when also present in the serum they were associated with protection against both infection and the development of illness. The antibody concentrations showed strong correlation with each other and with that of antibodies detected by the neutralisation test. Following HRV-2 infection, rises in HRV-2-specific IgA in sera detected by ELISA occurred more frequently than rises in neutralising antibody. These results suggest that the ELISA is a sensitive and reliable indicator of recent infection, as well as a predictor of homologous immune status.

Key words: immunoglobulin, human rhinovirus type 2, common cold, IgA, IgG

INTRODUCTION

Rhinoviruses are the major causative agents of the common cold [Couch, 1984]. Earlier studies suggested that, following infection, rhinovirus serotype-specific antibodies appear in both the serum and nasal secretions and that the presence of such antibodies confers long-term protection against re-infection by the homologous rhinovirus serotype [Doggett, 1965].

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These antibodies have traditionally been detected by neutralisation tests [Hamparian, 1979]. Recently, however, we have established a sensitive enzyme-linked immunosorbent assay that detects rhinovirus-specific antibodies in sera and unconcentrated nasal washings. This new assay is simple, rapid, and can differentiate between classes of rhinovirus-specific immunoglobulins. In a preliminary study involving 12 volunteers inoculated intranasally with human rhinovirus EL (HRV-EL), we showed that the presence of circulating rhinovirus-specific IgA, rather than IgG, protected volunteers from infection [Barclay and Al-Nakib, 1987].

In the present study we describe a more extensive analysis of the anti-human rhinovirus type 2 (anti-HRV-2) immunoglobulin responses in both sera and nasal washings measured by ELISA. Thus 71 volunteers were inoculated intranasally with HRV-2, and the course of infection was monitored both clinically and virologically. Our data confirm the importance of IgA anti-rhinovirus immunoglobulins in protection against re-infection and/or illness; we provide evidence that a rise in specific serum IgA following infection is a good indicator of a recent infection.

MATERIALS AND METHODS

Human Volunteers

Samples were obtained from 71 volunteers taking part in a separate study to test the therapeutic effect of zinc gluconate lozenges on colds caused by HRV-2 [Al-Nakib et al., 1987]. They were housed in isolation according to standard procedures at the Common Cold Unit [Beare and Reed, 1977].

Serum samples were collected before the trial and 3 weeks after rhinovirus inoculation. These were stored at −20°C.

Nasal washings for antibody measurement were collected before the trial by instilling 5 ml of phosphate-buffered saline (PBS) into each nostril. The volunteer then forcibly expelled the liquid into a pot containing glass beads. After vortexing for 15 sec to break up mucus, the effluents were centrifuged at 13,000 rpm in a microcentrifuge, and the supernatants were stored at −20°C. Nasal washings for virus isolation were taken from 2 to 5 days after inoculation using standard methods [Barclay and Al-Nakib, 1987]. These were added to an equal volume of nutrient broth and stored at −70°C.

Viruses were isolated by inoculation of Ohio HeLa cells. Samples of 0.1 ml nasal washing were inoculated into confluent test tube cultures, these were rolled at 33°C. After 7–10 days they were examined for the presence of virus cytopathic effect (CPE). Colds were assessed by a combination of tissue count and subjective and objective evaluation of severity of symptoms, that is, clinical score [Beare and Reed, 1977].

On the basis of virus isolation and these criteria, volunteers were divided into three groups for analysis as follows: 1) *colds group*: those from whom virus was isolated and who had clinical colds; 2) *subclinically infected group*: those from whom virus was isolated but who did not have colds; 3) *not infected group*: those from whom no virus was isolated and who did not have colds.

Rhinovirus Growth and Preparation

Human rhinovirus type 2 (HRV-2) was grown in Ohio HeLa cells and crude antigen preparations were made as previously described for HRV-EL [Barclay and

Al-Nakib, 1987]. Control antigens was prepared in the same way from uninfected monolayers.

