JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1980. p. 493-497
0095-1137/80/10-0493/05$02.00/0

Vol. 12, No. 4

Enzyme-Linked Immunosorbent Assay for Detection of Antibody in Volunteers Experimentally Infected with Human Coronavirus Strain 229E

CORNELIS A. KRAAIJEVELD,[1]† SYLVIA E. REED,[2] AND MALCOLM R. MACNAUGHTON[1]*

Division of Communicable Diseases, Clinical Research Centre, Harrow, Middlesex HA1 3UJ,[1] and Common Cold Unit, Harvard Hospital, Salisbury, Wiltshire SP2 8BW,[2] England

An enzyme-linked immunosorbent assay was developed for detecting antibody rises to human coronavirus strain 229E and related strains in paired sera from infected volunteers. There was a close correlation between development of colds and significant antibody rises detected by the enzyme-linked immunosorbent assay. Furthermore, the assay was more sensitive than a neutralization assay. This enzyme-linked immunosorbent assay is an easy, accurate, and sensitive assay for measuring significant antibody rises to human coronavirus strain 229E group viruses, and it could be useful in the clinical diagnosis of these infections.

Coronaviruses are a group of large, single-stranded ribonucleic acid viruses with lipid-containing envelopes ranging in diameter from 80 to 200 nm and possessing widely spaced, club-shaped surface projections of up to 20 nm in length (11, 17). More than 20 human coronavirus (HCV) strains that cause mild upper respiratory tract infections in humans have been isolated (3, 11; H. E. Larson, S. E. Reed, and D. A. J. Tyrrell, J. Med. Virol., in press). Two serological groups, the HCV 229E and HCV OC43 groups, that show no antigenic cross-reactions with each other (11, 14) have been recognized.

Antibodies to HCV 229E group (2, 4, 5, 7, 13) or HCV OC43 group (2, 5, 6, 8, 9, 13, 15) viruses have been detected in human sera by complement fixation, hemagglutination inhibition, neutralization, and more recently, by radial diffusion hemolysis and radioimmunoassay. These studies have shown that HCV infection is widespread in populations in Europe and the United States. Antibody rises have been found in human paired sera from natural (4, 5, 7, 8, 9, 13) and experimental (2) HCV infections by complement fixation, hemagglutination inhibition, and neutralization.

In this report we describe an enzyme-linked immunosorbent assay (ELISA), developed for animal sera (C. A. Kraaijeveld, M. H. Madge, and M. R. Macnaughton, J. Gen. Virol., in press), for the measurement of antibody rises in volunteers inoculated with HCV 229E and related strains. There was a close correlation between antibody rises detected by ELISA, clinical illness, and virus shedding from the volunteers. The assay was quicker and more sensitive than a micro-neutralization assay.

† Present address: Laboratory of Microbiology, State University, Utrecht, The Netherlands.

MATERIALS AND METHODS

Volunteers. The subjects were adults taking part in experiments at the Common Cold Unit, Salisbury. The general methods have been described (1, 16). In brief, volunteers in isolation received an intranasal inoculation of HCV or saline, and their clinical responses were assessed daily under double-blind conditions. At 6 days after inoculation the reactions were graded as nil, mild, moderate, or severe cold. Nasal washings for virus isolation were collected 3 and 4 days after inoculation by using Hanks' balanced salt solution, and sera were obtained 3 days before and about 21 days after inoculation. The experiments were approved by the Ethical Committee of the Harrow Health District (Clinical Research Council).

Viruses. HCV 229E was the prototype strain originally obtained from D. Hamre. The HCV 229E-related strains HCV PR, HCV TO, and HCV KI were isolated in tissue culture at the Common Cold Unit from nasal washings of subjects with natural colds (Larson et al., in press; S. E. Reed, manuscript in preparation; Macleod and Reed, unpublished data). HCV PR used for inoculation of volunteers was prepared from nasal washings of infected subjects and had not been passed in vitro.

Cell cultures and virological methods. The continuous cell line MRCc was originally obtained from A. F. Bradburne. The growth medium was Eagle basal medium with 10% newborn calf serum and antibiotics. Cultures inoculated with virus were maintained in L-15 medium with 2% fetal bovine or rabbit serum, diethylaminoethyl (DEAE)-dextran (15 μg/ml), and antibiotics.

