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CORONAVIRUS AND MULTIPLE SCLEROSIS

-EVALUATION OF A SEROLOGICAL STUDY-

by

Qingsi ZHENG, M.D.

A thesis submitted to the School of Graduate Studies
in partial fulfillment of the requirements for
the degree of
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Department of Microbiology and Immunology,
School of Medicine, Faculty of Health Sciences,
University of Ottawa,
Ottawa, Canada.

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UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

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ABSTRACT

The evaluation of a serological survey with statistical methods was carried out to determine if there was a relationship between human coronavirus (HCV/229E and persistent progeny VH) and multiple sclerosis (MS).

MS is a demyelinating disease of the CNS. The cause of MS is unclear, but in recent years both viruses and abnormal immune responses were thought to be involved in the etiology. Coronaviruses are one of the possible candidates. Since attempts to isolate viruses from patients with MS have all failed, epidemiological and serological methods were adopted.

From October, 1982 to May, 1983, 584 sera from 39 MS patients and control pairs were tested using an indirect immunofluorescence assay (IFA) method with HCV persistently infected cells (HV-1). The results showed no significant differences in the titres of serum antibodies in the two groups. This might suggest that coronaviruses are not associated with the cause of MS. However, our study indicated a significant association between colds and exacerbations of MS symptoms and there were significantly more HCV-related colds in the MS patients compared with their controls. This relevance of these findings needs further investigation.

A comparative antigenic analysis using plaque reduction and kinetic neutralization tests as well as immunodiffusion tests served to indicate that the VH persistent virus was antigenically distinct but related to the parental strain, 229E and supported the reliability and sensitivity of the IFA using HV-1 cells (VH virus). The use of the neutralization tests revealed that the antigenic differences were associated with the virion peplomer glycoprotein, E2.

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CORONAVIRUS AND MULTIPLE SCLEROSIS

- EVALUATION OF A SEROLOGICAL STUDY -

Prologue

A viral agent has been proposed as a cause of multiple sclerosis (MS), a chronic demyelinating disease of the central nervous system (CNS), but little substantive evidence has accumulated (Johnson and Herndon, 1974; Fraser, 1977; Cook and Dowling, 1980). Now there is growing evidence that abnormalities in immunologic control are operative in patients with MS although whether these changes are cause or effect of the disease is not clear (Lisak, 1980). Both mechanisms however, are not mutually exclusive; in fact, it would appear likely that they are sequential, the second being dependent on the first (Fischman, 1981; Johnson, 1982; McLeod, 1982).

Coronavirus may be involved in the etiology of MS and several reports of the presence of coronavirus-like particles in the CNS of MS patients have been published (Tanaka et al., 1976; Burks et al., 1980; Gerdes et al., 1981). Also, the JHM strain of Mouse Hepatitis Virus (MHV), a coronavirus, produces experimentally a demyelinating disease in mice similar to MS (Lucas et al., 1977; Nagashima et al., 1978). MHV persistent infections in cultured mouse cells have been reported (Lucas et al., 1977; Stohlman and Weiner, 1978; Hirano et al., 1981; Wege et al., 1982) and this in vitro murine system has been proposed as a suitable model for extrapolation to the human disease (Lucas et al., 1978).

Recently, we have participated in a serological study and an antigenic analysis of human coronavirus, strain 229E in association with MS. The aims of the study were:

1. To determine if an etiological relationship exists between coronavirus and MS by serological survey.

2. To undertake a comparative antigenic study of HCV/229E and the progeny VH coronavirus which is shed by the persistently infected HV-1 cell system established in our laboratory in 1979 (Chaloner-Larsson and Johnson-Lussenburg, 1981a&b).

The preliminary serological study was performed in 1980-1981 and followed by the case/control study in 1982-1983. My participation in this study started at the time the serological assays (IPA) were nearly completed and my role in this work was to undertake the statistical analyses of these results, in consultation with members of the Department of Epidemiology and Community Medicine, University of Ottawa. These results led to the next phase of my work which was to detect and evaluate the antigenic differences between the parent strain HCV/229E and its progeny persistent, VH virus. That these viruses differed antigenically was suggested as a result of the serological survey and other evidence which became apparent during this time.

The work described in this thesis is presented in such a way as to reflect the two perspectives of this study; the first being concerned with the statistical evaluation of the results, which included the test used, and the second being concerned with the antigenic analysis of the 229E and VH viruses which was relevant to the interpretation of the results of the case/control study.

Chapter I
INTRODUCTION

1. Multiple Sclerosis (MS).

MS is a demyelinating disease which affects the central nervous system (CNS). For over a century , MS has intrigued workers in all the neurological sciences, but it still remains a disease of unknown cause, inadequate treatment, and unpredictable outcome.

Whatever the cause, MS short-circuits the electrical messages that pass between the CNS and rest of the body by attacking a fatty substance called myelin, which insulates nerve fibers, and replacing it with sclera, or scar tissue. This causes tremours, weakness or paralysis and often impared vision and speech and loss of urinary control. The symptoms depend on the part of the nervous system affected. For example, MS in the spinal cord can cause weakness, numbness, pricking, or paralysis of the arms or legs. In the optic nerve, it may lead to uncontrolled eye movements or double vision. MS is considered a leading chronic disability among young adults, usually striking healthy whites between the ages of 20 and 40 (Garelik, 1985). It was reported that over 250,000 individuals in the United States alone suffer from MS (Waksman, 1981 a&b).

According to epidemiological investigations, MS has a unimodal age-specific onset curve. It rarely begins before the age of 15 or after the age of 50, although cases have been described in children as young as 2 (Waksman, 1981a). Age-specific onset curves show a sharp peak at age 30 for both men and women. Females with MS outnumber males by 1.5 to 1 (Acheson, 1977; Johnson, 1982).

The geographic distribution of MS is perhaps the most consistent

characteristic observed by epidemiologists. It is considered that the worldwide distribution of MS comprises three zones of frequency or risk (Kurtzke, 1984). The high risk zone, with prevalence rates of 30 and above per 100,000 population, includes northern and central Europe into the USSR, northern US and southern Canada, New Zealand, and south-eastern Australia. These regions are bounded by areas of medium frequency, with prevalence rates between 5 and 29 per 100,000, and include the southern US, south-western Norway and northern Scandinavia, and probably the USSR from the Ural Mountains into Siberia as well as the Ukraine. Except for Sicily which is reported to have a high rate, the entire Mediterranean basin from Spain to Israel - and possibly including Tunisia in northern Africa - are also regions of medium prevalence. Also in these medium risk zones fall most of Australia, perhaps Hawaii and the midportion of South America. Low frequency risk with prevalence rates below 5 per 100,000, characterizes all the other known areas of Asia and Africa, Alaska and Greenland, and the Caribbean region including Mexico and probably northern South America (Kurtzke, 1984).

These observations seem to suggest that MS prevalence is associated with latitude, but this relationship is not yet well established. There are wide variations in prevalence rates at the same latitude. For example, the prevalence of MS is reported to be very high throughout the British Isles and Scandinavia, but there are differences in reported among the Shetland Islands, the Orkney Islands, and the Faroe Islands and within sections of Denmark, Sweden, and Finland. In addition, repeated surveys for MS in Japan have failed to reveal a prevalence relevant to Japan's latitude. The prevalence is less than 10% of that expected when compared to northern Europe and North America. Further-

more, there are no differences among regions of Japan, which extends over an area of 15 northern latitudinal degrees. (Detels, 1978).

Several investigators have tried to correlate the prevalence of MS with factors such as temperature, altitude, pets, major dietary component (e.g., rice/wheat, sheep/cattle, high proteins/low proteins, etc.), solar irradiation, or trace metals. None of these correlations have been confirmed (Detels, 1978; Ellison, 1984; Norman, 1983; Cook, 1985; Lauer, 1984).

All high and medium-risk areas comprise predominantly white populations: MS is the white man's burden (Kurtzke, 1983). In America, blacks and Orientals, and possibly Indians, have much lower rates of MS than do whites, but each group still demonstrates the geographical gradients found for whites (Kurtzke, 1983).

Migration studies indicate that, on the whole, migrants retain much of the risk of their birthplace. MS death rates for migrants born in one risk area and dying in another are intermediate between those characteristic of their birthplace and their death residence regardless of the direction of the move. Prevalence studies for migrants from high to low risk areas indicate the age of adolescence to be critical for risk retention: those migrating beyond age 15 retain the MS risk of their birthplace; those migrants under 15 acquire the lower risk of their new residence. Several low to high studies show that those migrating in childhood or adolescence do in fact increase their risk of MS (Kurtzke, 1983,1985; Ellison, 1984; Lauer, 1984).

The migration data, plus the geographic distribution, serve to define MS as an acquired, exogenous (environmental) disease whose acquisition in ordinary circumstances takes place years before clinical

onset. In the native population in the Faroe Islands previously free of the disease, an outbreak of 24 cases of MS occurred between 1943 and 1960 following the period of British occupation. This was the first apparent point-source epidemic of the disease, and was explained by a putative infectious agent. This indicated that MS seemed not only to be acquired but also to be transmissible (Kurtzke and Hyllested, 1979; Kurtzke, 1980,1983,1985).

The cause of MS is still unclear despite numerous studies since the clinical disease was first described by Jean-Marie Charcot and an infectious cause was postulated by Pierre Marie (1884) more than a century ago (Johnson, 1975). No one knows what causes it. In recent years speculation has grown that the etiology of MS is viral. Another hypothesis ascribes it to a malfunction of the immune system.

Based on the virus theory, MS is caused by one or more slow-acting microorganisms, the symptoms of which appear years after infection or by a delayed reaction to a common virus. The exposure is believed to be at or before the time of puberty, and an incubation period of 3 to 30 years elapses before the onset of the disease. This long period of latency is consistent with other types of human demyelinating diseases associated with viral infections, such as postinfectious encephalomyelitis and progressive multifocal leucoencephalopathy. The remissions and exacerbations of the clinical signs and the multifocal areas of demyelination typical of MS are also consistent with a viral infection. The pattern of myelin loss in MS shows a wider zone of loss of the periaxonal myelin-associated glycoprotein than of the basic protein, which is distributed throughout myelin. The same pattern is seen in progressive multifocal leukoencephalopathy.

The viruses which have been suggested as etiological agents of MS

include poxviruses (Johnson, 1982), herpesviruses (Gudnadottir et al. 1964; Wrobleska et al., 1979), rhabdoviruses (Johnson, 1982), paramyxoviruses (ter Meulen et al., 1972; Field et al., 1972; Koprowski et al., 1975; Lampert, 1975), orthomyxoviruses (Johnson, 1982), coronaviruses (Burks et al., 1980; Tanaka et al., 1976), togaviruses (Johnson, 1982), picornaviruses (Friedmann and Lorch, 1985) , and a variety of "uncharacterized viruses" such as the Carp agent (Carp et al., 1972, 1977, 1978) and the SMON (subacute myelo-optico-neuropathy) or IM (Inoue-Melnick) agents (Inoue et al., 1984; Melnick, 1982; Melnick et al., 1984). They have been implicated by serological studies, morphological demonstrations of virus-like particles in tissue, and isolations of agents or transmission of disease in animal models. The first serological studies reported higher levels of antibody against measles virus in MS patients than in control groups (Adam and Imagawa, 1962). These findings have been confirmed in over 30 subsequent studies (Norrby, 1978). In another study, 23% of patients with MS had disproportionately ratios of high spinal fluid to serum antibody titres to two or more viruses, including measles virus (Norrby et al., 1974). This might suggest that either multiple viruses can cause MS or that in MS, pre-programmed B cells are non-specifically activated or released from immunoregulation after entering the CNS (Cremer et al.,1980). Thus the preponderance of measles antibody could simply reflect a larger population of peripheral B cells sensitized to measles than to other viruses (Johnson, 1982).

The immunological theory holds that the body's system of defenses may attack and destroy its own tissue. This may occur by mistake however, since viruses take over and change body cells when they invade. The immune system may thus destroy the cells which it now does not

recognize as self and in the process, hopefully eliminate the virus. Studies reporting abnormal cell- mediated immune (CMI) responses to virus are less well documented. Although there are reports of altered responses to a variety of viruses, these responses may correlate with disability rather than specific disease (Cook and Dowling, 1980). Also, abnormalities in immune responses are not found in every patient with MS, and they do not appear to be specific for MS because serum antibody increases, similar to those associated with measles virus antigens, have been documented in systemic lupus erythematosus and chronic active hepatitis (Johnson, 1982).

There have been some reports on morphological studies of particles in autopsy and biopsy tissue of patients with MS, and on attempts to isolate the agents in animals. But scientists have been unable to recover the same agent consistently, and in no case has isolation been confirmed in multiple laboratories. At present, no definitive evidence has incriminated a specific virus or other causes in the etiology of MS, but an increasing body of circumstantial and fragmentary data has accumulated and the role of a viral-like agent is still generally accepted.

2. Human coronavirus 229E and persistent progeny VH virus.

(i) General properties of coronavirus.

In 1968, coronaviruses were recognized as the agents responsible for a number of common diseases in a wide range of animals, including the common cold in humans (Almeida et al., 1968). These diseases were quite distinct; in some species the predominant illness was respiratory, in others gastrointestinal and in still others neurological. Lately, there has been evidence that coronaviruses can cause chronic, unrecog-

nized infections besides the acute diseases. It is believed that such persistent infections of humans and animals may be involved in a number of chronic and degenerative diseases (Wege et al., 1982).

The coronaviruses are enveloped with a particle diameter of 80-130 nm. The name for the group was adopted to describe the characteristic fringe of crown-like projections seen around the viruses by electron microscopy; these projections are much larger and rounded rather than sharp or pointed as is the case with the myxoviruses.

The particles contain an unsegmented genome of single-stranded RNA (MW $6\text{-}7 \times 10^6$). The helical nucleocapsid is 7-9 nm in diameter and matures in the cytoplasm by budding into cytoplasmic vesicles. The 3 chief proteins include a 60K phosphorylated nucleocapsid protein (NP), a 90K glycoprotein making up the petal-shaped structure (E2, peplomer) and a 23K glycoprotein (E1) embedded in the envelop lipid bilayer. During replication, viral antigens are found only in the cytoplasm of infected cells (Siddell et al., 1982).

Each strain of coronavirus restricts itself to its own animal species, creating difficulties in growing them in the laboratory, especially the human strains. But now cell systems have been developed and we have an understanding of the replication strategy of the virus. Some strains of human coronaviruses still require human embryonic tracheal or nasal organ cultures (HCV/OC43) whereas others will replicate in human embryonic lung, intestine or kidney cell cultures (HCV/229E). The optimal temperature for growth of the human strains is 33-35°C.

The Coronavirus Family includes two human respiratory strains, avian infectious bronchitis virus (IBV), mouse hepatitis virus (MHV), an enteritis virus of swine, and others. Antigenic cross reactions occur

between some human and some animal strains, but avian IBV appears to be unrelated to human agents. The avian and nonavian coronaviruses each appear to fall into two distinct and unrelated groups. In the case of IBV, at least eight different serotypes (Hopkins, 1974) have been recognized and these again fall into two groups (Darbyshire et al., 1979). The relationships are shown in Table 1. The location of antigenic sites on the coronavirion structural proteins has been investigated. Coronaviruses basically contain three major antigens, as has been shown by immunodiffusion experiments (Hajer and Storz, 1978; Yaseen and Johnson-Lussenburg, 1981). In human, porcine and murine systems the antigenic sites responsible for the induction of neutralizing antibodies are associated with the surface E2 glycoproteins (peplomers) (Garwes et al., 1978; Macnaughton and Davies, 1981; Schmidt and Kenny, 1981; Storz and Rott, 1981). The surface glycoproteins are also involved in complement fixation (CF) and hemagglutination inhibition (HI) reactions, however, not all strains hemagglutinate. The E1 and NP antigens are not involved in conferring protection and therefore, are not involved in neutralization tests but can be detected by CF, immunofluorescent (IF) and immunodiffusion (ID) tests using appropriate antibodies.

