CHAPTER 18

Human Coronavirus Infections

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I. INTRODUCTION AND HISTORY

The first report of a human coronavirus was in 1965 when Tyrrell and Bynoe (1965) isolated a virus from the nasal washings of a male child. The child had typical symptoms and signs of a common cold and the washing was found to be able to induce common colds in volunteers challenged intranasally. The virus, termed B814 (after the number of the nasal washing), could be cultivated in human embryo tracheal organ tissue but not in cell lines used at that time for growing other known etiologic agents of the common cold. At the same time, Hamre and Procknow (1966) were characterizing five "new" agents isolated from the respiratory tract of medical students with colds. One of these agents, strain 229E, was adapted to grow in WI-38 cells. Subsequently, Almeida and Tyrrell (1967) showed that these isolates were morphologically identical to the viruses of avian bronchitis and mouse hepatitis. McIntosh and colleagues (1967a), working at the National Institutes of Health in Bethesda, then isolated six morphologically related viruses that could not be adapted to cell monolayer culture but would grow in organ cultures. Two of these isolates, OC (for organ culture) 38 and 43 were then adapted to grow in suckling mice brain. The term "coronavirus," which described the characteristic morphology of these agents, was accepted in 1968 (Tyrrell *et al.*, 1968a).

II. HUMAN RESPIRATORY CORONAVIRUSES

There are a number of human respiratory coronaviruses described in the literature, but few have been well characterized (Table I). On the basis of

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The Coronaviridae, edited by Stuart G. Siddell, Plenum Press, New York, 1995.

TABLE I. Classification of Human Coronaviruses

Serogroup	Prototype	Virus
A	229E	229E
		LP
		PR
		TO
		KI
		PA
		AD
		Linder
		Others
B	OC43	OC43
		OC38
		OC44
Unclassified		B814
		692
		OC16
		OC37
		OC48
		HO
		GI
		RO

serological cross-reactivity, however, it is possible to classify most of them (Reed, 1984; Bradburne, 1970; McIntosh *et al.*, 1969). The two main serogroups are 229E-related and OC43-related and it is these prototype viruses that will be discussed in the rest of this chapter. Coronaviruslike particles have been seen in the stools of humans, but, as they have not been characterized, they will not be discussed in this chapter.

III. EPIDEMIOLOGY

Most epidemiological surveys of these viruses as agents of respiratory tract illness have been based on serology, using either complement fixation or hemagglutination-inhibition tests for the two prototype viruses 229E and OC43. As it is clear that serologically unrelated coronaviruses exist, the prevalence of these viruses is likely to be underestimated. Moreover, it is also certain that not all human respiratory coronaviruses have yet been adapted to tissue or organ culture. This conclusion is supported by a study based in London, England, in which coronaviruses were isolated, by tissue or organ culture, from 18.4% of patients with common colds. However, a further 13% of the isolates were able to induce colds in inoculated volunteers, although they could not be identified as any of the known "common cold" viruses (Larson *et al.*, 1980).

The most extensive epidemiological survey of OC43 infection has been the study of the community of Tecumseh, Michigan (Monto and Lim, 1974). A 4-year study involved looking for serological evidence of infection with OC43

in 910 persons in 269 families. A mean of 17.1% of individuals showed evidence of infection with OC43 in any one year. There was, however, a cycling of the frequency of occurrence: OC43 infections occurred in most years but there were peaks of infection every 3 years. Over 80% of infections occurred despite preexisting antibody with infections gradually diminishing with age. The peaks of infection took place in the winter–spring months. A study of 229E infections in the same community showed a mean annual rate of 7.7% infected persons (Cavallaro and Monto, 1970). This figure was half of that recorded in a 6-year study of Chicago medical students, in which there was also marked year-to-year variation with peaks of 35% incidence (Hamre and Beem, 1972). Nearly all 229E infections also occurred in the winter and spring months. The Tecumseh study showed that either 229E or OC43 was dominant in any one year.

Studies of coronaviruses as causes of clinical illness have shown that coronaviruses are second only to rhinoviruses as the causes of the common cold. In the United States and England, 229E and OC43 are responsible for 1 to 30% of all clinical cases (McIntosh *et al.*, 1970a,b; Isaacs *et al.*, 1983; Wenzel *et al.*, 1974; Kaye *et al.*, 1971; Owen-Hendley *et al.*, 1972; Bradburne *et al.*, 1967), with approximately an equal number of subclinical infections.