For preparation of antigens for ELISA, MRCc cell monolayers were washed free of bovine serum and inoculated with HCV at a multiplicity of infection of about 0.02 50% tissue culture doses (TCD_{50}) per cell. Cultures were incubated stationary at 33°C in medium

with 2% rabbit serum, the medium was changed after 24 h, and the cultures were harvested at 42 to 48 h by scraping the cells into the supernatant medium. Suspensions were then frozen and thawed once, pipetted thoroughly to disperse cell debris and centrifuged at 4,000 rpm for 30 min. The supernatant fluids, having infectivity titers of $10^{6.0}$ to $10^{7.0}$ TCD_{50} per ml were used as antigens. Control antigens were prepared from uninoculated cells by the same method.

Virus was detected in volunteers' nasal washings by inoculation of tubes containing semiconfluent monolayers of MRCc cells. Cultures were incubated at 33°C on a roller apparatus, and typical HCV 229E-like cytopathic effect was read between 4 and 7 days postinoculation.

Neutralizing antibody in volunteers' sera was titrated by a micromethod by using MRCc cells in L-15 medium with 2% fetal bovine serum. Sera were inactivated at 36°C for 30 min and diluted from 1:2 to 1:128 in 0.025-ml volumes in microtiter plates. Virus diluted to contain approximately 100 TCD_{50} per 0.025 ml was added, and the mixtures were incubated for 1 h at room temperature. A total of 2×10^4 MRCc cells suspended in 0.1 ml of maintenance medium were added to each well, the test was incubated at 33°C for 5 days, and the cytopathic effect was reached by using an inverted microscope. A fourfold or greater rise in titer between pre- and postinoculation serum samples was considered significant.

ELISA. The ELISA method used to detect antibodies in human sera was based on a method described previously for animal sera (Kraaijeveld et al., in press). Flat-bottomed polystyrene microtiter plates (Dynatech) were coated with duplicate 0.2-ml amounts of antigen diluted in 0.1 M carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at room temperature. After incubation the plates were washed four times with phosphate-buffered saline containing 0.05% Tween 20 and 0.02% sodium azide (PBST) and shaken dry. Portions of 0.2 ml of sera diluted in PBST were added to the wells and incubated for 4 h at room temperature. After incubation, the plates were washed four times in PBST and shaken dry. Anti-human immunoglobulin G, directed against heavy and light chains, and labeled with alkaline phosphatase conjugate (Miles Laboratories) at a dilution of 1:800, was added in 0.2-ml quantities and left overnight at room temperature. After four additional washes with PBST, 0.2 ml of phosphatase substrate, consisting of a 0.1% solution of *p*-nitrophenylphosphate in 10% (wt/vol) diethanolamine buffer (pH 9.8) with 0.02% sodium azide and 0.01% $MgCl_26H_2O$, was added to each well. Absorbance values were read after 30 min at 405 nm in a Flow Titertek Multiscan photometer.

RESULTS

Detection of antibody rises in human paired sera by ELISA. Paired sera were obtained from 15 volunteers who were inoculated with HCV PR, an HCV 229E-related strain. Antibody rises were measured by ELISA using HCV PR, HCV 229E, and HCV TO as antigens. Optimum dilutions of the HCV antigens and selected sera were determined by checkerboard titrations, and an antigen dilution of 1:200 and serum specimens diluted to 1:25, 1:50, 1:0, and 1:200 were used for all assays. These dilutions generally produced a satisfactory range of absorbance values for postinoculation sera and acceptably low values for preinoculation sera.

Table 1 shows ELISA absorbance values of HCV antigens against dilutions of paired sera from one of the volunteers (Table 2, no. 13). This was a typical result, and it shows ratios of postinoculation serum to preinoculation serum absorbance values of over 2 for all serum dilutions with HCV PR, HCV 229E, and HCV TO. In another paper (Kraaijeveld et al., in press), we considered that a postinoculation serum was positive for specific antibodies when the ratio of postinoculation to preinoculation serum absorbance values at the same dilution was 2 or more. We have used the same criterion for significant antibody rises in this study and conclude that the volunteer in Table 1 had a significant antibody rise against HCV 229E, HCV PR, and HCV TO after inoculation with HCV PR.