(ii) Human coronavirus 229E

229E is the human coronavirus prototype strain. In Chicago during the winter of 1962, five agents were isolated in primary human kidney cell cultures from specimens collected from medical students with a common cold (Hamre and Procknow, 1966). The viruses were ultimately adapted to WI38 cultures and exhibited a type of cytopathic effect (CPE) not previously seen. A prototype strain, 229E, was selected for

Table 1: Antigenic relationships of coronaviruses.*

Mammalian			
Group 1		**Group 2**	
HCV-229E and other isolates		HCV-OC43 and other isolates	
TGEV	one serotype	MHV	many serotypes, also related to RCV and SDAV
CCV	one serotype	BCV	one serotype
FIPV	one serotype	HEV	one serotype
Avian			
Group 3		**Group 4**	
IBV	at least 8 serotypes	TCV	one serotype
No cross-reactions with other strains		No cross-reactions with other strains	
Unclassified isolates			
Several isolates of HCV (and HECV); Porcine coronavirus, CV-777 and others; FECV; RTV; rabbit coronavirus; feline enteritis coronavirus.			

* Key to Abbreviations.

BCV.....bovine coronavirus
CCV.....canine coronavirus
FECV.....foal enteritis coronavirus
FIPV.....feline infectious peritonitis virus
HCV.....human coronavirus
HEV.....hemagglutinating encephalomyelitis virus
HECV.....human enteric coronavirus
IBV.....infectious bronchitis virus
MHV.....mouse hepatitis virus
RTV.....runde tick coronavirus
TCV.....turkey coronavirus
TGEV.....transmissible gastroenteritis virus

- From Wege et al.,1982

characterization and was found to be RNA-containing, ether-labile, and 89nm in diameter, but distinct serologically from any known myxo- or paramyxoviruses. Sera collected from the five medical students all exhibited a 4-fold rise in neutralizing antibody titer against 229E. The 229E strains were soon also demonstrated to have a similar structure on electron microscopy and to develop in infected cells by budding into cytoplasmic vesicles. As a result of the similarity of the human agents to IBV and also to mouse hepatitis virus (MHV), they were collectively considered to represent a group of vertebrate viruses antigenically and structurally distinct from the myxoviruses (Hamre and Beem, 1972; Tyrrell et al., 1975).

HCV/229E was originally isolated in cell culture and eventually adapted to human diploid lung cells (WI38). However, these diploid cells were not entirely suitable for primary isolation of 229E-like agents (Monto, 1984). Another cell system, L132, a heteroploid human lung line, has been reported to be more reliable for primary isolation of 229E (Bradburne and Tyrrell, 1969) and more recently, HFT (human fetal tonsil) cells have been used (Schmidt and Kenny, 1981).

(iii) Persistent HV-1 Infection and the Immunofluorescence Assay.

A persistent infection by human coronavirus 229E (HCV/229E) was established in the human continuous cell line (L132) in our laboratory in 1979. The L132 cells persistently infected with and continually shedding high titers of coronavirus have been maintained successfully since their establishment (Chaloner-Larsson and Johnson-Lussenburg, 1981a&b). To distinguish them from the standard HCV/229E:L132 system, the persistently infected cells have been termed HV-1 cells and the virus derived therefrom, VH virus. It is the only in vitro persistent

virus/host system of human coronaviruses.

The HV-1 cells have been continuously shedding virus through more then 400 infected cell passages performed at intervals of 2 days at the usual split ratio for normal uninfected cells of 1:3. Virus titers obtained by plaque assay (Kennedy and Johnson-Lussenburg, 1975/76) reveal amounts of cell released virus in the supernatant fluid (SNF) varying between 10^4 pfu/ml after splitting to 10^5 pfu/ml when the cells reach confluence. Total virus present in the whole cultures is consistently 10-fold higher (10^5-10^6 pfu/ml). Stocks of the persistently infected cells can successfully be stored at -80°C and revived without difficulty.

The mechanism of the establishment and maintenance of viral persistence in this system has been studied. The persistently infected cells are resistant to superinfection with standard HCV/229E but poliovirus replication appears to be entirely unaffected when compared to infection of normal L132 cells. Defective interfering (DI) particles and reverse transcriptase could not be detected. Virulence appears to be expressed more efficiently in normal cells at 33°C but the persistent virus shedding condition can only be maintained at 37°C (Chaloner-Larsson and Johnson-Lussenburg, 1981a & b). It has recently been shown that both alpha and beta interferon are involved in the maintenance of this persistent infection (Chudzio and Johnson-Lussenburg, unpublished results).

A reliable and routine indirect immunofluorescence assay (IFA) has been developed using the persistently infected cells HV-1. Monolayers of the already infected HV-1 cells were grown on 8-chamber LabTek slides and, after acetone fixation, stored at -20°C. The procedure of staining was standard (Pedersen et al., 1978). These prepared slides can be used

in laboratory studies for determining antigenic relationships of the coronaviruses and for the screening and titration of sera obtained from MS patients and controls. The IFA LabTek chamber system has also been used to study the mechanisms of persistence since the condition of cell monolayers and viral antigens can be readily evaluated by IFA and compared with virus production and cell viability at relevant intervals during experiments.

The specificity of the IFA reaction has been confirmed using known convalescent serum samples (pre- and post-exposure sera against both 229E and OC43 virus infections) which were provided by the Common Cold Research Unit, Salisbury, England, through the courtesy of Dr. S. Reed. There was no difference in the appearance of monolayers stained with absorbed, hyperimmune guinea pig serum (Yaseen and Johnson-Lussenburg, 1981) or the anti-229E convalescent (post-exposure) sera. There were very low titers associated with some of the OC43 sera, but since the titres of the paired sera were unchanged, it was assumed that there were a low levels of 229E antibody from a previous infection. Further confirmation of the specificity of the IFA reaction was obtained by using convalescent and hyperimmune animal sera from cats, dogs and pigs which had been exposed to feline infectious peritonitis virus, canine coronavirus and transmissible gastroenteritis virus respectively. Since these viruses belong to the same antigenic group as 229E (Table 1), positive IFA reactions would identify specific shared coronavirus antigens. Furthermore these sera should not cross react with the human host cell antigens thus providing a control for the fluorescent focus assay. These antisera, in addition to the standard guinea pig absorbed hyperimmune serum were included routinely on randomly selected slide squares as quality controls for both the specificity of the IFA reaction

and the condition of the HV-1 cell monolayers.

3. Multiple Sclerosis and Coronaviruses.

As previously mentioned, in recent years, epidemiological evidence and some clinical evidence have supported the viral hypothesis in the etiology of MS. Human coronavirus is one of the candidates.

The JHM strain of mouse hepatitis virus, a murine coronavirus, is known to cause a demyelinating-remyelinating disease in mice and rats which manifests as a disease similar to MS (Lucas et al, 1977; Nagashima et al, 1978; Wege et al, 1982; Johnson, 1984). Further support of coronavirus as the candidate etiologic agent in MS was provided by several reports of the presence of coronavirus-like particles in and the isolation of coronavirus from the CNS tissue of MS patients (Tanaka et al., 1976; Burks et al., 1980; Gerdes et al., 1981). In 1976, Tanaka et al. reported the presence of particles morphologically similar to coronaviruses in tissue removed at autopsy from an MS patient. The particles were observed in the cisternae 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 35nm, and an outer diameter of 55 to 65nm. The morphology of these particles most closely resembled that of mouse coronaviruses.

In another investigation, Burks et al. (1980) reported the isolation of coronaviruses from two MS patients after repeated passage of brain material in mice or 17 Cl-1 mouse cell culture. Using rabbit and guinea pig immune serum prepared against the isolates, these investigators reported that, in plaque neutralization experiments, neither isolate was related to human coronavirus (HCV) 229E, but that both were

serologically related to the mouse coronavirus A59 and the human OC43. These findings were later confirmd by the more sensitive technique of immunoprecipitation (Gerdes et al. 1981). Identical polypeptides were identified from infected 17 Cl-1 cell cultures with antisera specific for either the MS isolates, A59 or HCV OC43. But unfortunatly, these results did not clarify whether the MS viruses are of murine or human origin since the human OC43 and the MHV strains are antigenically related (Table 1), and mouse cells, in which MHV may be latent, were used for virus isolation.

Thus, so far, a viral cause for multiple sclerosis remains only an attractive hypothesis, not an established fact. None of the proposed candidate viruses have been shown to be unequivocally associated with the cause of MS.

Chapter II

MS CASE/CONTROL SEROLOGICAL STUDY

1. Background.

The possibility of human coronavirus as an etiological agent of MS has been considered because the disease it causes in humans fits the criteria required (common disease of childhood, Johnson, 1982) and because of its role in experimental demyelinating disease in rodents and because there have been some reports of its association with MS in humans. Coronavirus is one of the more common causes (16-20%) of the "common cold" in both adults and children (Bradburne et al., 1971; Kaye et al., 1971; McIntosh, 1974). In the case of these infections, it has been suggested that a transient meningoencephalitis may occur during the acute phase of the illness (Robb and Bond, 1979) providing the virus with access to those cells of CNS which are most likely involved in MS. The implication of a coronaviral agent in the etiology of MS by serological methods has been attempted by many workers with equivocal results (Cook and Dowling, 1980; Madden et al., 1981a & b; Leinikki et al, 1982; Hovanec and Planagan, 1983). Using a sensitive enzyme-linked immunoassay (ELISA) these workers reported no significant difference in the serum reactivity between MS patients and control groups. The conclusion by Madden et al. (1981b) that coronaviruses do not appear to be associated with the cause of MS has been disputed by Fleming et al. (1982). These workers and others (Bradburne and Somerset, 1972; Macnaughton, 1982) however found generally high proportions of positive serum reactors in each group. It should be mentioned here that in these studies, no attempts were made to document the prevalence of coronavirus infections in the communities and no information about seasonal factors was provided. Also,these sera were random, individual samples and not sequential repeats. Thus, there was no way to distinguish acute corona-

virus infections from the putative persistent viral involvement in MS.

In order to elucidate the etiological role of coronavirus in MS, the present investigation was undertaken to seek further serological evidence of a role for coronavirus in MS by comparing serum antibody titers among patients and controls. In the preliminary study, sera were obtained from 28 MS patients and 14 age/sex matched controls. The results of the microimmunodiffusion test in cellulose acetate (Johnson et al. 1964) which was used to detect 229E antibody, indicated more positive serum reactions in the MS group than in the controls (Table 2. Johnson-Lussenburg, unpublished results).

Table 2. Preliminary Immunodiffusion Study of Coronavirus Antibodies in MS patients and matched controls.*

	+	-	Total
MS Patients	25 (89%)	3	28
Controls	3 (21%)	11	14

* Groups significantly different, $P < 0.01$.

The presence of specific anti-229E antibodies in the positive sera was confirmed by the plaque reduction test on several selected sera. These sera were also tested by an immunofluorescence assay (IFA) using the persistently infected HV-1 cells. With this more sensitive assay, all the sera, both control and MS were positive at titers of 1/20 or greater. However, the titers of MS patients were higher; 60.7% of the MS sera (17/28) and 42.9% of the control sera (6/14) were positive at a 1/80 serum dilution. The geometric mean titers (GMT) for both groups were 65.01 for MS and 45.92 for controls and this difference was found to be of borderline significance ($P < 0.1$ by the Student's t-test; White, 1973). Since in this study, only small numbers of sera had been

obtained sporadically as they became available, it was not feasible to control for seasonal or other environmental factors. Therefore, a longitudinal, in depth serological study of MS patients was planned, with controls selected to meet the criteria necessary for statistical evaluation. Since coronavirus is one of the agents which causes the common cold, its cyclical pattern, seasonal incidence and other environmental factors had to be considered. To cover the putative "cold season" (late winter and early spring) a minimum 8 month period was considered essential. The case control study started in October of 1982 and ended in May of 1983. The aim of this study was to try to demonstrate a difference in the immune response of persons with MS compared to their controls as reflected by the levels of circulating HCV/229E antibody.

2. Materials and Methods.

(i) Subjects: Approval for the study was obtained from the Ethics Committee of the Faculty of Health Sciences, University of Ottawa and the Ottawa General Hospital. Through the Multiple Sclerosis Clinic at the Ottawa General Hospital and with the collaboration of its Director, Dr. R.F. Nelson, area MS patients were invited to participate in the study. They were requested to provide their own 'pal' controls; either a spouse, relative or close associate (neighbour, friend, colleague at work) who would be willing to participate for the duration of the 8 month study. A total 39 MS patients and appropriate controls volunteered making a total of 78 individuals which could be further sub-divided on the basis control types i.e. spouse controls (20) who were age and environmentally matched versus non-spouse controls (19) who were age and sex-matched and had a common community environment.

Two questionnaires (Appendix 2) were completed by all the partici-

pants; the first upon entry into the study, was designed to provide information about their respiratory infections, recent or otherwise, in addition to other relevant details such as age, sex, duration of disease (case), relationship to patient (control) etc. while the second, at the end of the study, was designed to obtain a few details about childhood and family background up to age 15 as well as further details of previous respiratory infections.

(ii) MS and control sera: In this study, a total of nearly 1200 human sera was collected. These included 584 out of a possible 624 for the MS/control group or 94% compliance by the participants. In addition, in an attempt to monitor the levels of HCV/229E antibodies in the general population to determine the prevalence of 229E respiratory infections, sera from two different sources were obtained as community controls. Through the courtesy of Dr. G. Rock of the Ottawa Red Cross Society, sera were obtained from 325 blood donors in 13 weekly batches of 25 over the late spring period, March to June, 1983. And through the courtesy of Dr. E. Rossier, Regional Virus Diagnostic Laboratory, Children's Hospital of Eastern Ontario, some 260 serum samples were randomly selected from several thousand available. Unfortunately these latter specimens were taken during the 1980/81 and 1981/82 seasons and were not therefore temporally matched.

HCV/229E positive control sera included absorbed hyperimmune guinea pig serum (Yaseen and Johnson-Lussenburg, 1981) and feline convalescent FIP antiserum as described previously.

All sera were heat inactivated at 56°C for 60 min. before use. Following initial IFA screening of 101 of the MS/control group sera at dilutions of 1/5 (24), then 1/10 (77), in which all specimens were positive, the remaining sera were screened at 1/20. All the positive

reactors were then titrated (1/20 - 1/160) using the IPA with coronavirus persistently infected cells. The sera were all coded and tested blind and the reactions were read by the same individual. The code was not broken until the end of the series. A limited number (11) of paired case/control sera were selected for further serological characterization using ELISA, neutralization and immunodiffusion analysis.

(iii) Immunofluorescence Assay (IFA): In this study, an indirect immunofluorescence assay (Pedersen et al., 1978) using the coronavirus persistently infected cells (HV-1) was used. Before cell culture, the 8-chamber LabTek (Flow Laboratories) slides were conditioned by adding 0.5 ml minimal essential medium (MEM) with 10% fetal calf serum (FCS) to each chamber and incubating at 37°C overnight. After removing the MEM from each chamber, 0.5 ml of the HV-1 cell suspension ($4-7.5 \times 10^4$ cells/ml), was added, the slide chambers were placed on wet filter paper in large glass petri dishes and incubated at 37°C in 5% CO_2 until the cells reached confluence (generally 24-30 hrs). After removing the medium and the upper plastic chamber, the slides, with the silicone gaskets still in place, were washed in PBS, fixed with acetone for 15-20 min., allowed to air dry, and stored at -20°C. These prepared slides have a minimum shelf life of 3 months at this temperature.

Briefly, the procedure for immunofluorescence staining was as follows: 50 ul of the antiserum dilution was added to each monolayer square (8 squares/slide = 8 different sera) and incubated in a moisture chamber at 37°C for one hour. The slides were then washed three times in PBS and allowed to dry. 50 ul/well of commercial preparations of appropriate anti-globulins conjugated with fluorescein isothiocyanate was then added and the slides again incubated at 37°C for one hour. After incubation, they were washed in PBS, counter-stained with 0.002%

Evans blue for 15 min. then blotted dry. The slides were examined by epifluorescence using a Leitz Laborlux II microscope.

(iv) ELISA: These tests were performed by C.M.Johnson-Lussenburg through the courtesy of J. Fleming, University of Southern California, Los Angeles, U.S.A. and were based on the method described by Sutter et al. (1980).

