

## Detection of Antibodies to Human Coronaviruses 229E and OC43 in the Sera of Multiple Sclerosis Patients and Normal Subjects

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Sera collected from 90 multiple sclerosis patients and 148 age-matched normal subjects were examined for the presence of antibodies against human coronaviruses (HCV) 229E and OC43 by enzyme immunoassay (EIA). The results demonstrated no significant difference between the MS patients and the normal subjects in their antibody titer to HCV 229E and HCV OC43. Further analysis of these 238 sera indicated that a stronger EIA reaction was generally observed against HCV OC43 (mean EIA value at an optical density of 492 nm = 0.896) than against HCV 229E (mean EIA value at an optical density of 492 nm = 0.346).

Multiple sclerosis (MS) is a demyelinating disease of unknown etiology which affects the central nervous system. Over 250,000 individuals in the United States alone suffer from MS (15). The epidemiological, clinical, and pathological features of MS are consistent with certain known viral infections (8). Many different viruses have been suggested as etiological agents of MS, including poxviruses, herpesviruses, rhabdoviruses, orthomyxoviruses, paramyxoviruses, and picornaviruses (1, 8, 14). Recently, the possibility of coronaviruses as etiological agents has been considered because of their role in demyelinating disease in rodents and some reports of association with MS in humans.

The JHM strain of mouse hepatitis virus, a murine coronavirus, is known to cause a demyelinating-remyelinating disease in mice and rats (2, 7, 12). Pathological examination of mice shows widespread demyelination of the brain and spinal cord characterized by the destruction of myelin with sparing of nerve fibers.

In 1976, Tanaka et al. (13) reported the presence of particles morphologically similar to coronaviruses in tissue removed at autopsy from an MS patient. The particles were observed in the cisterns of the rough endoplasmic reticulum of brain cells, the common site of coronavirus maturation. The particles were described as "doughnut shaped" with electron lucent centers, an inner diameter of 25 to 35 nm, and an outer diameter of 55 to 65 nm. The morphology of these particles most closely resembled that of mouse coronaviruses.

In another investigation, Burks et al. (4) reported the isolation of coronaviruses from two separate MS patients after repeated passage of brain material in mice or 17 Cl-1 cell cultures. Using immune serum prepared to the isolates in rabbits and guinea pigs in plaque neutralization experiments, these investigators reported that neither isolate was related to human coronavirus (HCV) 229E, but that both were serologically related to the mouse coronavirus A59 and HCV OC43. These findings were later confirmed by the more sensitive technique of immunoprecipitation (6). Identical polypeptides were precipitated from infected 17 Cl-1 cell cultures with antisera specific for either MS isolate, A59, and HCV OC43. The present investigation was undertaken to seek further serological evidence of a role for coronaviruses in MS by comparing serum antibody titers and incidence among patients and controls.

A diploid fibroblast cell line of human embryonic lung origin (M-7) was obtained from Microbiological Associates, Walkersville, Md. and grown in Eagle minimal essential medium with Earle salts (EMEM) supplemented with 4% newborn calf serum and 100 µg of streptomycin per ml and 100 U of penicillin per ml.

HCVs 229E (American Type Culture Collection) and OC43 (courtesy of G. Gerna, Pavia, Italy) were propagated in M-7 cells with serum-free EMEM with 100 µg of streptomycin per ml and 100 U of penicillin per ml. Virus infectivity was determined by plaque assay in M-7 cells.

Sera from patients with MS and other neurological diseases were supplied by F. Milgrom (State University of New York at Buffalo, Buffalo, N.Y.), W. Olszewski (Buffalo General Hos-

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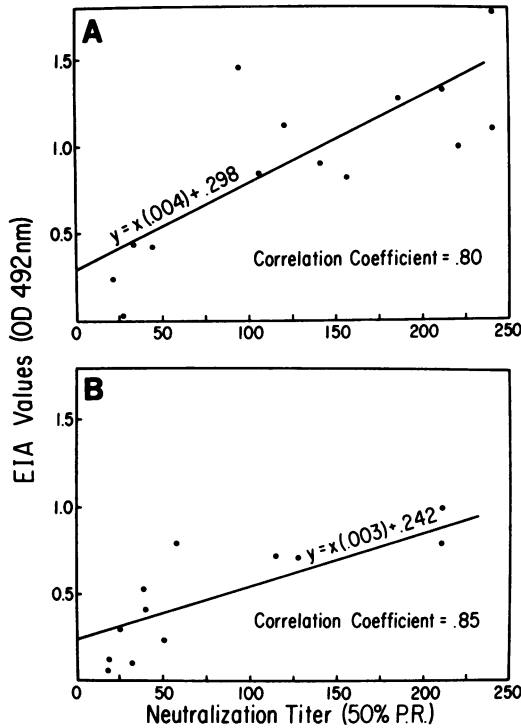