Preparation of Rabbit Anti-HRV-2 Hyperimmune Sera

Fifty millilitres of crude HRV-2 antigen with a titre of approximately $10^{8.0}$ $TCID_{50}$/ml was concentrated by ultracentrifugation and resuspending the virus pellet in 2 ml of PBS; 0.5 ml of this antigen was then emulsified in an equal volume of Freund's complete adjuvant (FCA) and used to inoculate one rabbit subcutaneously. Six weeks later, the rabbit was inoculated intramuscularly with an emulsion of 0.5 ml HRV-2 and 0.5 ml of Freund's incomplete adjuvant (FICA). Fourteen days later, the animal was bled out. The resulting hyperimmune serum had a neutralisation titre of 1:2,560 against 10^3 $TCID_{50}$ HRV-2. This was stored at −20°C.

Enzyme-Linked Immunosorbent Assay (ELISA) for HRV-2-Specific Immunoglobulins

The ELISA for HRV-2-specific immunoglobulins in sera and nasal secretions was an adaptation of that previously described for HRV-EL-specific immunoglobulins [Barclay and Al-Nakib, 1987]. That is, polystyrene round-bottomed micro ELISA plates (Nunc Immunoplates U type II) were incubated overnight at 4°C with 100 μl per well of a 1:1,000 dilution of rabbit anti-HRV-2 hyperimmune serum in carbonate/bicarbonate buffer, pH 9.6. Wells were drained and washed three times in PBS containing 0.05% Tween 20 (PBS-T). Then 100 μl of HRV-2 or control antigen (CAg) diluted 1:5 in PBS-T containing 1% bovine serum albumin (BSA) was added to alternate rows of wells. Plates were incubated for 2 hr at 37°C, then drained and washed as before.

One hundred microlitres of standard or test sample diluted in PBS-T containing 1% BSA and 5% CAg was added in duplicate to both HRV-2- and CAg-coated wells. As standard, a pool of known HRV-2-antibody-positive sera or nasal washings was titrated on each plate in one-half $\log_{10}$ dilution steps. Samples were diluted to $10^{-2.5}$ and $10^{-3.0}$, respectively, for the assay of specific IgA and IgG in sera and $10^{-2.0}$ for the detection of specific IgA in nasal washings.

Plates were then incubated for 2 hr at 37°C. After washing as before, 100 μl of either anti-human IgA alkaline phosphatase conjugate (Sigma) or anti-human IgG alkaline phosphatase conjugate (ICN) was added, diluted 1:1,000 in PBS-T with 1% BSA, and incubated overnight at 4°C. Plates were finally washed four times with PBS-T and 100 μl substrate (Sigma 104 *p*-nitrophenol phosphate tablets dissolved at 1 mg/ml in 10% diethanolamine buffer) was added to each well. Plates were incubated for 1 hr and optical densities (OD) at 405 nm were read in a Dynatech MR700 ELISA reader using a reference filter of 490 nm.

Calculation of Antibody Units in Volunteer Samples

Mean ODs from duplicate wells were corrected by subtracting the mean OD of CAg-coated wells. The serum standards were arbitrarily allotted 5.0 $\log_{10}$ units and the nasal washing standards, 4.0 $\log_{10}$ units. Using corrected ODs, from the titrations of these on each ELISA plate a standard curve was calculated. Corrected ODs for each sample were then read off on this standard curve. Thus each sample was awarded a number of $\log_{10}$ units, which related it to the known standards. Antibody units for HRV-2 specific IgA in nasal washings were further corrected by normalising for total

IgA in the sample. The ELISA for total IgA in nasal washings was performed as previously described by Callow [1985]. This normalisation resulted in a more significant difference between the specific IgA concentrations in nasal secretions for the three groups.

To establish the size of a significant rise in HRV-2-specific antibody following HRV-2 infection, paired sera from 12 volunteers who had received an intranasal inoculation of saline (no virus), and who had been housed in isolation exactly as the other volunteers, were tested for HRV-2 antibodies. A significant rise in HRV-2-specific antibody was then set as the mean difference between these paired sera plus 2 SD of that mean. Thus a significant rise for ELISA HRV-2-specific IgA in sera was a difference of 0.2 $\log_{10}$ units, and for IgG serum-specific antibody this value was 0.3 $\log_{10}$ units. A rise in HRV-2-neutralising antibody was considered significant if the difference was fourfold (i.e., 0.6 $\log_{10}$ steps) or greater.