Flat-bottomed polystyrene microtiter plates (96-well) were coated with suitable dilutions of 229E or VH antigen (infected cell lysates, clarified at 3000 rpm x 30 min.) and incubated overnight at 4°C. After incubation, the antigen was removed from the plates by "flicking" and they were allowed to air dry. The wells were then washed twice with ELISA medium (Appendix 1) and shaken to dry. Test and control serum dilutions were added to the wells and incubated for one hour at room temperature. After incubation, the plates were washed again (6X) and flicked dry. Staph A horse radish peroxidase conjugate (1/1500; Zymed, CA.) was added and incubated for exactly 30 min. at room temperature. Then, after 4-6X washes with ELISA medium, the freshly prepared enzyme substrate (O-phenylene diamine) was added to each well and incubated for 30 min. in the dark. The reaction was stopped by adding 2M H_2SO_4 and then absorbance values read at 490nm after 1 hour at room temperature using an ELISA reader (Dynatech Minireader II).

(v) Virus Neutralization Assay: Cell monolayers were inoculated with virus-antiserum mixtures previously incubated in a 37°C waterbath for 60 min. These were allowed to adsorb for 1 h. at room temperature before overlaying with agar medium (overlay #3, Appendix 1). Then they were incubated at 33°C for 5 to 6 days to allow development of plaques. The endpoint was expressed as the reciprocal of the dilution of serum which reduced the number of plaques by 50% as determined from a log/probit

plot (Child et al., 1983).

Other neutralization procedures used in this study will be described in detail in the next chapter.

(vi) Immunodiffusion: The technique used in our experiments was previously described by Johnson et al (1964). The cellulose acetate strips were cut transversely into pieces and soaked by immersing them in Tris buffer (pH 7.2; Appendix 1). These strips were then placed on a clean glass slide and the perspex template slipped into a central position along the wet surface eliminating the formation of air bubbles in the process. The prepared slides were placed in small moisture chambers (square petri dishes, containing damp filter paper) which were in turn placed into a larger moisture chamber. After filling the wells with the appropriate reactants, precipitation lines were allowed to develop over 40-48 hours at 23°C. Following incubation, the templates were removed under running tap water and the cellulose acetate strips were removed and washed in PBS for a minimum of four hours followed by a ten minute wash in distilled water. All immunodiffusion reactions were stained with thiazine red R and direct enlargements of the clarified and mounted reactions were made on F-3 Kodabromide paper using a Beseler photographic enlarger.

The antigen and antiserum preparations will be described in detail in the third chapter. Briefly, concentrated preparations of both HCV/229E and VH viruses were used as antigen and, in addition to the serum samples from the study participants, control hyperimmune and prebleed guinea pig sera were used.

(vii) Statistical methods: The statistical analysis was based on "Statistics in Medicine" (Colton, 1974). Group differences were assessed by the Student's-t test and analysis of variance methods (Appendix 1).

The MS and control groups were sub-divided on the basis of their common cold histories and other characteristics according to the information provided in both questionnaires (Appendix 2). Records of the occurrence of respiratory infections (colds) and/or MS exacerbations were obtained monthly throughout the study. Comparisons between cases and controls were done using conventional unpaired analysis. Paired analyses were used in comparing monthly changes in antibody titre. Coding of serum titres using the logarithmic transformation and calculation of the geometric mean titres (GMT) were performed according to White (1973).

Computer analyses were performed using the main computer of the University of Ottawa through the courtesy of Dr. S. Narod, Dept. of Epidemiology, University of Ottawa. BMDP statistical software (program 7D) was used for the selected tests.

3. Results

(i) Immunofluorescence Assay: It was important to maintain a consistently high quality of slides used in the IFA and care was taken to ensure the even distribution of the cell monolayers and the characteristic appearance of the FA-labelled cells in addition to routine monitoring of virus production by the persistently infected HV-1 cultures. There was an unpredictable tendency for the cells to congregate in the central areas of the squares and this was found to be due to accumulated static charges in the plastic chambers. Once the slides were placed on wet filter paper and kept in a humid atmosphere, this problem was eliminated. The characteristic appearance of the HV-1 cell monolayers used in the routine IFA is illustrated in Fig.1. This pattern of FA-labelled cells was consistent and there was no detectable

FIGURE 1A. Immunofluorescence assay using HV-1 persistently infected cells. The virus-infected green fluorescing cells are easily visible among the brownish red non-infected cells. (10X objective)

FIGURE 1B. Immunofluorescence assay using HV-1 persistently infected cells. Much of the characteristic green colour has been lost in the printing however, it can be seen that it is restricted to the single infected cells which are the typical reaction pattern. (40X objective)

COLOURED PICTURES
Images en couleur

A.

B.

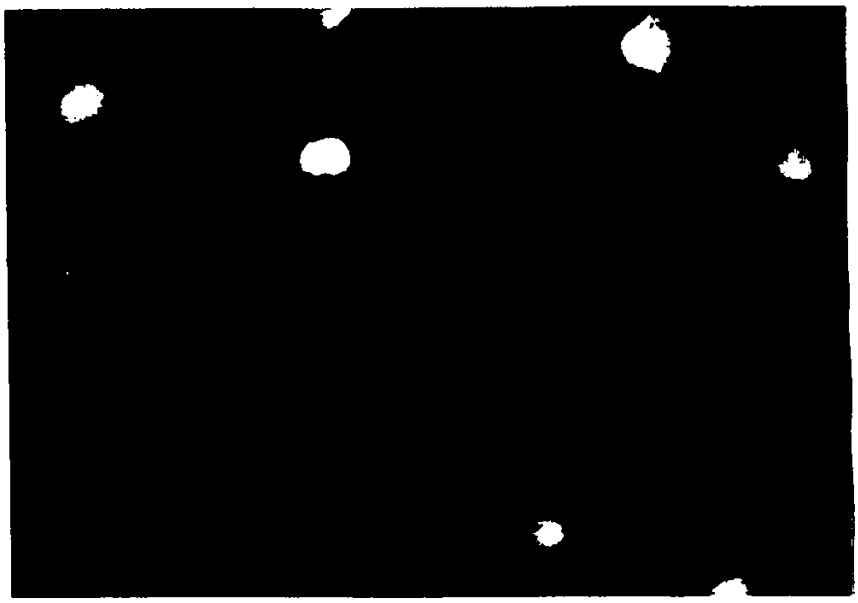


FIGURE 1.

difference between the control reactions using known positive antisera (absorbed guinea pig anti-229E hyperimmune or FIP convalescent sera) and positive human sera. Non-specific immunofluorescence was quenched using the Evan's blue counterstain, however, there was no difficulty in recognizing the characteristic coronavirus-infected cell pattern and distinguishing excessive non-specific reactions. There were very few borderline positive specimens when serum dilutions of 1/20 were used but when they occurred, the samples were re-tested. The titration endpoints were taken as the reciprocal of the highest dilution giving a 1+ virus-typical FA reaction using high power oculars (100X) with non-fluorescing immersion oil (Wild/Leitz, Canada).

(ii) Comparison of MS Cases and Controls. The characteristics of both groups are summarized in Table 3. The information on which this comparison is based was obtained from both questionnaires. It can be seen that, except for MS, both groups are remarkably similar which indicates that the control group was appropriate to the study. In the final questionnaire an attempt was made to document information believed at one time to be relevant to the development of MS; namely whether, during childhood and up to age 15, the participants had lived in a mainly rural or urban environment, had prolonged, close contact with animals such as family pets (dogs, cats) or farm animals or knew of schoolmates who subsequently developed MS. As can be seen in Table 3, there was no difference between the cases and the controls. However, there was a highly significant difference between the groups with respect to a family history of MS ($P < 0.01$). This finding is in agreement with other reports of a genetic predisposition to the disease (Kurtzke, 1980; Waksman, 1983).

Table 3. General Comparison of MS cases and controls

	Cases	Controls
Number	39	39
Controls: Spouse	20	20
Non-spouse	19	19
Sex: male	10 (25.6%)	15 (38.5%)
female	29 (74.4%)	24 (61.5%)
Average age	38.8 years	39.4 years
Siblings	3.15	3.26
Family history of MS	20.5%	2.6% (P<0.01)
.....Childhood history to age 15.....		
Urban inhabitant	28 (71.8%)	23 (60.5%)
Family pets:		
Dogs	59%	58%
Cats	56%	50%

(iii) Comparisons of average titers: The IFA titres for 584 MS/pal control sera are shown in Table 4 . The GMT for these two groups (all months combined) is almost the same (1.957±0.109 for the MS patient group and 1.950±0.127 for control group). The P value is 0.90 which indicates no statistically significant difference. Further analysis of the data obtained from the final questionnaire showed that the GMT was also not affected by the frequency of colds, age, or sex. Thus, these results suggest that there was no major difference in the frequency of colds reported by all the individuals in this study.

(iv) Monthly comparison of the Geometric Mean Titres: The monthly comparison of the GMT between the MS case/control groups is shown in Fig.2. The antibody levels for both groups peaked in December, then gradually decreased, but there were no significant differences in the seasonal patterns of these two groups; GMT of 2.42 for MS cases and 2.45 for the controls. The monthly average GMTs are summarized below in Table 5.

Table 5. Monthly Average Geometric Mean Titres.

	OCT	NOV	DEC	JAN	FEB	MAR	APR	MAY
Total	1.93	2.08	2.49	2.11	2.13	2.02	1.84	1.68
Controls	1.97	1.98	2.45	2.16	2.03	2.03	1.85	1.75
Cases	1.89	2.18	2.42	2.05	2.24	2.00	1.82	1.62

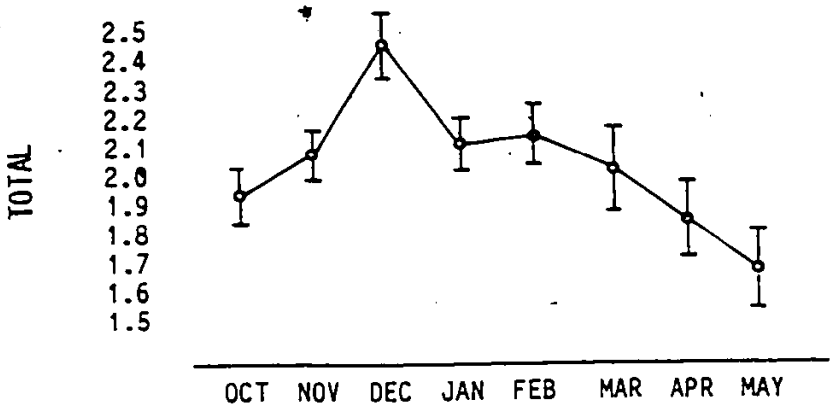
Table 4. Comparisons of GMT of average IFA titers of MS cases and controls

	Average titer	P value
Case	1.957±0.109	
Control	1.950±0.127	0.90
Cold history*		
Zero cold	1.830±0.66	
One cold	1.890±0.107	
Two colds	2.250±0.227	0.30
Three colds	1.966±0.203	
Age**		
< 44	1.940±0.113	
> 44	1.944±0.123	0.90
Sex		
Male	1.984±0.153	
Female	1.939±0.100	0.80

* Based on the information provided in the final questionnaire. The participants were divided into groups according to their reported frequencies of colds experienced during the previous 12 months.

** Age at which individuals segregated equally for statistical testing.

FIGURE 2.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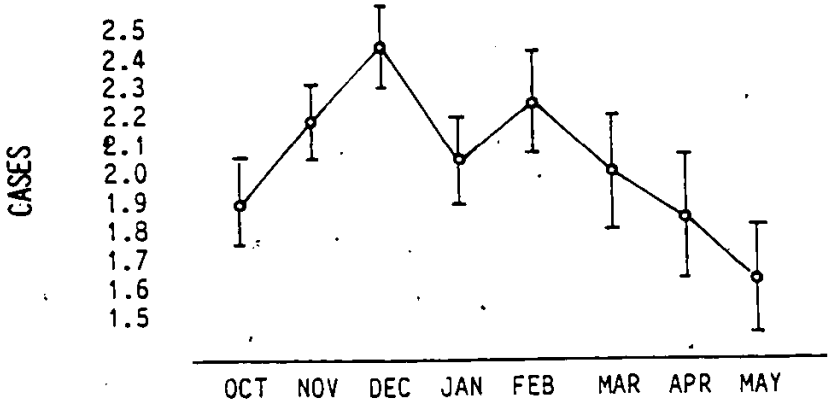
CASES
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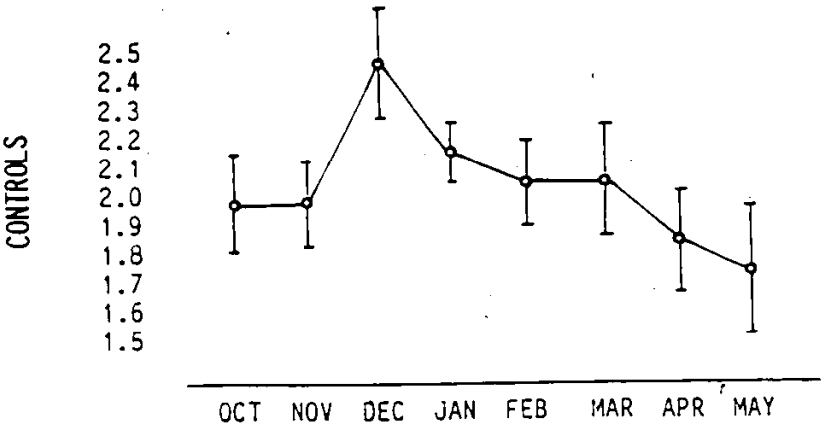
CONTROLS
2.5
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2.2
2.1
2.0
1.9
1.8
1.7
1.6
1.5
OCT NOV DEC JAN FEB MAR APR MAY

FIGURE 2. Monthly average geometric mean titres of MS case and control sera

A. Total

B. Cases

C. Controls

(v) Relation of Colds to Geometric Mean Titres: The analysis of the relationship between the GMT of MS patients and controls based on the colds which they experienced during the 8 month study period also showed no significant differences (Fig.3). The total number of colds in both groups was 40. The lack of significant correlation between the average titer and frequency of cold by month is indicated by Pearson's correlation coefficient of 0.733. The data in Table 6 showed that the average titres did not change with the occurrence of colds. The average titre for all months was 2.046±0.064; 2.114±0.115 for 'cold' months and 2.028±0.068 for 'no cold' months. A 'cold' month is defined as that month in which an individual experienced a cold versus a 'no cold' month when no cold was reported. To further test for an association, we examined MS patients' GMTs grouped according to the months before and after as well as the 'cold' month. As can be seen in Table 6, the average titres of these three groups were not significantly different. In summary, therefore, these results indicate that there was no group correlation of cold symptoms to titers in either the MS cases or between the cases and controls.

Table 6. Relationship between Colds and GMT of MS cases and controls.

	Average GMT	
All months	2.046 ± 0.064	
'Cold' months*	2.114 ± 0.115	
'No cold' months	2.028 ± 0.068	MS Patients subgroup
Month prior to cold	2.020 ± 0.109	2.00
Month of cold	2.114 ± 0.115	2.05
Month after cold	2.070 ± 0.122	2.28

* 'Cold' month is that month in which an individual experienced a cold as opposed to a 'no cold' month when no cold was reported.

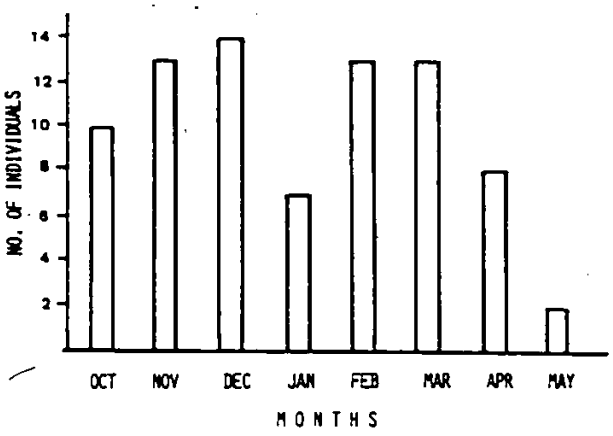
FIGURE 3.

FIGURE 3. Number of colds experienced by month.

A. All participants.

B. Comparison of MS patients and Controls.

A.