The highest ratio of postinoculation to preinoculation absorbance values obtained at any of the serum dilutions tested was called the ELISA ratio. The standard error in the calculation of ELISA ratios between the same serum dilutions in different assays was less than 5%, except for absorbance values of less than 0.1, which were sometimes obtained with serum dilutions of 1:100 or 1:200, and ratios were not calculated in these cases. Table 2 shows ELISA ratios of paired sera from volunteers inoculated with HCV PR against HCV antigens for the serum dilutions of 1:25, 1:50, 1:100, and 1:200. Significant antibody rises of 2 or more are underlined. There was a close correlation between ELISA ratios obtained with the antigens HCV PR, HCV 229E, and HCV TO, and significant antibody rises were detected in the same pairs by the antigens, with the exception of one case (no. 5).

Figure 1 shows a histogram of ELISA absorbance values in preinoculation sera from 42 volunteers tested against HCV PR. A wide spread in absorbance values was observed, suggesting that the volunteers had wide variations in quantities of existing antibody from previous HCV 229E group infections. Similar results were obtained when these sera were tested against HCV 229E and HCV TO. The hatched and unhatched areas on this histogram represent preinoculation sera from volunteers who respectively showed no significant and significant antibody rises to these viruses in their postinoculation sera. Generally, more significant antibody rises were found in volunteers with low levels of HCV

TABLE 1. *ELISA of HCV isolates against dilutions of a pair of human sera*[a]

HCV antigen[b]	ELISA absorbance values against the following serum dilution							
	1:25		1:50		1:100		1:200	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Control	0.14	0.16	0.11	0.12	0.07	0.09	0.09	0.06
PR	0.24	1.24	0.12	0.92	0.07	0.74	0.09	0.77
229E	0.21	1.09	0.13	0.97	0.13	0.77	0.13	0.67
TO	0.22	1.97	0.13	1.45	0.07	1.12	0.07	0.81

[a] Paired sera from a volunteer (Table 2, no. 13) inoculated with HCV PR.
[b] Antigen at a dilution of 1:200 was clarified supernatant from cells infected or not infected with appropriate HCV isolate.
[c] Pre, Preinoculation; post, postinoculation.

TABLE 2. *Antibody rises in human paired sera detected by neutralization and ELISA*

Human paired sera[a]	Symptoms of common cold	Clinical score	Virus shedding	Neutralization using HCV PR[b]	ELISA ratios using HCV antigens[c]			
					Control	PR	229E	TO
1		0	−	−	1.2	1.4	1.0	1.2
2		0	−	−	1.1	1.1	1.5	1.2
3		0	−	−	1.2	1.1	1.1	1.1
4		0	−	−	1.1	1.4	1.1	1.0
5	Mild	26	+	−	1.1	1.4	**2.0**	**3.4**
6	Mild	20	+	−	1.1	**3.6**	**3.2**	**3.1**
7	Moderate	29	+	−	1.1	**3.3**	**3.1**	**2.6**
8		0	+	+	1.4	**3.7**	**3.7**	**4.9**
9	Mild	18	+	+	1.0	**3.4**	**2.9**	**5.1**
10	Mild	24	+	+	1.2	**2.7**	**3.5**	**6.2**
11	Moderate	43	+	+	1.2	**2.4**	**2.2**	**2.8**
12	Moderate	43	+	+	1.0	**3.5**	**3.3**	**8.0**
13	Moderate	49	+	+	1.1	**7.7**	**7.5**	**11.4**
14	Moderate	53	+	+	0.9	**2.9**	**2.1**	**5.4**
15	Moderate	75	+	+	1.1	**5.8**	**4.2**	**9.3**

[a] Paired sera from volunteers given HCV PR.
[b] +, Significant antibody rises; −, no antibody rises.
[c] ELISA ratios were the highest ratios of postinoculation to preinoculation sera absorbance values obtained for any of the serum dilutions 1:25, 1:50, 1:100, and 1:200. Antigen at a dilution of 1:200 was clarified supernatant from cells infected or not infected with appropriate HCV isolate. Significant antibody rises are boldfaced.

antibody before inoculation than in volunteers with high HCV antibody levels before inoculation.