FIG. 1. Relationship between EIA measurements (OD<sub>492</sub>) and plaque reduction neutralization (50% plaque reduction) titers of 12 human sera against HCV OC43 (A) and HCV 229E (B).

pital, Buffalo, N.Y.), and L. D. Jacobs (Millard Fillmore Hospital, Buffalo, N.Y.). Sera from 148 age-matched normal subjects were obtained from the American Red Cross, Buffalo, N.Y. The sera were diluted 1:500 and absorbed by overnight incubation at 4°C on M-7 monolayers before use in enzyme immunoassay (EIA) or plaque neutralization.

Plaque neutralization assays were conducted with M-7 fibroblast monolayers in 24-well plates (Rochester Scientific co., Rochester, N.Y.). The method was similar to that described by Kennedy and Johnson-Lussenburg (10). The test serum was heat inactivated and absorbed by overnight incubation with washed M-7 cells. Serial four-fold dilutions of absorbed sera were made with phosphate-buffered saline (PBS; pH 7.2) as the diluent. The virus was diluted in serum-free EMEM with antibiotics, added to an equal volume of each serum dilution, and incubated for 1 h at 4°C. The final concentration of virus was 100 PFU/0.5 ml. After incubation, 0.1 ml of each sample per well (four wells per dilution) was added. The virus was allowed to adsorb for 1 h at 33°C followed by the addition of 1 ml of overlay medium consisting of EMEM, 0.6% Noble agar, 2% calf serum, 100 µg of bromo-

deoxyuridine, 200 µg of DEAE-dextran per ml, and antibiotics. Monolayers infected with 229E virus were fixed with 10% buffered Formalin after 3 days of incubation at 33°C and stained with 0.1% crystal violet. Fibroblasts infected with OC43 virus were fixed and stained after 5 days of incubation at 33°C.

To prepare antigens for EIA, we washed confluent monolayers of M-7 cells to remove serum and inoculated them with HCV OC43 or 229E at a multiplicity of infection of 0.01 PFU/cell. After adsorption at 37°C for 1 h, serum-free EMEM with antibiotics was added. The infected monolayers were incubated at 33°C for 48 h, and total virus was harvested by freezing and thawing the infected cells three times. Cellular debris was removed by centrifugation at 2,000 rpm for 20 min at 4°C, and the clarified lysates were used as antigen in the EIA procedure. The protein content of the antigen pools was determined by the Bradford (3) protein assay procedure (Bio-Rad Laboratories, Richmond, Calif.). The virus titer of the pools ranged from  $1.3 \times 10^5$  to  $2.2 \times 10^6$  PFU/ml with a mean titer of  $1.4 \times 10^6$  PFU/ml. A control antigen was prepared in a manner similar to that described above from uninfected M-7 cells.

The EIA method used was an indirect assay for the detection of antibodies to HCV 229E and HCV OC43. Test serum and peroxidase-conjugated goat anti-human immunoglobulin G (IgG) antisera, H and L chain specific (Northeast Biomedical Laboratories, South Windham, Maine), were diluted with PBS (pH 7.2) containing 0.05% Tween 20 and 1% bovine serum albumin (BSA). The plates were washed five times after each incubation period with PBS-BSA (PBS [pH 7.2], 0.05% Tween 20, 0.2% BSA).