NO. OF INDIVIDUALS
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12
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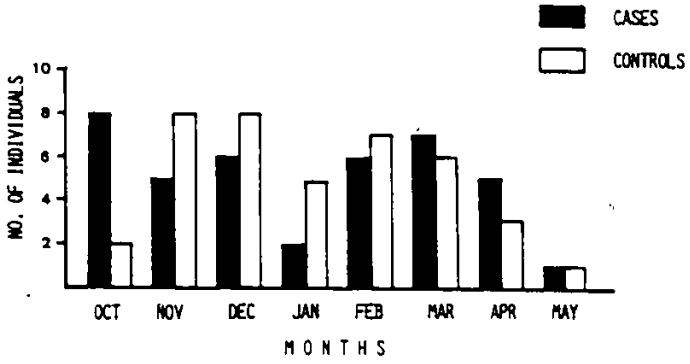
B.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(vi) The relation of MS exacerbations and colds: In a total 294 months of case observation, the monthly cold frequency was 13.3%, almost identical to that of 13.7% seen in the control population (40 colds/293 months). There were 19 exacerbations of MS and 39 episodes of colds and of the 19 MS exacerbations, 6 occurred in association with colds (Table 7). The rate of exacerbation was 3.38 times higher in 'cold' months than in the 'no cold' months ($p = 0.0284$, Fisher's exact test, one-tailed).

Table 7. Relationship of MS Exacerbations and Colds.

MS Exacerbation	Cold: Yes	Cold: No	
Yes	6	13	19
No	33	242	275
	39	255	294

$P < 0.05$

The above ratios are similar to those obtained by Sibley et al. (1985). 15% of the infections were associated with worsening of MS whereas 32% of MS exacerbations coincided with colds during this period. Although colds were most frequently reported during the winter months, there was little seasonality evident in the MS episodes.

(vii) Association of HCV/229E colds with MS cases and controls.

Within the colds experienced by the MS cases and controls it was possible to correlate the 4-fold or higher serological peaks identified by the BMDP statistical program. Since these peaks reflected the presence of HCV/229E antibodies, it was possible to determine the number

of HCV/229E caused colds within each group. These results are summarized in Fig.4. Of the total 80 colds (40 in each group), 46 (57.5%) were identified as being associated with HCV/229E, 28 with the cases group and 18 with the controls. Statistical analysis showed that there were significant differences between these groups ($P < 0.05$) and we concluded that there is a positive association of coronaviral infection with MS (Table 8).

Table 8 . Comparison of HCV/229E colds among MS cases and controls.

	HCV Cold +	HCV Cold −	
Case	28	12	40
Control	18	22	40
	46	34	80

$P < 0.05$

To determine whether there might be any bias between the spouse and non-spouse control groups (20 and 19 pairs respectively) which might reflect transmission of viral infections due to closer contact between spouses, we classified the subjects accordingly and again tested for coronavirus cold association. No significant differences were found in the numbers of coronaviral infections in either of these groups, confirming that the controls, whether spouse or non-spouse, were reliable and that the MS subjects were not in fact "over-controlled" as had been previously suspected.

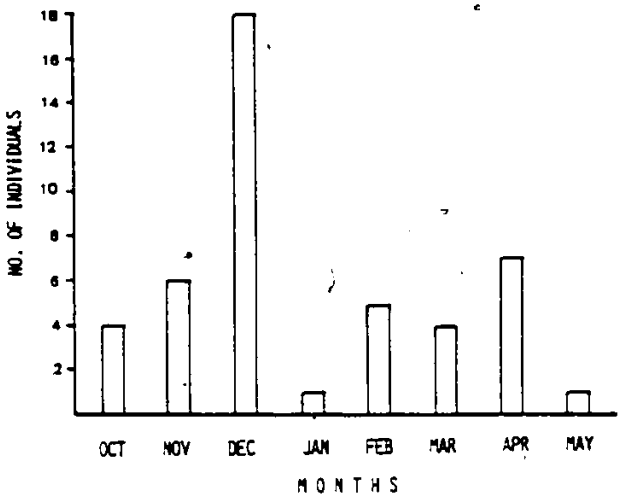
FIGURE 4.

FIGURE 4. Number of HCV/229E colds identified by four-fold rise in antibody titre.

A. All participants.

B. Comparison of MS patients and Controls.

A.


NO. OF INDIVIDUALS
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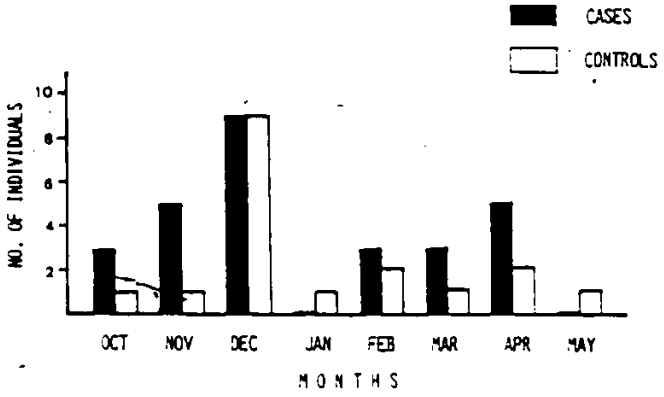
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B

(viii) Prevalence of HCV infection in the community. In a further series of IPA tests, we screened a number of sera from individuals in the general population as represented by Red Cross blood donors and persons whose sera had been sent to the Regional Virus Diagnostic Laboratory (query and diagnosis unknown). The first group of "community controls" were considered to be from normal, healthy adults who were unlikely to be suffering from a respiratory infection at the time of donating blood, however, clinical histories were not available. The 325 Red Cross sera were obtained in 13 weekly batches of 25 each, collected from the middle of March to mid June. They were screened at dilutions of 1/20 and the overall average rate of positivity was 40%. The proportion of positive sera in the first week was 48%. During the next 9 weeks, the percentage of positive sera ranged from 20-40 percent until the end of May when the rate began to climb. In the last three weeks there were 52%, 64% and 72% positives, respectively. Although the total period of time covered by this study was too short to draw any firm conclusions, these results tend to indicate an increase in coronavirus activity in the late spring/early summer of 1983.

The results of screening the serum samples which were randomly selected from among those sent to the Regional Virus Diagnostic Laboratory indicated that, during the 1980/81 specimen year, 41% of 100 sera, randomly selected from a total of 980, were positive, whereas for 1981/82 only 16% of 160 sera randomly selected from a total of 1568 were positive. Coronavirus infections are known to be cyclical in nature, and these result would tend to confirm this (Monto, 1984). Also, it could be that the OC43 strain of coronavirus was circulating and would not have been identified in our procedure.

Taken together, these findings clearly indicate the presence of

HCV/229E in the general community however, it was not possible to demonstrate seasonal incidence; neither was it possible to correlate the presence of antibodies with clinical respiratory disease. And, while these results served as a valuable control for the IFA used in our study since a good proportion of the sera were found to be negative at the 1/20 screening dilution, they did not provide the background information on the incidence of HCV/229E in the community which might have been relevant to the MS case/control study. We do not fully understand why both the cases and controls showed such a high rate of positivity (95-98% during any study month) but speculate that there was better opportunity for common exposures of and transmission between both the spouse and non-spouse controlled participants. Also it has been reported that the proportion of adults whose sera contained HCV antibodies approached 100% when sensitive immunological tests are used (Hovi et al., 1979; Macnaughton, 1982). These results served to emphasize the importance of the control population in this type of study.

(ix) Comparative serology of selected sera using different assays.

A comparison of the results of the preliminary serological survey using the immunodiffusion reaction with those of the sequential case/control study using the indirect immunofluorescence assay revealed a wide discrepancy both in the number of positive reactions but also in the statistical correlation between the MS cases and the controls. In order to determine the reliability of both tests, we compared the serological activity of a small number of MS case/control serum pairs which were selected to represent the following criteria:

IFA reactions - including clean reactors as well as those giving high levels of non-specific FA;

IFA titres - including high >1:1600, medium 1:800-1:1200, low <40 and negatives);

Cold history - including recent cold, cold within two years, no cold within 10 years or occurrence of cold during the study.

In total, 11 pairs of MS case/control sera were tested. In addition to the IFA, the assays included the immunodiffusion test (ID) using VH and 229E antigens, ELISA using VH and 229E preparations and neutralization (NT) using the plaque reduction test with 229E virus. The results are shown in Table 9. The summary of these results which is recorded in the bottom line of the table indicates that the highest proportion of positive sera were detected by NT, IFA and ID tests but although fewer were positive by ELISA, there were basically no differences demonstrated between the cases and controls by any of the tests. When we compared the results of three of the tests, as shown in Table 10, it was concluded that again there was no difference between the cases and controls, but there was a slightly better correlation between the IFA and NT results than either the IFA or NT with ELISA. The ID test results were excluded from this comparison because they were essentially all positive.

Since the type of antibody (neutralizing or precipitating) detected by the different serological tests was different depending on the viral antigens involved, the next step in evaluating these results was to classify the reactions according to the antigen detected.

It has been established that the viral antigen involved in neutralization is the peplomer, E2 protein only (Robb and Bond, 1979; Sturman and Holmes, 1983). The major antigen detected by IFA is the nucleoprotein (NP) which is present in the cells in which virus replication is

Table 9. Results of the serological tests comparing 11 selected pairs of MS case/control sera.

Pair Number	SEROLOGICAL TEST									
	NT(229E)*		IFA(VH)**		ELISA(VH)***		ELISA(229E)***		IDD	
	Case	Control	Case	Control	Case	Control	Case	Control	Case	Control
1	37.9	22.9	<20	>160	1000	800	1600	1600	-	+
2	0	19.5	160	>160	100	100	800	400	+	+
3	49.4	26.4	160	20	400	0	600	0	±	+
4	26.4	14.9	120	60	0	0	150	300	+	+
5	19.5	3.4	>160	60	0	400	200	800	+	+
6	34.5	34.5	20	>160	200	0	400	100	+	+
7	22.9	8.04	160/40	40	400	400	800	600	+	±
8	80.5	68.9	>160	40	0	0	0	0	+	+
9	19.5	26.4	80/120	40	400	400	800	400	±	±
10	11.5	60.9	160	60	120	150	>1600	1600	±	±
11	65.5	14.9	120	40	200	0	200	0	±	±
Reaction	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Summary	9/2	9/2	9/2	10/1	6/5	4/7	7/4	6/5	10/1	11/0

* % Surviving virus; the endpoint was taken as 50% plaque reduction.
** Reciprocal of highest + serum dilution; positive reactions are taken as >40.
*** Reciprocal of serum dilutions; positive reactions are >400 (229E) and >200 (VH).

Table 10. Comparison of the results of three serology tests.

Combined Results IFA/ELISA/NT	MS Cases	Controls	Total	%
All positive	5/11	5/11	10/22	45.5
2 of 3 positive	0/11	1/11	1/22	4.5
1 of 3 positive	6/11	5/11	11/22	50.0
All negative	0	0	0	0
Correlations:				
IFA with ELISA				
Both + or -	6/11	6/11	12/22	54.5
Different	5/11	5/11	10/22	45.5
IFA with NT				
Both + or -	8/11	9/11	17/22	77.3
Different	3/11	2/11	5/22	22.7
ELISA with NT				
Both + or -	7/11	7/11	14/22	63.6
Different	4/11	4/11	8/22	36.4

occurring. E2 might also be seen under these conditions however, it is not so abundant. The ELISA reaction is believed to be more specific for E2 antigen, however, solid evidence for this is lacking. The immunodiffusion reaction would be expected to detect all antigens, the strength of the reaction depending on the relevant proportions of reactants in the system. Therefore, it is only with the NT and the IFA tests that one can distinguish the antigens involved. The results of the four different serology tests were therefore evaluated according to the different viral antigens detected and further correlated to colds experienced by the subjects (Table 11). Since such colds would be expected to influence the type of antigens detectable, these were then examined in order to determine whether there was a relationship between the occurrence of colds and the identity of the antigen as defined by the serological test results. Because the colds reported by the subjects were not necessarily caused by HCV/229E, and no virus isolation studies to identify the viruses were undertaken, the analysis of the serological results was restricted to correlation between tests only, and could not be regarded as an evaluation of the reliability of the test. The relationship between the cold histories and the antigens detected is shown in Table 12.

Of the total 22 subjects, 13 (59%) experienced colds prior to the month of sampling and 9 did not. There were 5 MS case colds and 6 control colds which gave rise to NT and IFA antibodies. Using ELISA, similar positive reactions were found but there was an increase in the number of negative reactors. The NT and IFA results appeared to be inconsistent, however, since different antigens were involved in the reactions, and since we have obtained evidence of sub-clinical

Table 11. Cold history and antigens detected by different serological tests.

Test Subject		Sample Month	Cold History		Serological Test (Antigens detected)			
			Month Before	Earlier in Study	NT (E2)	IFA/VH (NP/E2)	ELISA E2&(NP)	ID (All)
1.	Case	May	Yes	No	+	-	++	-
	Control	"	Yes	No	+	++	+	+
2	Case	March	No	Nov.	+	+	+	+
	Control	"	No	Dec/Jan.	+	++	±	+
3.	Case	April	No	Oct/Nov.	±	+	+	±
	Control	"	Yes	No	+	-	-	+
4.	Case	March	No	Dec.(?)	+	+	-	+
	Control	"	No	Oct/Nov.	+	+	-	-
5.	Case	Feb.	No	No	+	++	-	+
	Control	"	Yes	No	+	+	+	+
6.	Case	Oct.	No	No	+	-	±	+
	Control	"	No	No	+	++	-	+
7.	Case	Nov.	No	No	+	+	+	+
	Control	"	No	No	+	±	+	±
8.	Case	Oct.	No	No	-	++	-	+
	Control	"	No	No	-	±	-	+
9.	Case	Nov.	Yes*	No	+	+	+	±
	Control	"	Yes	No	+	±	±	±
10.	Case	April	No	No	+	+	++	±
	Control	"	No	Feb.	-	+	+	+
11.	Case	Nov.	Yes	No	-	+	-	±
	Control	"	No	No	+	±	-	±

* MS exacerbation and cold.

Table 12. Relationship between cold history and antigens detected by three different serological tests.

Cold History	MS Case	Control	Total
Cold in month before sample	3/11	4/11	7/22
Cold earlier in the study	3/11	3/11	6/22
No colds reported	5/11	4/11	9/22
Correlations:			
Cold with NT (E2 antigen of 229E virus)			
Both positive	5/13	6/13	
Both negative	1/9	1/9	
Different	5	4	
Cold with IFA (NP and <E2 antigen of VH virus)			
Both positive	5/13	6/13	
Both negative	1/9	0	
Different	5	5	
Cold with ELISA (E2 predominantly, VH and 229E)			
Both positive	5/13	5/13	
Both negative	2/9	3/9	
Different	2	3	

infections in our previous analysis of colds and rising titres, it was concluded that these two tests together, were, in fact, more sensitive than the ELISA. There also were quantitative as well as qualitative differences in the antibodies detected by the different tests but, the numbers were too few for statistical evaluation. It was clear from these results however, that there were no significant differences between the MS cases and controls no matter which tests or antigenic preparations were used.

4. Discussion.

The aim of this study was to try to provide a link between MS and human coronavirus by demonstrating a difference in the immune response of persons with MS compared to their controls as reflected by the levels of circulating HCV/229E antibody. A preliminary study, using the micro immuno-diffusion test had revealed significant differences between a small number of MS patients and controls and it was necessary to provide more stringent evidence. Therefore, an in depth, sequential case/control study was designed to compensate for the fact that coronavirus 229E is a very common respiratory pathogen which causes repeated infections in both adults and children.

In order to control for virus exposure, it was decided that individuals who shared the household (spouse, friend or relative) and/or community (workplace, neighbours, friend) environment were required. The number of volunteers for this study was not large, however, in a sequential study of this kind, where each individual would provide a serum sample once a month over a period of 8 months, the number of specimens were considered adequate. This was supported by the high rate of compliance by all the participants, both the MS patients and their

'pal' controls throughout the entire 8 months of the study. In addition, when the individual group characteristics were examined, they were found to have very close similarity indicating that the controls were appropriate to the design of the study.