IV. DISEASE MANIFESTATIONS

Human respiratory coronaviruses are now well accepted as causes of upper respiratory tract illness and, in particular, the common cold. The viruses have been isolated from patients with the common cold and they produce common colds when inoculated into volunteers intranasally (Andrewes, 1962). There is a mean incubation period of 3 days (range 2–5 days) followed by an illness that lasts a mean of 6–7 days (range 2–18 days). The classical clinical illness is well known to all of us and consists of general malaise, headache, nasal discharge, sneezing, and a mild sore throat (Tyrrell *et al.*, 1993). Approximately one tenth of patients will also have a fever and one fifth will have a cough. Table II contrasts the clinical features of rhinovirus type 2-induced common colds, those induced by coronavirus 229E, and the illness caused by the influenza A virus (compiled from Lowenstein and Parrino, 1987; McIntosh *et al.*, 1973, 1974). It is not possible on an individual basis to distinguish rhinovirus colds from coronavirus colds. Although earlier studies suggested that there may be differences in the clinical symptomatology with different coronaviruses, this has not been substantiated.

Less well documented are lower respiratory tract infections associated with coronaviruses. A seroepidemiological study, using a complement fixation test, found that coronaviruses were less likely to be found in hospitalized children with lower respiratory tract infection than in controls with non-respiratory tract disease. From 565 children with lower respiratory tract infection there was evidence of infection with OC38 or OC43 in 3.5%, compared to 8.2% of 245 children in the group with nonrespiratory tract disease (McIntosh *et al.*, 1970a,b). In a later study, however, there was serological evidence of either 229E or OC43 infection in 8.2% of 417 hospitalized children under 18

TABLE II. Clinical Features of Rhinovirus, Coronavirus, and Influenza A Respiratory Tract Infection

Clinical feature	Coronavirus 229E (%)	Rhinovirus 2 (%)	Influenza A (%)
Fever	9–23	7–16	98
Nasal discharge and/or obstruction	94–100	64–100	20–30
Headache	32–85	28–50	85
General malaise	46–47	28–43	80
Sneezing	85	50	30
Sore throat	54–68	87–93	50–60
Cough	21–31	64–68	90
Hoarseness	12	57	10
Myalgia	9	21	60–75
Watery/sore eyes	29	43	60–70
Chills	18	21	90

months of age with lower respiratory tract disease (McIntosh *et al.*, 1974). The incidence of coronavirus infection was higher than that of other respiratory viruses, except parainfluenza virus type 3 and respiratory syncytial virus. It is unlikely that there is a direct infection of the lower respiratory tract with coronaviruses and any association is most probably through secondary phenomena. Though these secondary phenomena are yet to be defined, a link between coronavirus infection of the upper respiratory tract and wheezing attacks has been shown in several studies over the last 20 years (Isaacs *et al.*, 1983; McIntosh *et al.*, 1973; S. L. Johnston *et al.*, unpublished data). At particular risk are asthmatic children, in which up to 30% of acute wheezing episodes may be due to coronavirus infection. Increased airways resistance has been shown to occur in the upper respiratory tract in nonatopic individuals with colds (Bende *et al.*, 1989; Akerlund, 1993) and asthmatic individuals are likely to be at greater risk.

Nonrespiratory tract illnesses have also been associated with coronaviruses and include multiple sclerosis, pancreatitis, thyroiditis, pericarditis, nephropathy, and infectious mononucleosis (Riski and Hovi, 1980; Apostolov and Spasic, 1975; Arnold *et al.*, 1981). The association with multiple sclerosis has been of particular interest since coronaviruslike particles were seen in the postmortem brain of a patient who died with the disease (Tanaka *et al.*, 1976). Subsequently, OC43-related coronaviruses were isolated from the brain material of two multiple sclerosis patients (Burks *et al.*, 1980), although it is likely that these isolates were murine coronaviruses present in the mice used for cultivation (Weiss, 1983; Gerdes *et al.*, 1981; Fleming *et al.*, 1988). Seroepidemiological studies trying to ascertain an association of coronaviruses with multiple sclerosis have been conflicting (Madden *et al.*, 1981; Salmi *et al.*, 1982; Hovanec and Flanagan, 1983; Leinikki *et al.*, 1981). Gene detection has failed to detect OC43 (Sorensen *et al.*, 1986) but has suggested a neurotropism for 229E (Stewart *et al.*, 1992). It is clear that further research in this area is needed.