Correlation of antibody rises with clinical illness. Significant antibody rises detected by ELISA in the paired sera were compared with significant antibody rises found by neutralization and with the volunteers' clinical responses (Table 2). There was a good correlation between the symptoms and clinical score, virus shedding, and antibody rises detected by ELISA. High preinoculation ELISA absorbance values also appeared to correlate with immunity to infection (Fig. 1). Ten of the volunteers had some form of clinical illness, and an additional volunteer (no. 8), although having no symptoms, shed virus. All these volunteers had significant antigody rises on infection detectable by ELISA. However, neutralization tests with HCV PR did not detect significant antibody rises in three volunteers (no. 5, 6, and 7), who had mild or moderate colds, shed virus, or had significant antibody rises by ELISA.

Antibody rises in human paired sera using homologous and heterologous antigens by ELISA. Paired sera from volunteers inoculated with HCV 229E and two related strains, HCT TO and HCV KI, as well as HCV PR, were tested by ELISA with antigens HCV PR, HCV 229E, and HCV TO. Significant antibody rises were detected in sera of volunteers who developed colds with homologous or heterologous antigens.

Studies using paired sera from volunteers infected with HCV 229E and antigen prepared from HCV OC43 grown in suckling mouse brain (12) and mouse hepatitis virus strain 3 grown in mouse embryo fibroblasts (10) did not detect

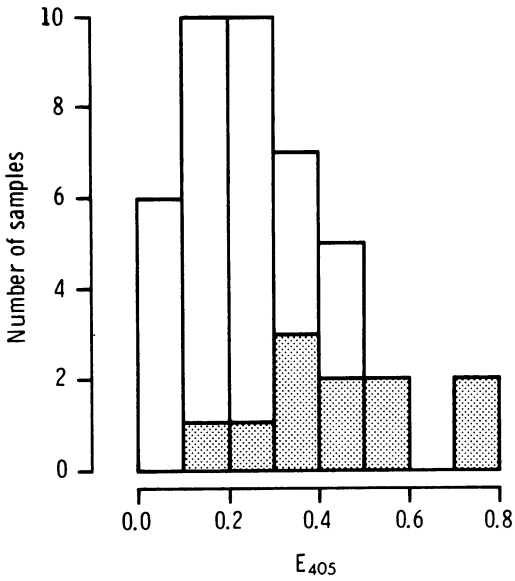

FIG. 1. *ELISA absorbance values obtained at E_{405} after 30 min using 1:200 dilutions of preinoculation sera from 43 volunteers givne HCV PR, HCV 229E, HCV TO, or HCV KI. Unhatched areas represent cases in which the paired sera showed a significant antibody rise by ELISA by using one or more of the antigens, and hatched areas represent cases in which no such rises were found.*

any antibody rises. Furthermore, no antibody rises were detected by using HCV PR as antigen with paired sera from volunteers inoculated with saline, influenza virus, or rhinovirus. In none of these cases was an ELISA ratio of more than 1:5 observed.

DISCUSSION

In this paper we have described an ELISA, using relatively impure HCV antigens, for detecting antibody rises to HCV 229E group viruses in paired sera from experimentally infected volunteers. There was a correlation between development of colds and significant antibody rises, and ELISA detected more significant antibody rises than did neutralization. ELISA appears to be applicable for detecting antibodies to several viruses related to HCV 229E when a single HCV 229E group virus was used as antigen, and to measuring antibody rises both in primary infections and in infections in volunteers with preexisting antibody. However, initial ELISAs have not been able to detect antibody rises in volunteers infected with HCV 229E when HCV OC43 was used as antigen. It would be interesting to compare our ELISA for HCV 229E group viruses with a radioimmunoassay similar to that described for HCV OC43 (6). These results confirm the suggestion that there are at least two distinct HCV antigenic groups (11, 14).

It would be expected that, using whole virus particles as antigens, our ELISA would detect only antibodies to surface structures. However, as the relatively crude virus preparations used contained mixtures of whole particles and subviral structures, including internal components (Macnaughton and Davies, unpublished data), it is not clear what antigenic determinants were involved in our ELISA. Although, in general, ELISA and neutralization detected antibody rises in the same volunteers, there is no reason to assume that the same antibodies were necessarily being measured by the two tests. Studies are in progress using a number of purified subviral components from HCV 229E group viruses as antigens in ELISA against human paired sera to identify the antibodies produced during infection and to determine the antigenic relationships between these viruses.