The indirect immunofluorescence assay instead of the immuno diffusion test was selected as the serological test because it offered the advantage of recognizing the specificity of the antigen/antibody reactions both in terms of the pattern of viral replication in the host cells, but also, the potential to distinguish between viral antigens. At the time this study was initiated, a simplified IF procedure had been developed which was based upon cells persistently infected with HCV/229E. At that time, the evidence indicated that both VH, the persistent virus and 229E, the laboratory strain from which it was derived, were antigenically similar. Therefore, the IFA using the persistently infected cells was adopted as the routine serological test.

The results of this study showed no statistically significant differences in the serological reactions between MS patients and controls with respect to the persistent VH virus in the IFA. Several possible reasons for this negative finding might be suggested.

First and foremost, the sample size was too small to resolve potential differences between the patients and their controls. This is particularly the case with such a high level of positive reactors in both groups and the circulation of coronaviruses in the community. If there was indeed any difference to be detected by serology, far greater numbers would be required to compensate for normal serum fluctuations in addition to responses to repeated virus exposures.

Secondly, the IFA using the persistently infected cells might not

be the most appropriate test for this type of study. This is especially relevant since it was recently demonstrated that there were considerable antigenic differences between the parental 229E and the VH virus used in the IFA. These will be discussed in the next chapter, but because of these antigenic differences, VH virus appears to be more reactive with NP antibodies than with E2 antibodies. This conclusion is supported by the results of the comparative serology of the few selected sera in which it appeared that the neutralization test complemented the IFA with respect to the serological response to colds. More neutralization tests are required however, to confirm this observation.

On the basis of the comparative tests, it appeared that the ELISA results did not provide any additional information about the validity of the IFA. This was mainly because the identity of the viral antigens could not distinguished but also because, by the nature of the test, non-coronaviral reactions such as those against host cell antigens or contaminants might occur giving rise to false positives.

The difference between the results of the preliminary survey using the immunodiffusion reaction with 229E antigen and the results of the case/control study using the IFA with VH virus may be associated with antigenic differences in the two viruses. Although the limited comparative study using both VH and 229E antigens did not reveal any major differences, this also requires further investigation.

Finally, it might be concluded that there is no association between MS and coronavirus 229E. It is considered that, to date, the results are not clear cut enough to rule out coronaviruses. The two findings which support this opinion are the significant association of colds with exacerbation of MS and the equally significant association of coronavirus 229E infection with MS patients in comparison to controls.

Although these latter findings are not strong evidence of a definite, etiological link between coronavirus and MS, they do provide tenuous support for a positive association between 229E infection and MS which requires further elucidation.

Chapter III.

ANTIGENIC RELATIONSHIPS OF 229E AND VH HUMAN CORONAVIRUSES

1. Background.

The persistent infection of a human cell line, L132 with the human coronavirus (HCV) strain 229E was successfully established in 1979 (Chaloner-Larsson and Johnson-Lussenburg, 1981a) and to distinguish it from the standard HCV/229E:L132 system, the persistently infected cells have been termed HV-1 cells and the persistent virus derived therefrom was called VH. Preliminary tests to characterize these viruses showed that both the 229E standard virus and the VH persistent virus were neutralized by hyperimmune anti-229E guinea pig serum. Based on this persistent HV-1 cell line, a sensitive and reliable indirect immunofluorescence assay (IFA) was developed and adopted as the routine test for serological studies.

Using this IFA technique with its VH virus, the MS case/control serological study described in Chapter 2 was carried out in 1982-1983 with the objective of determining whether human coronavirus 229E could be implicated as an etiological agent in multiple sclerosis. The results of the study were not equivocal; showing no significant difference between antibody titres of MS patients and controls. This result was in contrast to the preliminary serological study using both the immunodiffusion test and the IFA, in which 229E antigen preparations were used for the preliminary screening. There were significantly higher titres (ID test $p<0.01$; IFA, $p<0.1$) in the MS group as compared to the controls.

One explanation for this discrepancy would be that the two viruses,

229E and VH were not as similar antigenically as was previously thought. Recent information has been obtained which supports the existence of such antigenic differences.

First, in molecular hybridization studies comparing the RNA genomes of the two viruses, it has been established that the VH genome is larger than the 229E genome, by about 30% (McLeod, D.A., M.Sc. Thesis, University of Ottawa, 1985). It might be expected that such differences would be reflected in the viral structural protein antigens.

Secondly, the results of IFA studies using HCV monoclonal antibodies obtained through the courtesy of J. Fleming, University of Southern California, demonstrated that the internal NP antigens were shared by both viruses but the E2 peplomer (surface) antigens were different (Johnson-Lussenburg, unpublished results).

Finally, in neutralization tests to measure antibody titres of the control anti-229E guinea pig sera used in the IFA, it was found that serum neutralization titres obtained with 229E virus were considerably lower that those obtained with VH virus.

The aim of the present study therefore, was to further explore the nature of these differences especially where relevant to the interpretation of the results of the serological survey. In order to understand the antigenic relationships two types of neutralization tests were used, both of which were specific for antigenic determinants of the E2 peplomer antigen and could be measured in terms of characteristic biological activity by plaque assay.

Virus neutralization tests measure the loss of virus infectivity due to the binding of antibody molecules to specific antigenic determinants (epitopes) on the surface of the infectious virion. In these tests, serial dilutions of serum are mixed with constant, known

amounts of virus. After an appropriate incubation period, the mixtures are inoculated into cell cultures and monitored for development of characteristic viral cytopathology. The serum antibody titre is defined as the reciprocal of the highest dilution of serum to inhibit virus multiplication. In general, the neutralization test is one of the more specific of all the virus-antibody reactions since it is associated with the biological activity of the virus (Mandel, 1979; van Regenmortel, 1981).

There are several types of neutralization tests; the metabolic inhibition test, the plaque reduction test and the kinetic neutralization test to name a few. Plaque reduction tests may be used for detecting cross reactions between the antigenic components of viruses which may be indistiguishable by standard neutralization tests which rely on the development of virus cytopathic effect in cell cultures. The sensitive kinetic neutralization test which measures the speed of virus neutralization by antibodies is particularly suitable for determining small antigenic differences between closely related virus strains (Childs et al., 1983).

In order to understand the antigenic relationship between the HCV/229E and persistent progeny VH viruses, and to determine if they were antigenically distinct, both were examined for cross-reactivity by the plaque reduction and the kinetic neutralization tests using immune sera prepared against each strain. Immunodiffusion analyses were also used first to characterize and then to compare the viral antigenic components as well as to evaluate the reacting antibodies present in the immune sera.

2. Materials and methods:

(i) Cells and Viruses: L132 cells, a human embryonic lung cell line, were used throughout this study for virus production and assay according to procedures previously described (Kennedy and Johnson-Lussenburg, 1975/76; Chaloner-Larsson and Johnson-Lussenburg, 1981a&b). The cells were passaged at split ratios of 1:3 every 2 to 3 days and used between passage numbers 25 and 52. HCV/229E was originally obtained from Dr. A.Z.Kapikian at the U.S. National Institutes of Health, Bethesda, MD. It was reported that it had been passed in Wl-38 cells and, after an initial two passages in Wl-38 cells in this laboratory, has been cultivated only in L132 cells. The VH virus is the persistent coronavirus shed by HV-1 cells which were originally L132 cells infected with HCV/229E virus. The HV-1 persistently infected cells are maintained at 37°C and were passaged regularly at 1:3 split ratios every 2 - 3 days. These cells served as the source of VH virus.

(ii) Plaque Assay: Both 229E and VH viruses were plaque assayed on L132 cells following standard procedures which have been described previously (Kennedy and Johnson-Lussenburg, 1975/76).

(iii) Virus production: A diagram showing batch production of HCV/229E and VH viruses is shown in Fig. 5 and will be described briefly. Monolayers of L132 cells which were 70-80% confluent within 2 days of splitting were used. 24 hours before infection, the monolayers were washed with PBS, the standard growth medium (MEM with 10% fetal calf serum (FCS)) was replaced with serum free M-199 and incubation was continued at 37°C. 0.33ml of virus inoculum having a titer of 10^6 to 10^8 pfu/ml (MOI of 1.0 to 0.01 approx.) was added and allowed to adsorb for 60 minutes at room temperature. Then, 10-12 ml of M199 without FCS

FIGURE 5.

FIGURE 6.

FIGURE 5. Flow diagram showing virus preparation protocol.

FIGURE 6. Flow diagram to illustrate antigen preparation.

FIGURE 5.

VIRUS PREPARATION.

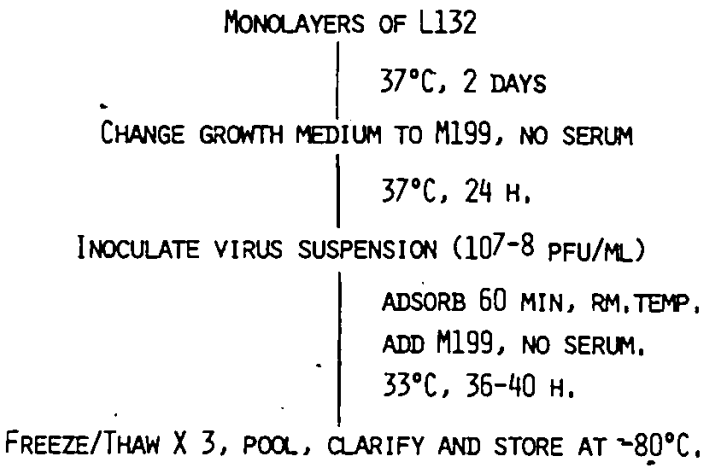
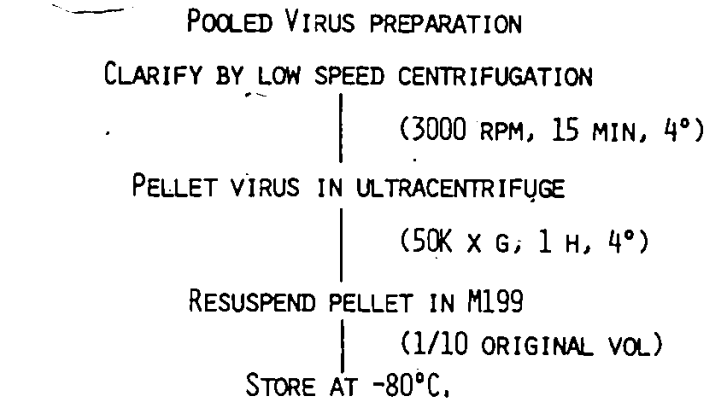


FIGURE 6.

ANTIGEN PREPARATION.



was added and incubation continued at 33°C for 36-40 hours. Then the cells were subjected to 3 cycles of freezing and thawing to release the virus from the cells, the suspensions were pooled, clarified by low speed centrifugation (3000 rpm for 30 min.), aliquoted into small lots and stored at -80°C. This preparation was used in the neutralization studies or for antigen preparation.

(iv) Preparation of Antigens: After the three freeze-thaw cycles and clarification by centrifugation at low speed at 4°C to remove cell debris, virus was pelleted by centrifugation at 50,000 x g for 60 min. at 4°C (Fig. 6). The pelleted virus was resuspended in 1/10 the original volume of serum-free M199 and stored at -80°C. This semi-purified preparation was used for immunizing guinea pigs.

More concentrated antigen was needed for the immunodiffusion reaction, therefore two cycles of low and high speed centrifugation (differential centrifugation) were performed, the final semi-purified preparation being concentrated 100-fold. In order to ensure that all viral components were available to participate in the immunodiffusion reaction, the virus in the concentrate was disrupted by adding sodium deoxycholate (1% final concentration) and incubating the mixure for two hours at room temperature (Yaseen and Johnson-Lussenburg, 1981).

(v) Preparation of antisera: 250-350 gm female guinea pigs were used. and immunized with either 229E or VH virus preparations according to standard procedures (Yaseen and Johnson-Lussenburg, 1981). After test bleeds to evaluate that antibody production was satisfactory, the animals were exsanguinated. The sera were pooled and stored at -20°C. 229E-specific immunoglobulins were prepared by ammonium sulfate precipitation according to standard procedures (Campbell et al., 1963).

(vi) The Plaque Reduction (PR) test: The procedures for both the plaque reduction and kinetic neutralization tests used in this study were based on the method described by Habel (1969). They are illustrated diagrammatically in Fig. 7A and 7B.

In the PR test, two-fold serial dilutions of antiserum were prepared in serum-free M199. These were mixed with equal volumes of a suspension of 229E or VH viruses diluted to contain about 200-400 p.f.u./ml. After one hour incubation, 0.33ml of the mixture was added to an L132 cell monolayer. After one hour of adsorption at room temperature, the agar overlay was added to the cells which were then incubated at 33°C for 5-6 days to develop plaques. The serum titre was taken as the reciprocal of the highest serum dilution which reduced the number of plaques by 50%. Virus and cell controls were included in the assays.

(vii) The Kinetic Neutralization (KN) test: This test measures the speed of virus neutralization by antibodies and is considered to be very sensitive for detecting small antigenic cross-reactions (Childs et al., 1983). In the KN test, stock virus was diluted in serum-free M199 to contain approximately 10^5 to 10^6 p.f.u./ml. The antiserum was diluted to that concentration found by preliminary PR neutralization tests to permit survival of 10^{-1} to 10^{-2} of the homologous virus preparation. Equal volumes of the diluted virus and the properly diluted antiserum were then mixed and incubated at 37°C. At 0, 5, 15, 30, 60, and 90 minutes after mixing, 0.2 ml samples were removed by pipet and inmediatly expelled into 1.8 ml cold serum-free M199, mixed thoroughly and then diluted in two serial 10-fold steps to 1/100 and 1/1000. 0.33 ml of these dilutions were inoculated onto cell monlayers and incubated under agar for plaque development (Fig. 7B).

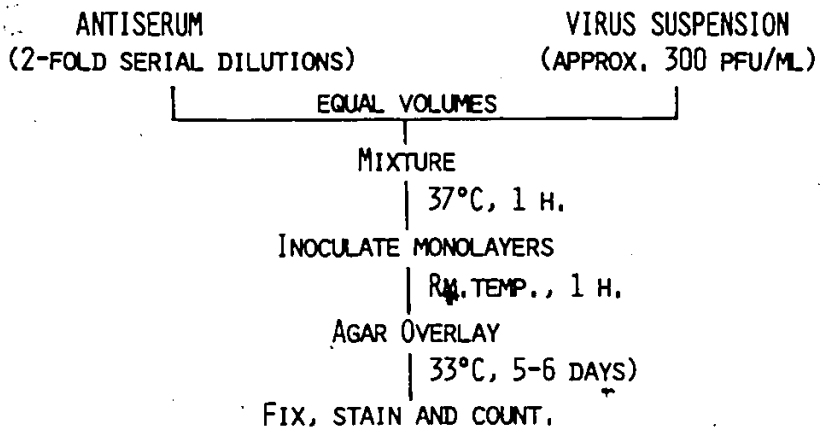
FIGURE 7 A&B

FIGURE 7A. Flow diagram to illustrate the plaque reduction test procedure.

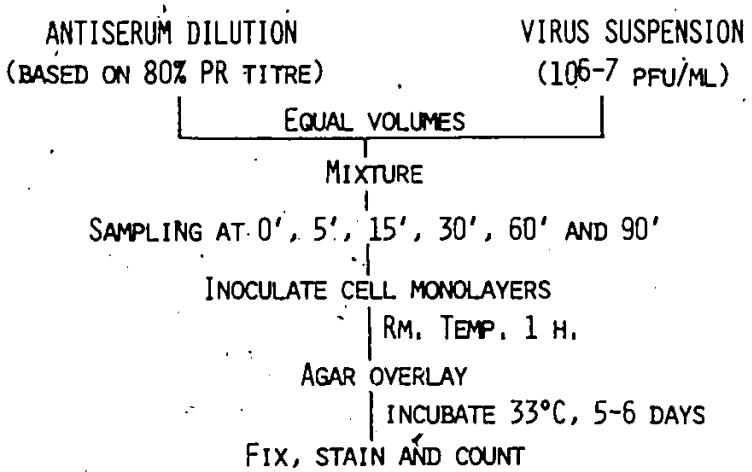
FIGURE 7B. Flow diagram illustrating the kinetic neutralization test procedure.