Microneutralisation Test

Neutralising antibodies in sera were detected by microneutralisation tests against a challenge of 100 $TCID_{50}$ homologous rhinovirus as previously described [Barclay and Al-Nakib, 1987].

Statistical Methods

The statistical analyses were performed on an IBM XT computer, using the programme, Statistical Package for Personal Computers (SPP, Patrick Royston, Clinical Research Centre, Northwick Park Hospital, Harrow, England). For comparing the frequencies of antibody rises in the three volunteer groups, a χ^2 test was used, with Yates' correction. For examining the significance of the differences in antibody concentration between the groups, a rank analysis of variance was used; for comparing antibody concentrations of different types, or the magnitude of antibody rises, Spearman's rank correlation coefficient was calculated.

RESULTS

Occurrence of Infection and Symptoms

Of the 71 volunteers who were intranasally inoculated with HRV-2, 13 (18.3%) had colds, 36 (50.7%) had subclinical infections, and 22 (31%) did not become infected.

Relation of Pre-Inoculation Antibodies to Infection and Symptoms

Figure 1 shows a) the mean HRV-2-neutralising antibody, b) specific IgG and c) IgA in the serum, and d) specific IgA in nasal secretions (normalized for total IgA) in pre-inoculation samples from the three groups of volunteers.

The presence of HRV-2-specific antibody in the serum appears to confer protection against both infection and symptoms. Thus volunteers who did not get infected had more HRV-2-specific antibody in their sera (IgA and IgG antibodies) than those who had subclinical infections or those who were infected and developed colds. Those who developed colds had the lowest amount of HRV-2-specific antibody in their sera. These differences in amounts of HRV-2-specific serum antibody between the three groups were highly significant for ELISA-specific IgA and neutralising antibody ($P < .001$) but were not significant for ELISA-specific IgG. Volunteers

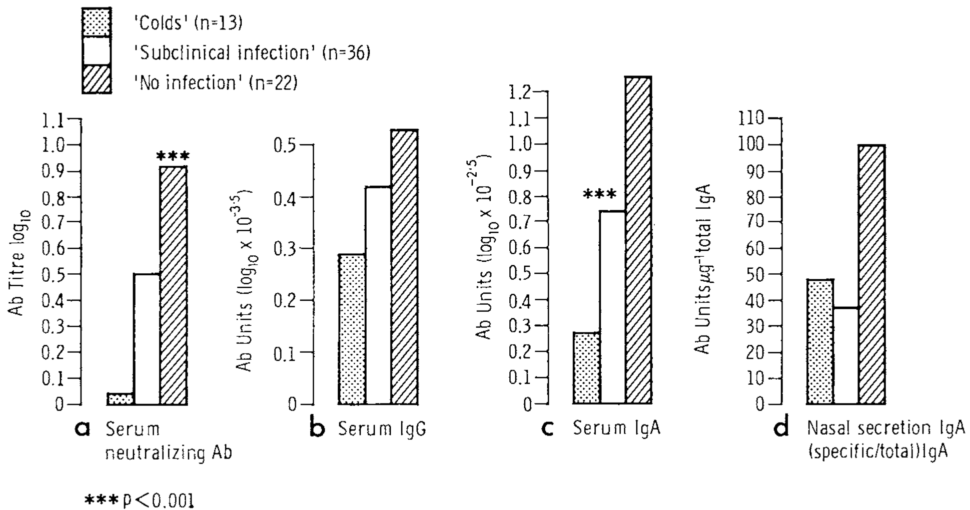


Fig. 1. HRV-specific antibodies in pre-inoculation sera and nasal secretions. **a:** Geometric mean titres of pre-inoculation HRV-2 serum neutralising antibody, **b:** ELISA HRV-2-specific serum IgG, **c:** IgA, and **d:** nasal secretion IgA for three groups of volunteers. Volunteers were divided into these three groups according to their subsequent response to HRV-2 inoculation, as described in the text.