Portions (300 µl) of viral antigen (10 µg/ml) in carbonate-bicarbonate buffer (pH 9.6) were added to one-half of the inner 60 wells of Microelisa substrate plates (Dynatech Laboratories, Inc.,

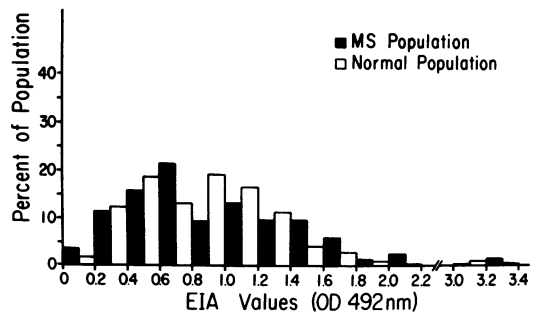


FIG. 2. EIA values (OD<sub>492</sub>) of sera from 90 MS patients and 148 normal subjects reacted with HCV OC43 antigen.

TABLE 1. Detection of coronavirus antibody in human sera by EIA

Serum from:	No. (%) with antibodies to 229E and OC43, respectively, of:					
	+ <sup>a</sup>	-	-	+ <sup>b</sup>	+	-
Normal subjects ( <i>n</i> = 148)	0 (0)		58 (39.2)		85 (57.4)	5 (3.4)
MS patients ( <i>n</i> = 90)	1 (1.1)		40 (44.4)		44 (48.9)	5 (5.5)
Neurological patients <sup>c</sup> ( <i>n</i> = 11)	0 (0)		6 (54.5)		3 (27.3)	2 (18.2)

<sup>a</sup> EIA values (OD<sub>492</sub>) > 0.242.

<sup>b</sup> EIA values (OD<sub>492</sub>) > 0.298.

<sup>c</sup> Diagnoses included Guillain-Barre syndrome, acute sciatica (2), acute-stage Bell's palsy, amyotrophic lateral sclerosis, peripheral neuropathy, and unspecified neuritis (5).

Alexandria, Va.). A control antigen of M-7 lysate diluted to the same concentration was added to the remaining wells. The plates were incubated at 4°C overnight and then treated with carbonate-bicarbonate buffer (pH 9.6) containing 0.5% BSA for 1 h at 37°C. After the plates were washed, absorbed test serum (1:1,000) was added to duplicate wells (300 µl/well) and incubated at 37°C for 1 h. After being washed, 300 µl of conjugate diluted 1:8,000 was added to each well and incubated for 1 h at 37°C. Conjugate controls were included on each plate. A 300-µl amount of diluted substrate (*o*-phenylenediamine dihydrochloride; Sigma Chemical Co., St. Louis, Mo.) was added to each well and incubated at 37°C for 30 min. The reaction was stopped by the addition of 50 µl of 2.5 M H<sub>2</sub>SO<sub>4</sub>. The optical density at 492 nm (OD<sub>492</sub>) was read. The EIA values for each serum assayed were determined by the formula EIA value (OD<sub>492</sub>) = mean ( $\bar{X}$ ) OD<sub>492</sub> with viral antigen -  $\bar{X}$  OD<sub>492</sub> with M-7 cell lysate antigen. Day-to-day variations in the EIA ( $\pm$  1 standard deviation) were corrected by adjusting the EIA values (OD<sub>492</sub>) by the value produced by a positive control serum included in each assay.

Fourteen sera from normal subjects were tested by EIA and plaque neutralization for the detection of antibodies to HCV 229E and HCV OC43. The correlation coefficient for comparison with HCV OC43 was 0.80 (Fig. 1A), and for HCV 229E it was 0.85 (Fig. 1B). The cut-off value for antibody activity against OC43 was an OD<sub>492</sub> of 0.298. This value was based on the comparison of EIA and neutralization data. Similarly, anti-229E antibody was indicated by OD<sub>492</sub> values greater than 0.242.

Serum samples taken from 90 MS patients and 148 age-matched normal subjects were then assayed for antibodies to HCV 229E and HCV OC43 by EIA. The results obtained when these two populations were tested by EIA for anti-OC43 activity are shown in Fig. 2. The data demonstrate that the MS and normal popula-

tions reacted similarly to HCV OC43. A total of 84 (93%) MS patients had antibodies to HCV OC43 in their sera (Table 1). A similar analysis of 148 normal subjects indicated the presence of anti-OC43 activity in the sera of 143 (96%) normal subjects.