FIGURE 7

A. PLAQUE REDUCTION TEST.



B. KINETIC NEUTRALIZATION TEST.



3. Results.

(i) Plaque reduction neutralization test: The results of a typical PR test of 229E and VH viruses neutralized by anti-229E serum are shown in Table 13. It can be seen that the titre of anti-229E in the homologous system was nearly 28,000 when estimated using a log probit plot as described by Childs et al.(1983). On the other hand, essentially no VH virus could be detected at all serum dilutions indicating that VH virus was neutralized more efficiently than the homologous 229E. Unfortunately the quantity of this anti-229E preparation was limited and the supply was exhausted before we were able to determine the exact PR titre for the heterologous system. On the basis of our tests however, it must be at least in the 50,000 range.

When the anti-VH serum was used, VH virus was again more efficiently neutralized although the PR titres were not as high for either of the viruses (Table 14). From the log probit plot, the 50% PR titre of anti-VH serum against the homologous VH virus was estimated to be nearly 21,000 whereas when the heterologous 229E virus was used, the 50% PR titre was slightly less than 9,000 (Fig.8).

When the immunoglobulin fraction of the newly prepared anti-229E guinea pig serum was tested, once again, it was found that the VH virus was more efficiently neutralized. In this experiment, in order to compare the persistent virus shed by the HV-1 cells with the VH virus which had been propagated through 2-3 acute infections in the process of preparing large batches, supernatant fluid from the HV-1 cultures, containing around 105 p.f.u./ml was included. In this experiment it was termed HV virus. When the results were plotted (Fig. 9), there was still a marked difference between the 50% PR titres of the heterologous

Table 13. Plaque reduction neutralization test of 229E and VH using guinea pig anti-229E serum

Serum Dilution	Plaques	
	229E (%)	VH
1/3200	0 (0)	0
1/6400	9 (13.24)	1
1/12800	11 (16.18)	0
1/25600	31 (45.59)	1
Control	68 (100)	125

Original virus titer: 229E $2.7X10^6$pfu/ml
VH $8.4X10^4$pfu/ml

Table 14. Plaque reduction neutralization test of 229E and VH viruses using guinea pig anti-VH serum.

Serum Dilution	Plaques 229E* (%)	Plaques VH** (%)
1/1600	2 (0.62)	1 (0.13)
1/3200	19 (5.86)	10 (1.3)
1/6400	94 (29.01)	103 (13.4)
1/12800	244 (75.3)	255 (33.2)
control	324 (100)	768 (100)

* Original 229E titer: $2.18X10^5$pfu/ml
** Original VH titer: $1.54X10^7$pfu/ml

FIGURE 8.

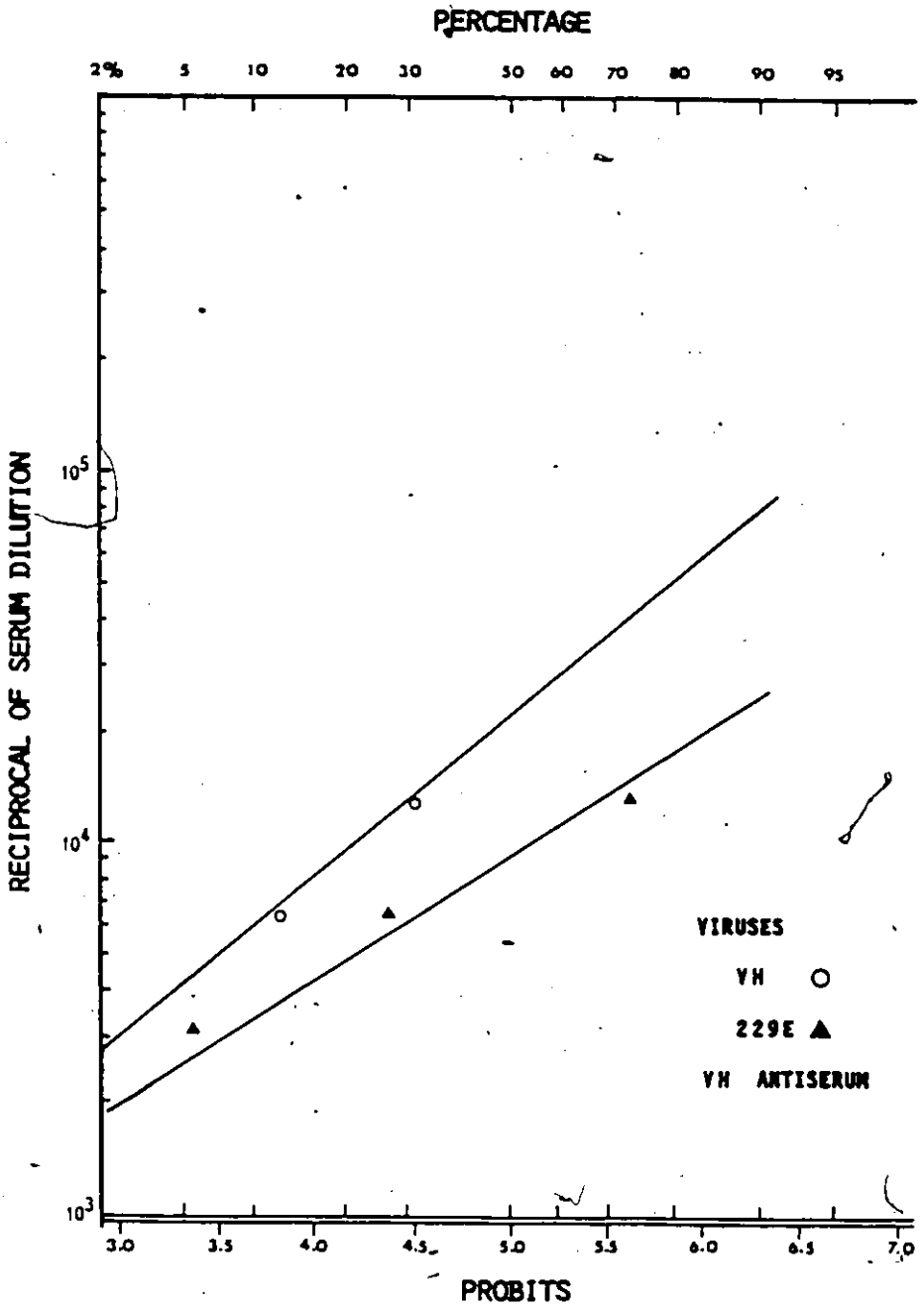
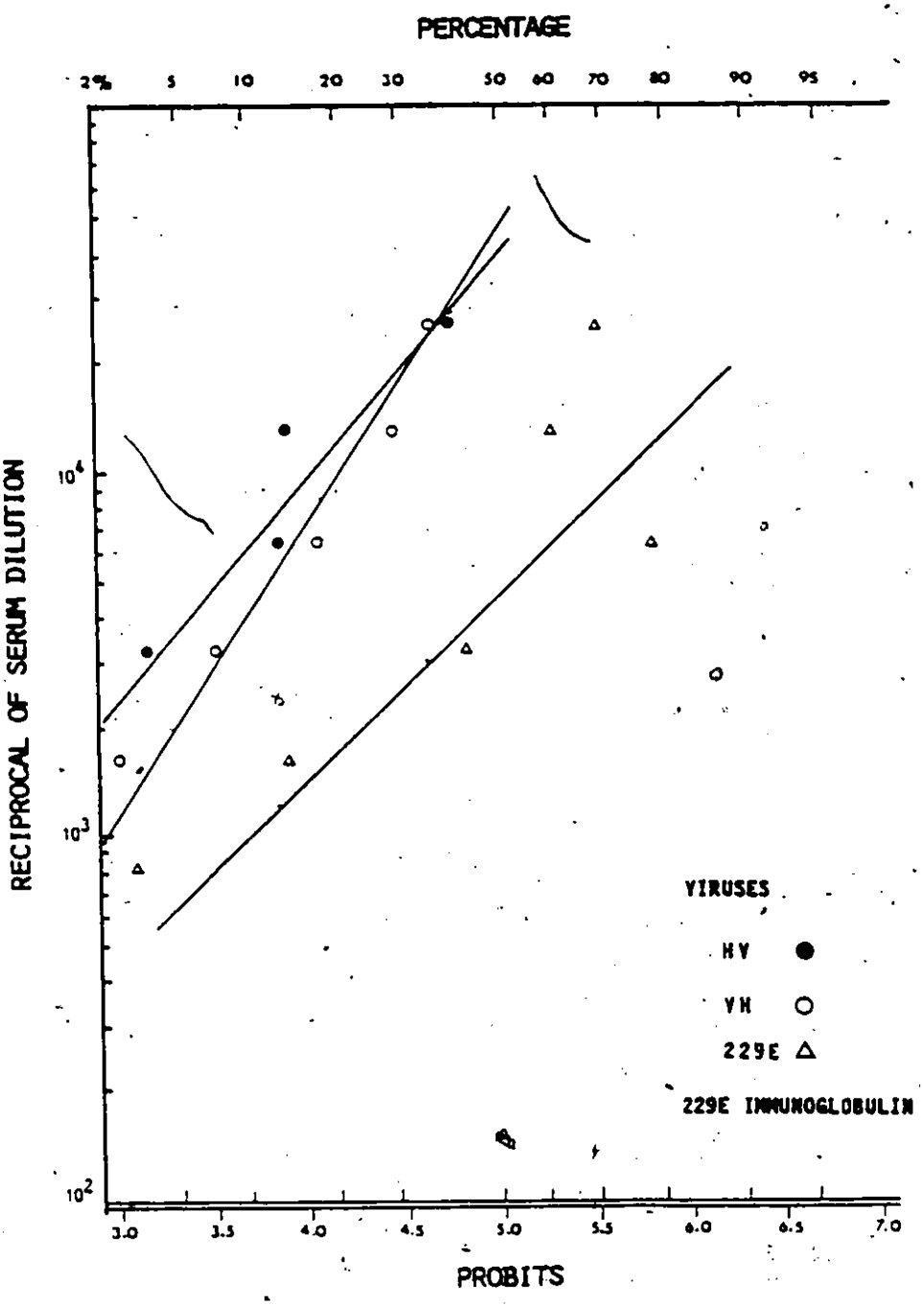

PERCENTAGE
2% 5 10 20 30 50 60 70 80 90 95
RECIPROCAL OF SERUM DILUTION
10^5
10^4
10^3
VIRUSES
VH
229E
VH ANTISERUM
3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0
PROBITS

FIGURE 8. Log-probit plot of the results of the plaque reduction test of 229E and VH viruses neutralized by anti-VH serum.

FIGURE 9.

FIGURE 9. Log-probit plot of the results of the plaque reduction test of 229E and VH viruses neutralized by anti-229E immunoglobulin.


PERCENTAGE
2% 5 10 20 30 50 60 70 80 90 95
RECIPROCAL OF SERUM DILUTION
10^4
10^3
10^2
3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0
PROBITS
VIRUSES
HV
VH
229E
229E IMMUNOGLOBULIN

compared to the homologous viruses (13,800, 13,000, 4,600 for VH, HV and 229E viruses respectively). It was also evident that there was essentially no difference between the VH and the HV viruses. These results have been summarized in Table 15, below.

Table 15: Summary of Plaque Reduction neutralization results comparing 229E, VH and HV coronaviruses.

Virus	Antiserum		
	229E	229E/Ig*	VH
229E	28,000	4,600	9,000
VH	>50,000**	13,800	21,000
HV	NT***	13,000	NT

* The 229E/Ig was prepared from a different antiserum than the 229E used in these experiments.
** Lowest estimate based on PR result (Table 13).
*** Not tested.

(ii) Kinetic Neutralization Tests: As can be seen in Table 11 and Fig. 10, the KN test confirmed the results of the PR test and revealed that the infectivity of VH virus was almost entirely eliminated within the first 15 minutes. On the other hand, in the homologous test, 50% neutralization of 229E infectivity required 30 minutes and even after 90 minutes, infectious virus was still present.

The results of the KN test using anti-VH sera are presented in Table 12 and Fig. 11. Again the homologous virus was neutralized faster than the heterologous 229E, and the VH antiserum effectively neutralized the heterologous 229E virus.

(iii) Effect of thermal inactivation of virus during KN tests. In these KN experiments, the procedure called for incubation of the virus-

Table 16. Kinetic neutralization of 229E and VH viruses by guinea pig anti-229E serum (1/6400).

Time (minutes)	Plaques	
	229E (%)	VH (%)
0'	335 (100)	2240 (100)
5'	198 (59.1)	34 (1.5)
15'	262 (78.2)	2 (0.09)
30'	163 (48.7)	15 (0.67)
60'	12 (3.6)	1 (0.04)
90'	3 (0.9)	0

FIGURE 10.

FIGURE 10. Kinetic neutralization of 229E and VH viruses using anti-229E serum.

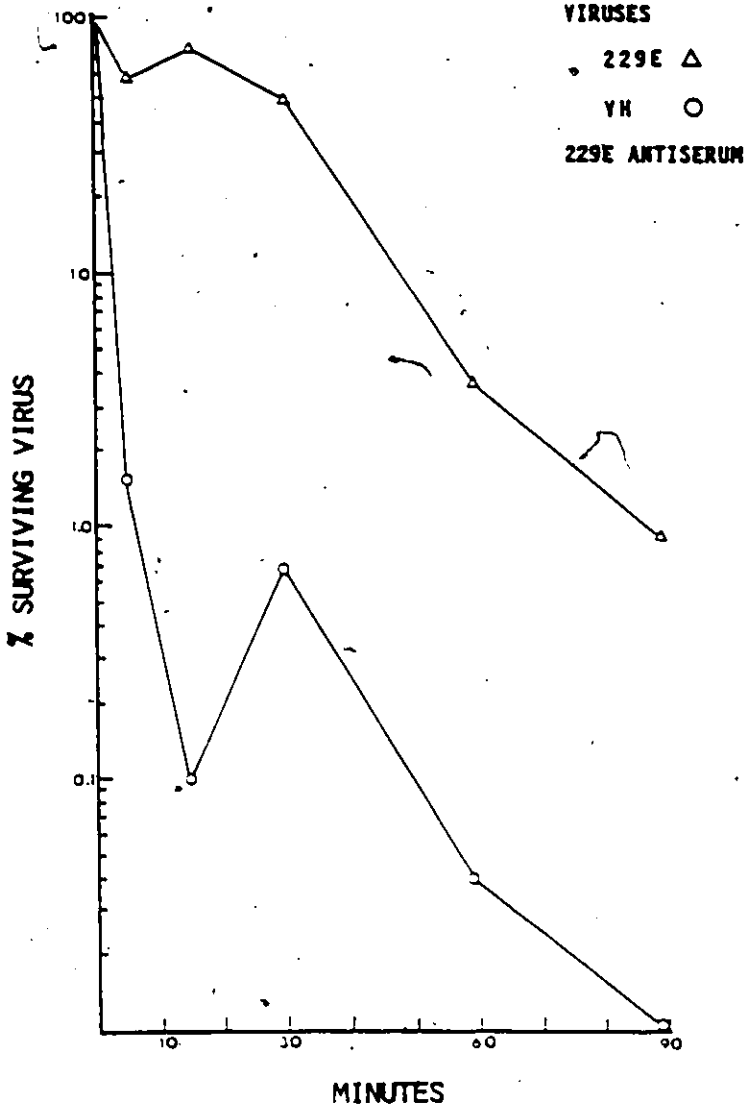

VIRUSES
229E △
YH ○
229E ANTISERUM
% SURVIVING VIRUS
100
10
1.0
0.1
10
30
60
90
MINUTES

Table 17. Kinetic neutralization test of 229E and VH viruses by guinea pig anti-VH serum.

Time (minutes)	Plaques	
	229E (%)	VH (%)
0'	69 (100)	381 (100)
5'	45 (65.22)	119 (31.23)
15'	25 (36.23)	46 (12.07)
30'	13 (18.84)	12 (3.15)
60'	3 (4.35)	1 (0.26)
90'	4 (5.79)	0

FIGURE 11.