TABLE I. Frequency of Rises of HRV-2 Specific Antibody After HRV-2 Inoculation

	Group			
Antibody	Colds	Subclinically infected[a]	Not infected[a]	χ^2 *P* value
Neutralising	11/13	24/36	3/22	<.001
ELISA-serum-specific IgA	11/13	27/36	7/22	<.001
ELISA-serum-specific IgG	3/13	16/36	4/22	NS

[a]Infection determined by isolation of infectious virus from nasal washing samples.
NS = not significant.

who were not infected had significantly more HRV-2-specific IgA in pre-inoculation nasal washings ($P < .05$) than those who became infected whether or not they had colds. However, the differences between the concentrations of this antibody when the three groups were considered individually, or between the *colds* group and the *subclinically infected* group were not significant. Therefore, although the presence of local specific antibodies appears to protect against infection, they do not appear to prevent illness caused by the infection.

Occurrence of Rises in Serum HRV-2-Specific Antibodies

Table I shows the number of volunteers in each of the three groups who had a significant rise in HRV-2-specific serum antibody following HRV-2 intranasal inoculation. The frequencies of rises in neutralising antibody and ELISA-serum-specific IgA were significantly different in the three groups ($P < .001$), the proportion of rises being highest in the *colds group*, and least in the *not infected group*. The difference in frequency of rises in specific IgG in the three groups was not significantly different.

Thirty-six of the 71 volunteers showed rises in both ELISA-serum-specific IgA and neutralising antibody, but only two volunteers showed rises by neutralisation and

not by ELISA. In contrast, nine volunteers showed rises by ELISA and not by neutralisation (Table II). Thus the rises in ELISA-serum-specific IgA were slightly more frequent than for neutralising antibody (45 of 71 or 63.4% vs. 38 of 71 or 53.5%), suggesting that the former might be a more sensitive test for detecting such rises.

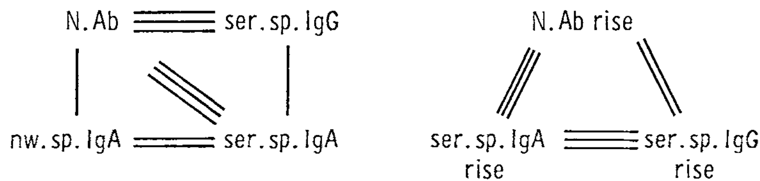
Correlations Between HRV-2-Specific Antibodies

The concentrations of the various HRV-2-specific antibodies were tested for correlations with each other and with the size of antibody rises. Substantial correlations were found between the concentrations of serum antibodies and between rises in these antibodies, as shown in Figure 2a. Generally the presence of, or a rise in, neutralising or ELISA serum IgG or IgA antibody was significantly associated with the presence of, or rise in, the other rhinovirus-specific antibodies. There was also a positive correlation ($P < .01$) between the presence of HRV-2-specific IgA in nasal secretion and that in the serum.

In addition, a negative correlation was found between the concentration of specific IgA in nasal secretion and the size of the rise in specific IgA in serum

TABLE II. Number of Rises of ELISA Serum HRV-2-Specific IgA and of HRV-2-Neutralising Antibody After HRV-2 Inoculation

		ELISA serum specific IgA rise		
		No rise	Rise	Total
Neutralising antibody rise	No rise	24	9	33
	Rise	2	36	38
	Total	26	45	71



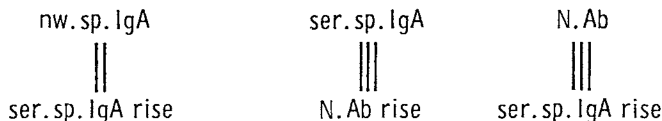


Fig. 2. Correlations between HRV-2-specific antibodies and antibody rises. Significance of **a:** positive and **b:** negative correlations are indicated by the number of lines: three lines = $P < .001$; two lines = $P < .01$; one line = $P < .05$. HRV-2-specific antibodies are abbreviated as follows: N.Ab = neutralising antibody; ser. sp. IgG = serum specific IgG; ser. sp. IgA = serum specific IgA; nw. sp. IgA = nasal secretion specific IgA.

($P < .01$) (Fig. 2b). Thus volunteers with low amounts of pre-inoculation HRV-2-specific IgA in nasal secretions were those most likely to show a significant rise in serum-specific IgA.