The role of coronaviruses in causing diseases outside the respiratory tract has been doubted, in part, because there has been no evidence that the virus can

spread from the nasal mucosa. Using the polymerase chain reaction, however, it has recently been possible to show that there is a short viremic phase of 229E, in at least some experimentally inoculated volunteers (S. Myint, unpublished data). The identification of the 229E receptor as a metalloprotease that is found on the surface of cells in many tissue types would also suggest a possible involvement of human coronaviruses in diseases other than just the common cold.

V. PATHOGENESIS AND IMMUNE RESPONSE

Little is known about the detailed pathogenic mechanisms in human coronavirus infection, principally because there is no animal model of infection. Even the predominant mode of transmission is uncertain. Infection can be induced experimentally by direct inoculation of virus into the nose, but this is unlikely to be the natural route. By analogy with rhinoviruses it is likely that infection is either by aerosols or fomites (Editorial, 1988). In support of this view is the finding that 229E survives well in an atmosphere of high humidity and low temperature (Ijaz *et al.*, 1985).

Once in the nose, the virus is thought to enter the cell via a specific receptor, aminopeptidase N. Replication is optimal at 32–33 °C, the temperature in the superficial layers of the nasal mucosa. This results in sloughing of the superficial nasal epithelium and a proteinaceous exudate. Serum antibody levels rise after about a week, but it is not clear whether it is this response or cell-mediated mechanisms that clear the infection (Callow *et al.*, 1990). Certainly, there is some correlation of the severity and likelihood of disease with preexisting serum antibody, but the mere presence of such antibody is not protective (Callow, 1985). Serum antibody levels peak about 2 weeks after infection and decline to low or undetectable levels at 12–18 months. Reinfections are common.

VI. DIAGNOSIS

Because of the trivial and temporary nature of common colds, the detection of coronavirus infections has not been attempted in routine diagnostic laboratories. This situation is unlikely to change unless antiviral therapy becomes available. Although most techniques available to the diagnostic virologist have been used to detect human respiratory coronaviruses, even in research laboratories the range of tests employed by any one center tends to be limited. This chapter will give a synopsis of the range of methods used, but the reader is referred elsewhere for details of methods (Myint and Tyrrell, 1994).

A. Organ Cultures

The method, or modifications of it, developed by Tyrrell and Bynoe (1965) to isolate B814 is still used by some laboratories but is hampered by the diffi-

culty of obtaining human embryonic tracheal tissue. It is the best method available for primary isolation of the broadest range of respiratory coronaviruses.

Tracheal tissue is taken from 14- to 24-week embryos and planted in sterile plastic Petri dishes containing 199 medium. The tissue is immersed with cilia uppermost. Virus can be inoculated onto the cilia and then incubated at 33 °C for up to 10 days. Viral replication is indicated by cessation of ciliary activity and confirmed by interference with another virus (echovirus, parainfluenza, or Sendai). Electron microscopy was used originally to confirm the isolates as coronavirus but other tests such as virus neutralization are now employed.

Trachea obtained from 5- to 9-month-old fetuses have also been shown to support the growth of coronaviruses, and different media recipes can also be used (McIntosh *et al.*, 1967a,b).

B. Mouse Brain Culture

OC38 and OC43 (but not B814, OC16, OC37, OC44, or OC48) have been adapted to grow in suckling mice brains (McIntosh *et al.*, 1967a,b, 1970; Tyrrell *et al.*, 1968b). The mice can be inoculated intracerebrally or via the peritoneum, with encephalitis occurring in some several days later. In the initial description of McIntosh and colleagues, CD-1 Swiss mice were used, with encephalitis occurring 11–15 days after inoculation with virus that had been passaged several times in organ culture. After the fourth passage, the time to illness was reduced to 40–60 hr. Evidence of infection was not found in other organs (e.g., liver, heart, or lungs). Virus can be prepared from brain suspensions by clarification through low-speed centrifugation and then adsorption to and elution from group O erythrocytes. Virus may be visualized by electron microscopy. Although this is not used for primary isolation of virus, this method is still a commonly used means of preparing OC43 antigen for serological assays.

C. Cell Culture

Cell cultures have proved to be unreliable for the primary isolation of all human respiratory coronaviruses, but certain strains have been adapted to growth in them. The 229E and related strains grow well in a continuous heteropoid cell line termed C16, as they were the 16th clone of MRC-C cells that was selected (Philpotts, 1983). In the original description of these cells from the MRC Common Cold Unit in Salisbury, the morphology showed a mixture of fibroblastic and epithelioid cells. The former constituted three quarters of the cell population. These cells were contaminated with organisms detected by Hoechst stain 33258, presumably *Mycoplasma* spp. Thus, C16 cells would arguably be the cell line of choice for the isolation of 229E, but frequent passage of these cells results in a increasing proportion of the epithelioid content and a consequent reduction in the ability of the cell line to sustain replication of virus.