The results given in Fig. 3 and summarized in Table 1 were obtained when these same sera were tested by EIA for activity to HCV 229E. The results obtained also indicate no significant difference in the antibody response to HCV 229E between MS patients and the normal control population. Table 1 shows that 45 (50%) MS patients had antibodies to HCV 229E in their sera. Antibody activity to HCV 229E was detected in 85 (57%) normal subjects. Chi-square analysis demonstrated no difference between the two populations with respect to OC43 ( $\chi^2 = 1.37$ ,  $\alpha = 0.01$ ) or 229E ( $\chi^2 = 1.25$ ,  $\alpha = 0.01$ ).

A comparison of Fig. 2 and 3 suggested a difference in response to HCV 229E and HCV OC43. The results with HCV 229E were skewed toward lower OD<sub>492</sub> values (Fig. 3). The maximum EIA value (OD<sub>492</sub>) produced with HCV 229E was 1.600 with sera from normal individuals or MS patients. The mean EIA value (OD<sub>492</sub>) with HCV 229E was 0.346. EIA values (OD<sub>492</sub>) observed with HCV OC43 virus ranged from 0 to 3.4 with a mean EIA value (OD<sub>492</sub>) of 0.896. The antibody response elicited against HCV OC43 displayed a bimodal distribution (Fig. 2). Chi-square analysis of the data shown in Table 1 indicates that the antibody response to HCV OC43 differed from that to HCV 229E in both the MS patients ( $\chi^2 = 41.61$ ) and the normal subjects ( $\chi^2 = 64.22$ ) assayed by the EIA in this survey ( $\alpha = 0.01$ ). The linear correlation coefficient of EIA values (OD<sub>492</sub>) between the OC43 and 229E reactivities of 238 sera was 0.221. This value indicates very little, if any, cross-reactivity between the antigens.

Sera taken from 11 patients with neurological diseases other than MS were also examined by EIA for antibodies to HCV 229E or HCV OC43.

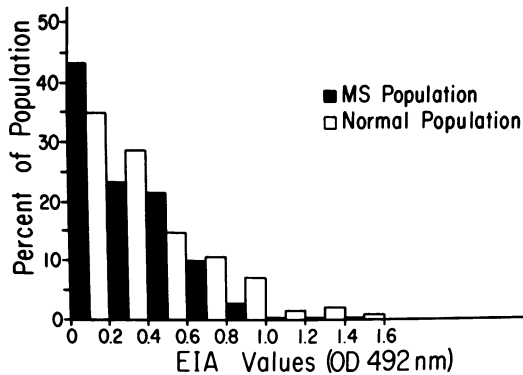


FIG. 3. EIA values (OD<sub>492</sub>) of sera from 90 MS patients and 148 normal subjects reacted with HCV 229E antigen.

The results obtained in these assays are summarized in Table 1. These patients responded to the virus strains in a manner similar to that of both MS patients and normal subjects.

The EIA assays indicated a difference in response to HCV 229E and HCV OC43. An examination of 238 sera (90 MS patients and 148 normal controls) by the EIA showed that a stronger reaction was generally produced to HCV OC43 than to HCV 229E. The apparent difference in antibody titers could be a result of a difference in the sensitivity of the EIA tests for OC43 and 229E. However, an examination of plaque neutralization data showed that the geometric mean neutralizing titer for HCV OC43 was 94.2 compared with a titer of 66.6 for HCV 229E when 12 random sera were tested against the viruses. Hendley et al. (7) had reported similar data for an 8-year serological investigation conducted between 1962 and 1970. Kaye and his associates (9) have reported a 33% reinfection rate with HCV OC43. These reports suggested that a disparity in antibody reactivity to these viruses exists in adults as a result of differences in the frequency of antigenic exposure. Nonetheless, some of the difference noted in the present study may be due to a lower sensitivity of the test for 229E antibody.

An examination of EIA values (OD<sub>492</sub>) indicated that MS patients had antibody levels comparable to those in normal individuals and patients with neurological diseases other than MS. These results agree with the data obtained by Leinikki et al. (11), who have recently reported finding no significant difference in serum reactivity between MS patients and control groups in regard to HCV 229E, HCV OC43, and mouse coronavirus A59.

The results reported here do not support the hypothesis of a role for HCV OC43 or HCV

229E in MS. Although EIA procedures theoretically should detect antibodies to a multiplicity of viral antigens (not just surface glycoproteins), it is still possible that a difference in response may exist in MS with respect to some other antigen of coronaviruses which was not detected by the EIA procedure used.

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