Similarly, there was also a negative correlation between the concentration of specific IgA in pre-inoculation sera and rises in rhinovirus-neutralising antibody and vice versa ($P < .001$). This suggests that a low specific IgA or neutralising antibody concentration in pre-inoculation sera was associated with subsequent large rises in both neutralising antibody and specific IgA following inoculation with HRV-2.

DISCUSSION

We have found a significant association between the presence of neutralising antibody and ELISA-serum-specific IgA and protection against re-infection and illness. Thus, volunteers who had high levels of HRV-2-specific IgA or neutralising antibody in their sera did not get infected or develop symptoms of colds when inoculated with HRV-2. In contrast, those who had lower levels of specific IgA or neutralising antibody had subclinical infections, and those who had little or no antibody became infected and developed colds. Interestingly, the presence of HRV-2-specific IgA in nasal secretions was only associated with protection against infection but not illness. Our findings are in general agreement with those for another virus that causes common colds—coronavirus 229E [Callow, 1985]. However, in that study, serum-specific IgG appeared to be at least as effective in preventing infection and disease as serum-specific IgA. Furthermore, coronavirus-specific IgA in nasal secretion, while significantly shortening the period of virus shedding, also appeared to reduce symptoms. Recently, a similar study involving samples obtained from volunteers inoculated with another rhinovirus serotype (HRV-9) and tested by ELISA for homologous antibodies, appears to confirm the association between ELISA-serum-specific IgA and protection against reinfection (Callow and Sergeant, unpublished data).

The standard serological test for a recent rhinovirus infection is the detection of a rise in neutralising antibody. This study showed that rises in circulating rhinovirus-specific IgA were at least as reliable for this purpose. In fact, the present ELISA detected rises in IgA in three subclinically infected and four uninfected volunteers, in whose sera the neutralisation test failed to detect such rises. These ELISA antibody rises may have been "false," since the "uninfected" volunteers did not excrete virus. In a separate study in our laboratory, however, several nasal washings from these HRV-2-inoculated volunteers from which no virus was isolated (i.e., the *not infected* group) contained HRV-2 antigen detectable by a biotin/streptavidin bridge ELISA system. Thus, although they were not excreting infectious virus that could be isolated in Ohio HeLa cells, they were actively producing viral antigens, which suggests that viral replication may have been taking place.

The size of rises in HRV-2-specific IgA in the serum after virus inoculation showed a significant negative correlation with the concentration of specific IgA in the pre-inoculation nasal secretions and with the presence of serum neutralising antibody, indicating that volunteers who were most susceptible to infection and illness, in fact, showed the largest antibody response.

The small number of serum-specific IgG rises detected by the ELISA and the weak correlation between the presence of pre-inoculation IgG and protection against

re-infection might be explained by the fact that, generally, the ELISA detects large amounts of specific IgG in all volunteer pre-inoculation sera so far tested. We suggested previously that this may be due to the use of crude tissue culture fluid harvests as antigens in the ELISA that contain a mixture of both *full* or *native* virus particles and empty capsids. The latter have been shown to cross-react with antisera against heterologous serotypes [Lonberg-Holm and Yin, 1973]. Thus, IgG persisting from previous infections with other rhinovirus serotypes could cross-react in the ELISA. We have recently obtained evidence that purification of the homotypic full virus particles on sucrose density gradients for use as antigens in the assay improves the specificity of the test (Barclay, unpublished data). This cross-reactivity does not appear to be a problem when an IgA class-specific conjugate is used, presumably because IgA antibody responses are of short duration and do not persist as long as those of the IgG class.

In conclusion, the ELISA described in this study is an accurate predictor of the immune status of an individual to the homologous rhinovirus and can be a reliable diagnostic assay detecting recent rhinovirus infection by measuring significant rises in specific IgA. Thus it could be useful in evaluating the efficacy of any novel rhinovirus vaccine or antiviral compound in the future by measuring the incidence of infection following challenge with the homologous virus. We are now studying the possibility that this ELISA system can be modified to detect rises to other rhinovirus serotypes.

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