Many laboratories have continued to utilize a cell line that was originally used in the work of Hamre and Procknow (1966): a human diploid cell strain from Wistar Institute, so-called WI-38 cells. Although 229E was readily adapted to this cell line, primary isolation was in human kidney cells, and the authors noted that WI-38 cells may not be ideal for primary isolation.

Apart from C16 and Wi-38 cells, many other cell lines have been used to grow individual virus strains (Hamre and Procknow, 1966; Reed, 1984; Larson *et al.*, 1980; Hamre *et al.*, 1967; Kapikian *et al.*, 1969; Bradburne, 1969, 1972; Schmidt *et al.*, 1979; Schmidt and Kenny, 1982; Tyrrell *et al.*, 1979; Chaloner-Larsson and Johnson-Lussenberg, 1981; Bruckova *et al.*, 1970). These are summarized in Table III. These viruses do not grow well in cell types commonly used for the isolation of other respiratory viruses such as HEp-2 or Rhesus monkey kidney, which makes routine identification unlikely. It is, moreover, clear that the ideal cell line for isolation and propagation is not yet available and other methods of diagnosis have to be applied.

D. Electron Microscopy and Immune Electron Microscopy

Electron microscopy of nasal washings is impractical as the virus load is usually below the level of sensitivity of standard methods. It has been used, however, to detect virus in tissue sections such as in mouse brain culture of OC43. It is also the means by which human enteric coronaviruses (HECVs) have been detected. Negative staining with tungsten has usually been the method of choice (Almeida and Tyrrell, 1967; Tyrrell and Bynoe, 1965), but molybdenum and uranium salts have also been used. The size of the particles appear greater if uranium salts are used in place of tungsten (Davies and MacNaughton, 1979).

TABLE III. Cell Lines and Strains Used for the Cultivation of Human Coronaviruses

Cell type	Virus	Primary isolation or adaptation
Human embryonic kidney	229E	Primary
C16 (see text)	229E	Adaptation ≥ primary
Human embryonic lung fibroblast, WI-38	229E	Adaptation ≥ primary
Human embryonic lung fibroblast, MRC-c	229E and OC43	Adaptation > primary (OC43, adaptation only)
Human embryonic lung epithelium, L132	229E and OC43	Adaptation > primary
Human embryonic intestinal fibroblast, MA177	229E	Primary isolation of some strains
Human type II pneumocytes	229E	Primary > adaptation
Human fetal tonsil fibroblast	229E and OC43	Adaptation
Human embryonic rhabdomyosarcoma	229E and OC43	Adaptation
Primary monkey kidney	OC43	Adaptation
Rhesus monkey kidney epithelium, LLC-MK2	OC43	Adaptation
Continuous green monkey kidney epithelioid, BSC-1	OC43	Adaptation

An attempt to enhance electron microscopy by utilizing antibody concentration of cultured virus has been used successfully to detect 692 virus in washings from an adult with upper respiratory tract infection (Kapikian *et al.*, 1973). Nasal washings were passaged through both cell culture and tracheal organ culture, and the resulting supernatant was then incubated with convalescent serum from the same patient. After centrifugation the pellet was then examined on a Formvar-carbon-coated grid. Aggregates of virus were clearly discernible. Supernatant that had been incubated with phosphate-buffered saline, instead of convalescent serum, was also examined but virus particles were not seen.

E. Immunofluorescence

An immunofluorescence method has been developed and applied to the detection of 229E and OC43 in nasopharyngeal secretions and washings (McIntosh *et al.*, 1967a). Sera were raised in rabbits against mouse brain-derived OC43 and cell culture-grown 229E and used in an indirect fluorescence assay. This test was able to detect homologous coronavirus antigens in nasal washings from infected volunteers, though cross-reactivity was noted with the 229E antiserum in washings from volunteers who had been inoculated with OC43 and OC44. No nasopharyngeal aspirates from 106 children who were hospitalized with respiratory tract infection had detectable coronavirus antigen by this method. It is difficult to ascertain whether this was due to a lack of sensitivity, as paired sera collected from 66 children during the study period did not show evidence of coronavirus infection.