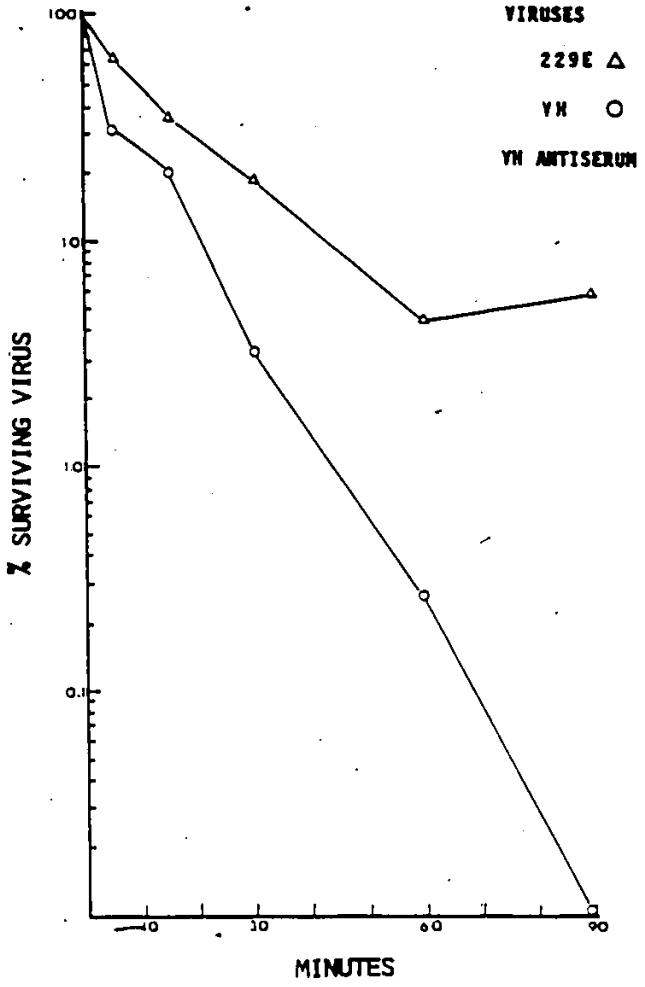

VIRUSES
229E △
YH ○
YH ANTISERUM
100
10
1.0
0.1
% SURVIVING VIRUS
30
60
90
MINUTES

FIGURE 11. Kinetic neutralization of 229E and VH viruses using anti-VH serum.

antibody mixtures at 37°C. Coronaviruses are easily inactivated on storage, even at refrigerator temperatures, and it is for this reason that the virus adsorption step in infecting cells is always at room temperature. Therefore, it was considered necessary to determine if the differences in antibody sensitivity of these viruses might be due to differences in susceptibility to thermal inactivation at the higher incubation temperature. As can be seen in Table 13, the results of the preliminary experiment showed that in fact, VH virus did not survive as well as 229E at 37°C but it did fare better than when antibody was present. At 23°C., both viruses were equally inactivated by nearly 40% after 90 minutes.

The results of the KN test using the newly prepared anti-229E immunoglobulin are shown in Table 19 and Fig. 12. Clearly neutralization of both viruses was much slower but, after 60 minutes, it was apparent that 20% more VH virus was neutralized than 229E. These results showed therefore, that there were differences in the sensitivity to neutralization of these viruses which were enhanced by thermal inactivation of the virus.

The neutralization of virus infectivity occurs through an interaction with antibodies and the outer virion surface components. In the case of coronaviruses these are the E2 peplomer glycoproteins and the results of both the PR and KN neutralization studies indicate that, in the case of 229E and VH, the E2 antigens are antigenically distinct but interrelated.

(iv) Homologous and Heterologous Immunodiffusion Reactions. Further antigenic characterization of the two viruses was carried out using the immunodiffusion reaction in cellulose acetate which was described in the Material and Methods section of Chapter II. In addition to providing

Table 18. Thermal inactivation of 229E and VH viruses.

Temperature	Virus	Virus titre 0'	Virus titre 90'	% Surviving Virus
37°C	229E	$10x10^5$	$5.5x10^5$	55
	VH	$3x10^7$	$1 x10^7$	33.3
23°C	229E	$3x10^6$	$1.8x10^6$	60
	VH	$2x10^7$	$1.3x10^7$	65

Table 19. Kinetic neutralization of 229E and VH viruses by 229E Immunoglobulin 23°C.

Time (minutes)	Number of Plaques 229E (%)	VH (%)
0'	109 (100)	246 (100)
5'	60 (55.05)	217 (88.21)
15'	71 (65.14)	153 (62.19)
30'	66 (60.55)	141 (57.31)
60'	67 (61.47)	109 (44.31)
90'	48 (44.04)	54 (21.95)

FIGURE 12.

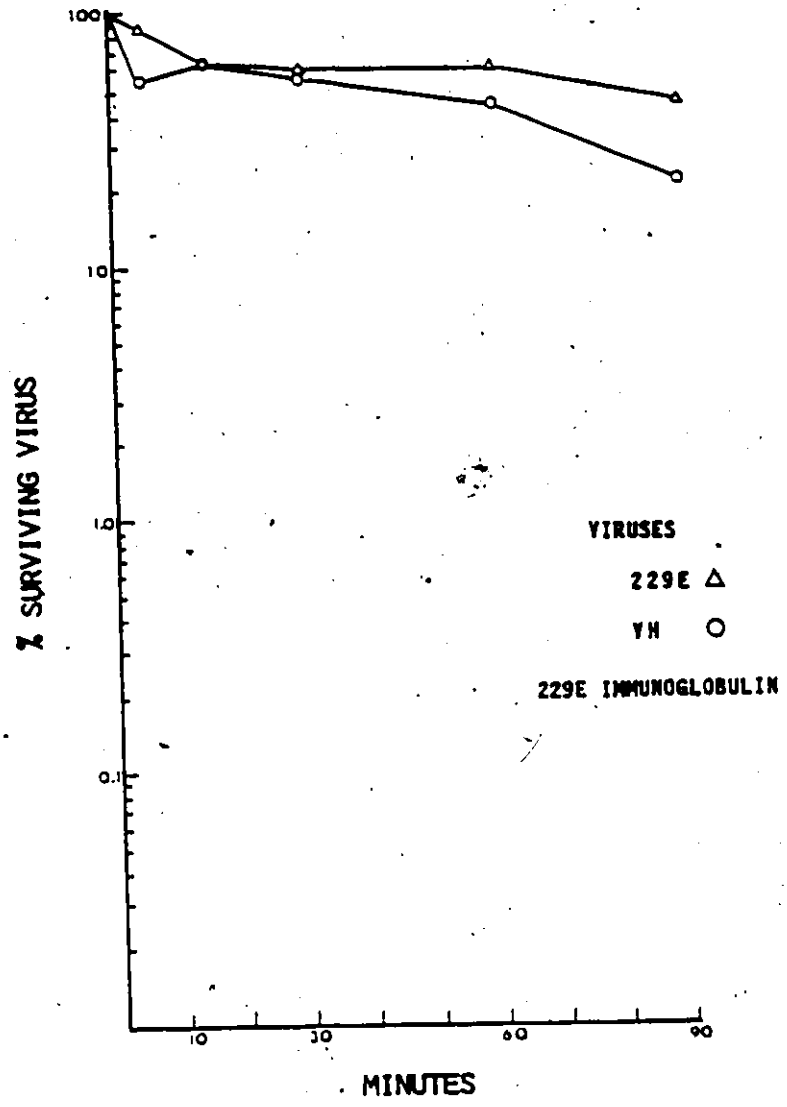

100
10
1.0
0.1
% SURVIVING VIRUS
10
30
60
90
MINUTES
VIRUSES
229E Δ
YH O
229E IMMUNOGLOBULIN

FIGURE 12. Kinetic neutralization of 229E and VH viruses using anti-229E immunoglobulin at 23°C.

information on the antigenic relationships between 229E and VH, these reactions were useful for the evaluation of the antiserum preparations. The results of several representative reactions are shown in Figs. 13, 14 and 15. It can be seen in Fig. 13B that the immunoglobulin preparations (Ig) have not detected as many antigenic components compared to the untreated antiserum (Fig. 13A), and that host antigens (L132) are present in the virus preparations and are detected and identified by host antiserum (L132/As) components. There are slight differences in the intensities of the precipitin lines in the homologous reactions, however, since they show complete reactions of identity, there are no differences in the antigenic characteristics of this major viral component. The reactions with the VH/Ig reveal an extra antigenic component present in both VH and 229E preparations. The reactions shown in Fig. 13A confirm the existence of the extra VH component (arrowed) which is only slightly detectable by the 229E As.

Further reactions were then carried out to evaluate the specificity of the immunodiffusion test when used to identify coronavirus antibodies in the sera obtained in the MS case/control study. Selected reactions are presented in Fig. 14 and 15. The control reactions, included in each slide, consistently confirmed the presence of the extra VH component. In Fig. 14A and B, the major precipitating components detected in the control antiserum reactions are not detected by the study sera, and are believed to be due to host antigens present in the 229E twice concentrated antigen. In these reactions, serum numbers SS, 330 (Fig. 14A) and 33 (Fig. 14B) would be classified as negative.

When the VH antigen was used in the ID test, again, positive lines developed due to serum antibodies in the study sera and these were identified as being VH specific (Fig. 15A; Sera 462, 119 and TC).

FIGURE 13.

FIGURE 13. Homologous and heterologous immunodiffusion reactions of 229E and VH antigens and antisera.

A. In the upper wells, there are 3 reacting components of VH Ag, all linking to heterologous VH Ag/ 229E As. But homologous 229E Ag/229E As does not link clearly with the heterologous reaction (partial identity ?). There is a 4th VH homologous component not found with the 229E reaction (arrowed).

B. The reaction patterns are similar to A. using immunoglobulin (Ig) instead of whole serum (As). The homologous VH Ag/VH Ig line 1 is very faint and line 4 is not visible. VH Ig detects an extra component in the 229E Ag preparation and Host Ag is detected by 229E Ig.

FIGURE 14.

FIGURE 14. Immunodiffusion test of selected MS patient and control sera with 229E antigen.

A. Serum numbers 25 and 441 are positive and identified as 229E because they line with the heterologous VH As/229E Ag line. The 229E Ig virus component line closest to the serum well is very weak. Serum numbers SS and 330 would be classified as negative in a screening test.

B. A reaction similar to A, in which 33 is negative and the other three, 29, 116 and 12 are positive and again there is no reaction of identity with the host antigenic component identified by both antisera (A and B).

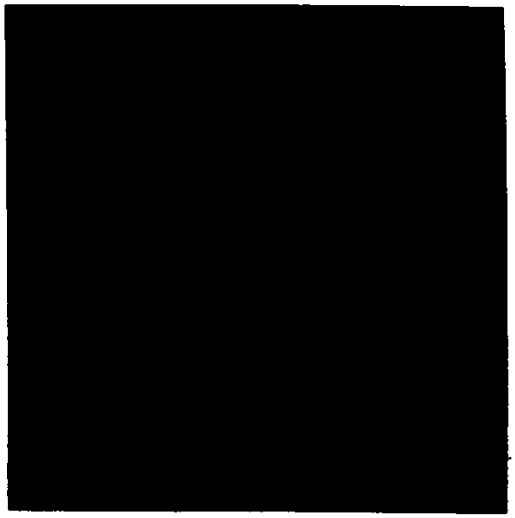


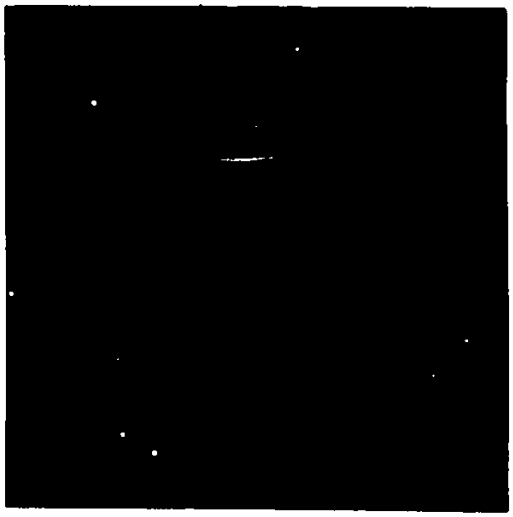
FIGURE 15.

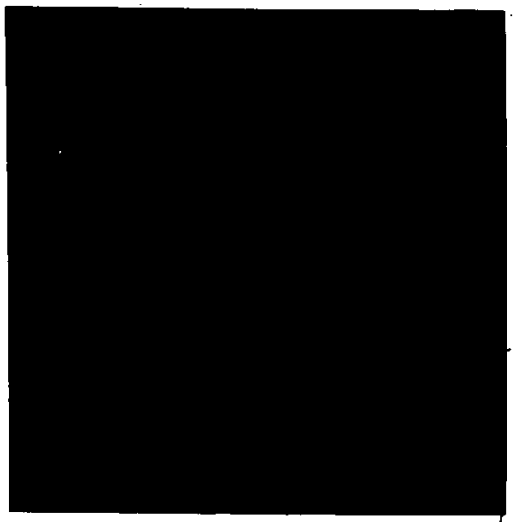
FIGURE 15. Immunodiffusion test of selected sera with VH and 229E antigen

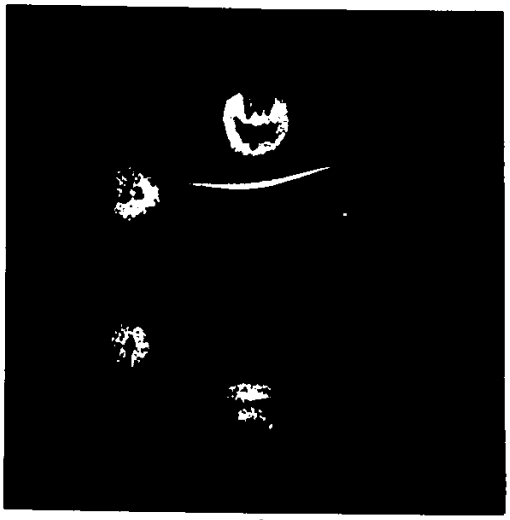
A. Immunodiffusion reaction lines present in sera 462, 119, 105 and TC link in a reaction of identity with the VH As precipitin lines.

B. Immunodiffusion reaction illustrating similar positive reactions as in A also reveals the dilution of viral reacting component in 229E Ig.

C. Reaction showing the decreased intensity of precipitin lines reflecting loss of reacting components from VH Ig compared to VH As due to the dilution of the serum during preparation of immunoglobulins. All the test sera in this reaction are positive and clearly link with the virus precipitin line.







These reactions were confirmed using the concentrated 229E antigen and the reactions were more definite as shown in Fig. 15B. In this reaction, it can also be seen that the 229E Ig preparation is not as effective as the 229E untreated antiserum for detecting the viral components. In the reaction shown in Fig. 15B, the untreated VH antiserum (VH As) was also more effective in detecting a 229E viral component which was also detected by all four of the study sera.

In summary, it would seem that the immunodiffusion reaction for the detection and identification of coronaviral antibodies in human sera is reliable, but that further work to identify the antigenic component involved is required.

4. Discussion.

The aim of this study on the antigenic relationships between the 229E and VH viruses was to try to characterize the antigenic differences which had been suggested in other experimental work. It was also hoped that this might help us to understand the difference between the results of the preliminary immunodiffusion study using 229E concentrated antigen and the later MS case/control IFA study using VH virus in the HV-1 persistently infected cells.

The two different types of neutralization tests (PR and KN) have demonstrated that the neutralizing antigens of the two viruses are distinct but related. These antigens are associated with the E2 surface peplomer glycoprotein and they were also found to be different in the IFA tests using monoclonal antibody. In our experiments, VH virus was more efficiently neutralized than 229E by both anti-229E and anti-VH. This would imply that both viruses have similar epitopes which are recognized by the neutralizing antibodies and these results might

suggest that the neutralizing epitopes on the VH virus are more exposed, or available to the antibody molecules thus making the VH virus more readily inactivated or that there is simply a quantitative difference in the amounts of reacting epitope on each virion.

Since the VH antiserum neutralized 229E virus to almost the same extent as the homologous antiserum (Figs. 10 & 11), we do not feel that differences in relative amounts of E2 antigen are involved. Instead, it is suggested that there might be structural differences between the E2 glycoproteins and that those present on the 229E virion may be masked by surface host material, glycosaminoglycan (GAG; Fig. 16) which is not present on the VH virion.