In conclusion, we have described an easily performed, accurate, sensitive assay for detecting significant antibody rises to HCV 229E group viruses by using relatively impure HCV 229E group viruses as antigens. This ELISA should prove useful for a clinical diagnosis of at least some HCV infections. Further studies are in progress to extend this assay to other HCVs.

ACKNOWLEDGMENTS

We thank Nicola Jones and Barrie Tyler for skilled technical assistance, J. W. Craig for care and assessment of volunteers, and D. A. J. Tyrrell for useful discussions.

LITERATURE CITED

1. **Beare, A. S., and S. E. Reed.** 1977. The study of antiviral compounds in volunteers, p. 27-35. *In* J. S. Oxford (ed.), Chemoprophylaxis and virus infections of the respiratory tract. CRC Press, Cleveland.
2. **Bradburne, A. F., and B. A. Somerset.** 1972. Coronavirus antibody titres in sera of healthy adults and experimentally infected volunteers. J. Hyg. **70**:235-244.
3. **Bradburne, A. F., and D. A. J. Tyrrell.** 1971. Coronaviruses of man. Progr. Med. Virol. **13**:373-403.
4. **Cavallaro, J. J., and A. S. Monto.** 1970. Community-wide outbreak of infection with a 229E-like coronavirus in Tecumseh, Michigan. J. Infect. Dis. **122**:272-279.
5. **Hendley, J. O., H. B. Fishburne, and J. M. Gwaltney, Jr.** 1972. Coronavirus infections in working adults. Eight-year study with 229E and OC43. Am. Rev. Respir. Dis. **105**:805-811.
6. **Hovi, T., H. Kainulainen, B. Ziola, and A. Salmi.** 1979. OC43 strain-related coronavirus antibodies in different age groups. J. Med. Virol. **3**:313-320.
7. **Kapikian, A. Z., H. D. James, Jr., S. J. Kelly, J. H. Dees, H. C. Turner, K. McIntosh, H. W. Kim, R. H. Parrott, M. M. Vincent, and R. M. Chanock.** 1969. Isolation from man of "avian infectious bronchitis virus-like" viruses (coronaviruses) similar to 229E virus, with some epidemiological observations. J. Infect. Dis. **119**: 282-290.
8. **Kaye, H. S., and W. R. Dowdle.** 1969. Some characteristics of hemagglutination of certain strains of "IBV-

like" virus. J. Infect. Dis. **120**:576–581.

9. **Kaye, H. S., H. B. Marsh, and W. R. Dowdle.** 1971. Seroepidemiologic survey of coronavirus (strain OC43). related infections in a children's population. Am. J. Epidemiol. **94**:43–49.
10. **Macnaughton, M. R.** 1980. The polypeptides of human and murine coronaviruses. Arch. Virol. **63**:75–80.
11. **McIntosh, K.** 1974. Coronaviruses: a comparative review. Curr. Top. Microbiol. Immunol. **63**:85–129.
12. **McIntosh, K., W. B. Becker, and R. M. Chanock.** 1967. Growth in suckling-mouse brain of "IBV-like" virus from patients with upper respiratory tract disease. Proc. Natl. Acad. Sci. U.S.A. **58**:2268–2273.
13. **McIntosh, K., A. Z. Kapikian, K. A. Hardison, J. W. Hartley, R. M. Chanock.** 1969. Seroepidemiologic studies of coronavirus infection in adults and children. Am. J. Epidemiol. **91**:585–592.
14. **Pedersen, N. C., J. Ward, and W. L. Mengeling.** 1978. Antigenic relationships of the feline infectious peritonitis virus to coronaviruses of other species. Arch. Virol. **58**:45–53.
15. **Riski, H., and T. Estola.** 1974. Occurrence of antibodies to human coronavirus OC43 in Finland. Scand. J. Infect. Dis. **6**:325–327.
16. **Tyrrell, D. A. J.** 1963. The use of volunteers. Am. Rev. Respir. Dis. **88**:128–134.
17. **Tyrrell, D. A. J., D. J. Alexander, J. D. Almeida, C. H. Cunningham, B. C. Easterday, D. J. Garwes, J. C. Hierholzer, A. Kapikian, M. R. Macnaughton, and K. McIntosh.** 1978. Coronaviridae: second report. Intervirology **10**:321–328.