F. Enzyme-Linked Immunoassay

An enzyme-linked immunosorbent assay (ELISA) method based on purified 229E and HECV CV-Paris (which has cross-reactivity with OC43) has been used to diagnose infections in children (Isaacs *et al.*, 1983; MacNaughton *et al.*, 1983). The ELISA method is a modification of that described for antibody detection using rabbit antisera (see Section VI.I). In a study of 30 children aged 6 months to 6 years, 159 samples were collected: 111 nose swabs, 11 throat swabs, and 55 nasopharyngeal aspirates. Of these, 34.2% of the nose swabs, but only 18.2% of the throat swabs and nasopharyngeal aspirates, were positive for either 229E or OC43. No comparison with serology was attempted, but the positivity rate would suggest that this ELISA was a sensitive test.

G. Nucleic Acid Hybridization

The first application of gene detection methods to detecting human coronaviruses was developed for 229E using Northern hybridization (Myint *et al.*, 1990). A cDNA that encoded the entire nucleocapsid gene for 229E was ligated

into a Riboprobe (Promega) vector, pGEM-1, from which ^{32}P-labeled full-length transcripts could be generated. These transcripts could be made as sense or antisense, depending on whether an SP6 or T7 promoter as used. This method has been applied to the detection of 229E in nasal washings from inoculated volunteers (Myint *et al.*, 1989) and has been shown to be at least as sensitive as culture. There is also the advantage of a diagnostic result being available within 48 hr. An interesting observation was that the probe method was able to detect virus for longer than cell culture in sequential samples from the volunteers. This probe method will not detect OC43 and attempts to remove the radioactive-labeling by incorporating biotin or digoxigein into transcripts have led to significant loss of sensitivity (S. Myint, unpublished data).

H. Reverse Transcription–Polymerase Chain Reaction

With the advent of gene and probe amplification strategies, it was to be expected that these methods would be seen as advantageous for viruses that are difficult to cultivate. Gene amplification methods based on "nested" priming have been shown to be a sensitive and specific means of detecting both 229E and OC43 (Myint *et al.*, 1994). Serotype-specific nested primers were designed from the known sequences of the nucleocapsid genes of 229E and OC43. The inner primers were, in particular, chosen to produce a small fragment of about 100 base pairs for maximum sensitivity. RNA is extracted using an acid-phenol/guanidinium isothiocyanate procedure followed by reverse transcription using murine Moloney leukemia virus reverse transcription. Two 20-cycle amplification steps are then used with the outer and inner sets of primers, respectively. The sensitivity of the assay appears to be much greater than that of cell culture or probe methods and each primer pair appears to be either 229E or OC43 specific. The use of this method has greatly enhanced the diagnostic yield of coronaviruses in clinical material from asthmatic children (S. L. Johnston *et al.*, unpublished data). The assay is, at least, as sensitive as a combination of culture and serology for diagnosing infection and is more specific. Reverse transcription–polymerase chain reaction is likely to become the method of choice for direct virus detection.

I. Serological Methods

Because of the lack of reliable detection methods prior to the development of those based on gene detection, most epidemiological studies have used serological assays to determine evidence of coronavirus infection. The most widely used and sensitive format is the enzyme-linked immunoassay. The assay was first described for strain 229E (Kraaijeveld *et al.*, 1980; MacNaughton, 1982) but has since been adapted for detection of antibodies to OC43 (Schmidt, 1984). The 229E assay uses antigen that is grown in cell monolayers and then clarified. The OC43 test uses mouse-brain-derived antigen. Rabbit antisera have been used for both 229E and OC43 tests. The specificities of the assays are

similar to that of counterimmunoelectrophoresis, neutralization, and complement fixation assays but sensitivity is over 1000-fold greater. In volunteer studies, the 229E assay has shown a close correlation between clinical illness and virus shedding. It has been the principal method for determining the occurrence and frequency of coronaviruses infections in serological surveys, but recent data suggest that some false-positive and false-negative reactions occur (Myint *et al.*, 1994). The 229E assay also detects antibody rises to some 229E-like viruses (PR, KI, and TO).

Other serological test formats have also been used: indirect hemagglutination (Kaye *et al.*, 1972) and immune-adherence hemagglutination (Gerna *et al.*, 1978) for 229E antibody; rapid microneutralization (Gerna *et al.*, 1979) and plague-reduction (Gerna *et al.*, 1980) for OC43 antibody; and immunofluorescence (Monto and Rhodes, 1977), complement fixation (Hovi, 1978), and single radial hemolysis (Hierholzer and Tannock, 1977; Riski *et al.*, 1977) for both 229E and OC43 antibody. These test formats have been superseded by the ELISA test.

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