Glycosaminoglycans are secreted by host cells and have been co-purified with several different coronaviruses, e.g. TGEV, MHV-A59. They are known to be on the external surfaces of the viral envelope since they can be removed by protease treatment of intact virions (Sturman and Holmes, 1983). They tend to aggregate spontaneously with like molecules to form large complexes. These complexes may mask the surface receptors of the virion and interfere not only with neutralization but also with virus infectivity. Since VH virus replicates more efficiently in L132 cells that 229E, producing considerably greater amounts of virus (titres 2 logs higher) in a shorter period of time (4 days instead of 6; (Chaloner-Larsson and Johnson-Lussenburg, 1981a & b)), it is suggested that GAG is not present on the surface of the VH virions. Therefore, we can further suggest that the neutralizing antibodies, not being impeded by GAG substances, attach more quickly to the receptors of the surface of VH virion eliminating the infectivity of VH virus. In the case of 229E virus GAG acts as a mask of the neutralizing epitopes on the surface of the virion interfering with their recognition by neutralizing

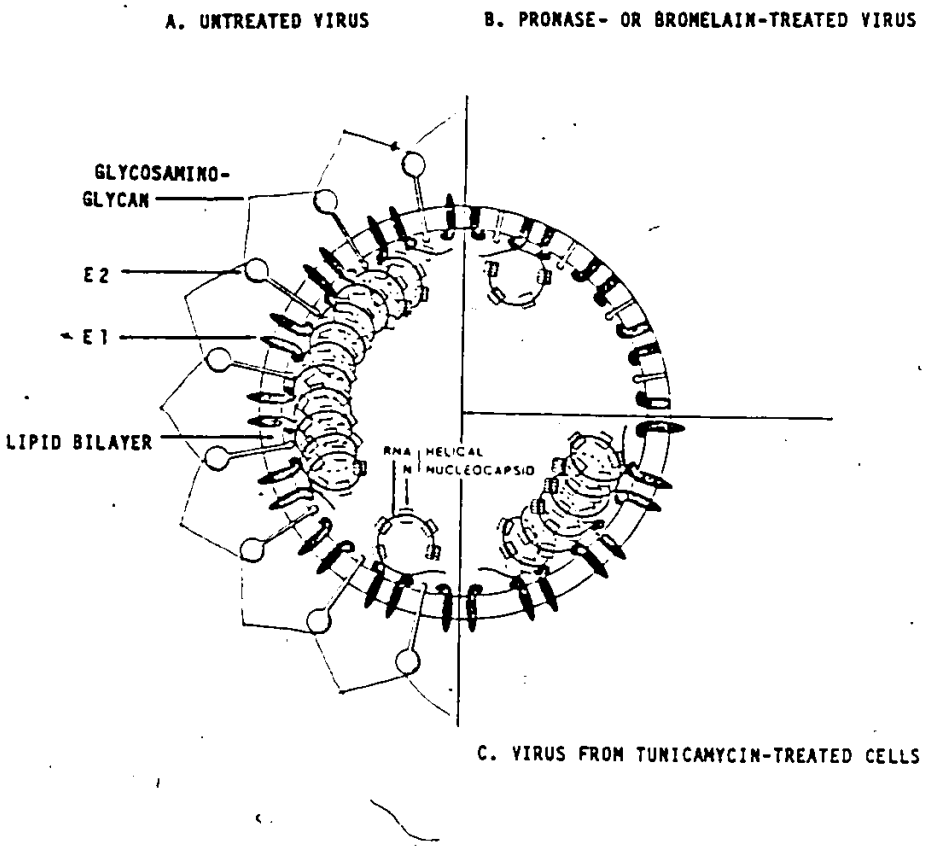
FIGURE 16.

FIGURE 16. Structural model of a coronavirion. This model is based on the structure of MHV and is excerpted from Sturman and Holmes, (1983).

A. The envelope of the intact virion contains two envelope glycoproteins, E1 and E2, in a lipid bilayer. The helical nucleocapsid is made up of a single, long, +RNA with the nucleocapsid protein, NP. Glycosaminoglycan is associated with the viral envelope and is diagrammatically illustrated.

B. Treatment with proteases removes most of the virion membrane projections, leaving the components within the lipid bilayer and the nucleocapsid intact.

C. Virions released from cells treated with tunicamycin lack E2 but contain normal amounts of glycosylated E1 and nucleocapsid. They are not infectious.



(Figure taken from Sturman and Holmes, 1983)

antibodies thus requiring more antibody molecules to neutralize the infectivity of 229E virus. These suggestions are clearly speculative and further evidence is required for their support. However, such information should facilitate studies designed to understand the molecular basis of the diverse acute, chronic and latent pathogenic processes induced by coronaviruses.

The results obtained in the immunodiffusion experiments did not demonstrate any major differences between the VH and 229E viruses which could explain the differences between the two serological survey studies. Therefore, we are of the opinion, that the differences reflect the different seasons and emphasize the importance of relevant controls and sample size. Overall, the immunodiffusion results are supported by the more stringent IFA study but the support is more tenuous. We also conclude that, while the IFA test using the VH antigen is both reliable and sensitive, it is advisable to include the neutralization test to identify E2 antibodies which are regarded as evidence of recent coronaviral infection.

Chapter IV.

GENERAL CONCLUSIONS

The objective of this study was to determine if there is an etiological relationship between human coronavirus 229E (HCV/229E) and MS. MS is one of the major diseases of the CNS affecting man and its etiology is unknown. There are considered to be two possibilities; one is a continuing viral infection of cells in CNS and the other is associated with an abnormal immune response with an autoimmune attack on the CNS. A yiral infection may set the stage for the autoimmune response and may trigger the abnormal immune reaction. A number of types of coronaviruses are known to establish persistent infections in their natural hosts and several murine strains can cause dymyelinating disease experimentally. Also, coronaviruses are very common pathogens in all their natural hosts and in humans usually cause relatively mild upper respiratory infections (common cold). These characterisitics make human coronavirus a good candidate to be considered as a factor in the developmeht of MS. Since it was not feasible to undertake isolation studies, the indirect serological approach was used to try to demonstrate an association between HCV/229E and patients with MS.

There were two major components to this study. One was an evaluation on a MS case/control serological study; the other was a comparative analysis of the antigens of HCV/229E and persistent progeny virus VH which were relevant to the interpretation of the survey results.

The 8-month longitudinal serological survey followed a preliminary one, in which the results showed a significantly higher positive percentage of immunodiffusion reactors with 229E antigen in the MS group (89%) compared with the control group (21%). The total number of sera

collected for this work was 42, 28 MS and 14 controls. The latter were matched for age and sex but not for season. Nor was there any clinical history available for the control subjects.

For the comparative antigenic investigation, two types of neutralization tests as well as immunodiffusion reactions were used to characterize the relationships between both the viruses. Although it was clearly demonstrated that these viruses were antigenically distinct but related there was no evidence that these differences should invalidate the use of IFA with the persistently infected cells (VH virus).

In the 8-month study, a total 584 sera obtained at monthly intervals from the same 39 MS case/pal subjects were tested by IFA using HV-1 persistently infected cells. And great care was taken to select controls who were most likely to be exposed to the same environmental conditions as the patients. The results of this study showed no significant differences in the titres of coronavirus antibodies in the sera from patients with multiple sclerosis and matched controls. The GMT of both groups was almost the same. This suggested that there were no significant link between MS and HCV (229E and VH coronaviruses).

But, examination of the data on colds experienced by the participants during the period of the study revealed several interesting facts. It was found that there was a statistically significant association between the occurrence of colds and exacerbation of MS symptoms in the patients. Furthermore, when four-fold rising titres associated with the reported cold was used to identify HCV/229E-VH as the infecting virus, there were significantly more colds associated with 229E-VH in the MS patients than among the controls. The significance of these findings in relation to a coronavirus etiology in MS is certainly inconclusive. However, they are suggestive and support the need for further investiga-

tions aimed at providing more direct evidence such as detection and identification of viral components in patient specimens. Sensitive techniques using genetic probes to detect viral genome sequences would be highly relevant in this regard.

A further study suggested by these results and related to the role of viral persistence in MS is concerned with the fact that the mechanisms of maintenance of the HV-1 persistently infected cells involves the presence of both interferon alpha and beta. It would be of great interest to extend our studies to see whether abnormal levels of serum IFNs are associated with either colds or MS patients or both. Further studies aimed at elucidating the role of the interferons and temperature in the establishment of the HCV persistent state. This is of even greater importance because the virus which is continually shed by the persistently infected cells appears to be more virulent than its original parent strain. It is also important to know whether IFN was responsible for this change especially since IFN is being considered for use as a cure for the common cold.

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APPENDIX 1

Special formulations used in this investigation.

1. Overlay #3

medium 199 (5x)	120 ml
water sterile	240 ml
sodium bicarbonate (5.6%)	21.4 ml
BudR (1%)	3 ml
DEAE-dextrose (4%)	3 ml
Fetal Calf Serum	12 ml
antibiotics (P.S.N.)	0.6 ml

2. Immunodiffusion buffer:

Tris (hydroxymethyl) Aminomethane	9.3 g
Nacl	7.0 g
Distilled water	1000 ml
Sodium azide	0.2%

Adjust pH with 1N HCl to 7.4-7.6

3. Thiazine Red R (stain for immunodiffusion reactions in cellulose acetate):

Thiazine Red R	0.1%
Acetic acid (Glacial)	1.0%

Thiazine red is dissolved in boiling water (distilled). The mixture is allowed to cool before acetic acid is added to a final concentration of 1.0%. The stain is then filtered to remove undissolved stain crystals.

APPENDIX 2

A. Tests used in Statistical Analysis

1. Chi-square test:

The Chi-square test can be used for the comparison of two proportions in paired or independent samples.

The general formula for the chi-square is :

$$X^2 (df) = (O-E)^2 / E$$

Where O = observed count in a category
E = expected count in that category if the null hypothesis is true
df = degrees of freedom (for fourfold table, df=1)

For example, the data about the relationship of MS exacerbation and cold are calculated as follows:

MS exacerbation	cold: yes	cold: no	
yes	6	13	19
no	33	242	275
	39	255	294

The E values for these four categories are:

$$\frac{19 \times 39}{294} = 2.52 \qquad \frac{19 \times 255}{294} = 16.48$$

$$\frac{39 \times 275}{294} = 36.48 \qquad \frac{255 \times 275}{294} = 238.52$$

In the case of E<5, the continuity correction of the chi-square formula called Yates' correction should be used.

$$X_c^2 (df) = \Sigma (|O-E| -0.5)^2 / E$$

$$X_c^2 (1\ df) = \frac{(|6-2.52|-0.5)^2}{2.52} + \frac{(|13-16.48|-0.5)^2}{16.48} + \frac{(|33-36.48|-0.5)^2}{36.48} + \frac{(|242-238.52|-0.5)^2}{238.52}$$

$$= 3.52 + 0.54 + 0.24 + 0.04 = \underline{4.34}$$

x^2 (1) 0.01 = 6.63 x^2 (1) 0.05 = 3.84

The calculated chi-square figure (4.34) falls between these two values. $6.63 > x^2 > 3.84$ $\underline{0.01<P<0.05}$

This indicated that there is a significant relation between MS exacerbation and cold.

For detail see Statistics in Medicine (Colton, 1974).

2. t-test

The t-test can be used for comparison of means of two samples. The formula is:

$$t_{n1+n2-2} = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{S^2(1/n1+1/n2)}}$$

Where $\bar{X}_1$ = The mean of sample I

$\bar{X}_2$ = The mean of sample II

n1 = The numbers in sample I
n2 = The numbers in sample II
df = n1 + n(2 - 2)
S^2 = an estimate of the standard deviation based on both samples. It can be calculated by equation:

$$S^2 = \frac{\Sigma(\bar{X}_1-\bar{X}_1)^2 + \Sigma(\bar{X}_2-\bar{X}_2)^2}{n1 + n2 - 2}$$

MS/CORONAVIRUS STUDY QUESTIONNAIRE #1

DATE.....

NAME:..... DATE OF BIRTH.....

ADDRESS.....

.....

TELEPHONE (home)..... (work).....

How long have you had MS?..... OR If you are a Control member of this study, who is your partner?

.....

****

	YES	NO
1. Have you had a cold recently, (i.e. within the last month)?	___	___
If YES, which of the following smymptoms did you have?		
Stuffy nose	___	___
Headache	___	___
Sore throat	___	___
Sneezing	___	___
Coughing	___	___
Fever	___	___
Muscle aches & pains	___	___
Upset stomach	___	___
Diarrhoea	___	___
How long did your cold last? 2 - 3 days	___	___
3 days to a week	___	___
longer than a week	___	___
If NO, can you remember the last time you had a cold?	___	___
Please give the appropriate date.....		
2. Do you get colds often?	___	___
If YES, how often? -Monthly	___	___
-every 2 or 3 months	___	___
-once or twice a year	___	___
3. Is there a season when you seem more likely to catch a cold?	___	___
If YES, which one(s)? - Spring	___	___
- Summer	___	___
- Fall	___	___
- Winter	___	___

If you have any questions about this questionnaire, or any other aspect of this Study, Dr. Johnson-Lussenburg will be glad to discuss them with you, now if you have time, or later by telephone ..... 737-6528 (work) or 824-1642 (home).

MS/CORONAVIRUS STUDY FINAL QUESTIONNAIRE

DATE..... .....

NAME:.....

PRESENT ADDRESS:.....

.....

PLACE OF BIRTH:.....

Was your home mainly RURAL....., or URBAN.....?

How long did you live there?.....year.

If you moved before age 15, how often.....?

Please list the places you lived up to age 15/16 with approximate dates:

.....

FAMILY; How many BROTHERS:.....and/or SISTERS:.....do you have?

Did you have PETS at home:.....? DOGS:.....CATS:..... OTHER:.....

Do any other members of your family have MS?.....

Brother;..... Sister:.....Uncles:.....Aunt.....Cousin:.....

Did you all live in the same town or area?.....

FRIENDS: Have any of your childhood friends developed MS?.....

How many?..... Were they neighbours?.....and/or Classmates.....

The following questions relate to upper respiratory infections (colds, sore throat, flu) and multiple sclerosis.

1) How many colds have you had this past winter?
   0, 1, 2, 3, more than 3

2) Do you have more colds then your spouse, children or friends?
   yes _____ no _____

3) Since you developed M.S. have you had more colds? ____ fewer colds? ____
   or no change ____?

4) Since you developed M.S. does it take longer to get over a cold?
   yes _____ no _____

5) Have you had cold or flu shots this past year? _____

Please mail this questionnaire back to Dr. Johnson-Lussenburg using the attached stamped envelope.

If you have any questions about this questionnaire please call Dr. Johnson-Lussenburg at 737-6528 (work) or 824-1642 (home).

(francais au verso)

HÔPITAL GÉNÉRAL D'OTTAWA
OTTAWA GENERAL HOSPITAL
501 CHEMIN SMYTH ROAD, OTTAWA ONTARIO, K1H 8L6
(613) 737-7777

PATIENT CONSENT FORM

NAME OF STUDY: CORONAVIRUS / MS STUDY

INVESTIGATOR(S): C.M. Johnson-Lussenburg. Ph.D. and R. Nelson, M.D.,F.R.C.P.(C)

PATIENT'S NAME: _____

I, _____, agree to participate in the above study and hereby authorize Dr(s). Nelson and Johnson-Lussenburg, the physician(s) and investigator(s) to proceed with the following ~~examinations and/or to administer these treatments~~:

- taking one blood sample by venipuncture each month for a period of 8 months.

I have received a detailed explanation of the examinations to be conducted ~~and treatment to be given~~ and I understand their nature and purpose.

I have had a detailed description of all the known side effects and risks related to the examinations and treatments. I have also received a description of any benefits that may be expected from these examinations and treatments. Alternative forms of examinatic and treatments have been disclosed to me.

I have been given an opportunity to ask questions ~~concerning the examinations and treatments involved~~ and the questions which I have asked have been adequately answered.

I have been told that I am able to withdraw my consent and to stop my participation in the study at any time and for any reason.

With full knowledge of this, I voluntarily consent to participate in the study.

DATE: _____ PATIENT'S SIGNATURE: _____

WITNESS: _____ WITNESS: _____
(investigator)