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Viruses Causing Common Respiratory Infections in Man. III. Respiratory Syncytial Viruses and Coronaviruses

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These sections on respiratory syncytial viruses and coronaviruses comprise the third of five installments that will be published in *The Journal* during 1973–1974. The first and second installments were in the March and September 1973 issues, respectively. After all sections have appeared, the compendium will be available as a hard-cover book from the University of Chicago Press. Reprints will not be distributed by the authors.

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I. IDENTITY

A. Name

Respiratory syncytial (RS) virus [2].

B. Initial Isolate

Isolates

Chimpanzee coryza agent (CCA) [1, 2].

1. *Clinical specimen.* Throat swabs from infants at Johns Hopkins Hospital, Baltimore, Maryland [2].

2. *Clinical illness.* Bronchopneumonia and laryngotracheobronchitis [2].

3. *Isolation system.* KB cells [2].

4. *Serologic confirmation of infection.* There was a rise in CF antibody from 1:4 to 1:80 and in neutralizing antibody from 1:4 to 1:64 in the first human subject from whom RS was isolated [2].

C. Other Isolates

CCA (Sue) [1], Long and Snyder strains [2], and Randall (Chicago 1966) [4]. Strain CH-18537, isolated in 1962, had some antigenic difference but was closely related to the prototype strain [26]. Subsequently, many isolates have been reported from different locales in the United States, Great Britain, Scandinavia, most other European countries, Russia, Japan, and Australia.

D. Classification

1. *Virus group.* Paramyxovirus by structure and properties; no hemagglutination has been demonstrated [24]. RS is more closely related to parainfluenza than to influenza virus.

2. *Related but distinct viruses.* Newcastle disease and parainfluenza viruses are antigenically different from RS, but they have similar internal particle structure, production of syncytium in tissue culture, and eosinophilic inclusions [23].

Related Viruses

3. *Identification and differentiation.* By complement-fixation (CF) and neutralization tests, RS virus is antigenically distinguishable from influenza A, B, or C, parainfluenza types 1, 2, 3, 4, mumps, Newcastle disease, measles, reoviruses, coxsackie viruses A9 and B 1–4, echo viruses 2 and 6, SV_{59}, and psittacosis viruses [1, 26].

4. *Natural hosts.* Man, chimpanzee, and cows [1, 2, 82, 122].

II. CHARACTERIZATION

A. Physical Properties

1. *Size.* RS virus was estimated, from sucrose density gradient centrifugation studies, to be 90–120 nm in diameter [2]; viral particles in infected cells measured 65 nm by electron microscopy. Particles negatively stained with phosphotungstic acid measured 120–300 nm [23]. Some viral particles were as large as 860 nm [25]. *Size*

2. *Structure.* In ultrathin sections of infected tissue culture, electron microscopy revealed viruslike particles in vacuoles or invaginations within the cytoplasm [14], but complete particles were not found in the cell cytoplasm [21]. The enveloping membrane has been described as fringed and the nucleoprotein strand as having a herring-bone appearance, with a mean diameter of 13.5 nm [80]. The pitch of the helix measured 64 A [69, 95]. *Structure*

   Purified preparations of virus resemble myxoviruses with a helical nuclear structure and spike formations around the capsid. These spikes have a length of 130–170 A and a width of 40–70 A. After treatment with ether, a double-contoured basal membrane, 70–100 A in width, was observed [25]. Treatment with Tween 80 resulted in the detection of a "rosette" particle similar to that found with Newcastle disease and Sendai viruses [45].

3. *Heat stability.* At 55 C and *p*H 7.8, 10% of RS viruses survived for 5 min [39]. At 37 C, the infectivity titer dropped 1 log/24 hr. This was not a first order reaction. At 4 C, there was little change for five days followed by a decrease for 30 days, at which time no virus was detected. After slow freezing to − 30 C, no virus was detectable when tested after five days. At − 50 C, there was an initial decrease in virus titer, which then remained constant for 48 days. After rapid freezing to − 70 C in veal infusion broth, there was no loss in titer [4]. Addition of 45% sucrose and storage at − 70 C resulted in a stabilization of the titer of infectious virus for more than two years. Glycerine may also be used to stabilize the virus during storage [44]. At − 20 C, survival was poor; it was better at 4 C [87, 104]. *Stability*

   The inactivation of RS virus by drying at 20 C is dependent on the relative humidity. Initially, inactivation is maximal at a relative humidity of 40%; later, the virus is most rapidly inactivated at a relative humidity of 30%–80%. It is suggested that inactivation occurs by two processes with different kinetics [102].

4. *pH stability.* The infectivity titer of RS virus is decreased after incubation overnight at *p*H 3.0, but infectious virus can be recovered after such exposure [28]. The optimal *p*H for storage is 7.5. The virus survives better at a *p*H approaching 8.0 than at *p*H < 7.0 [39].

5. *Other physical properties.* Density (hydrated) values are 1.19 (in sucrose) and 1.26 g/cm^3 (in CsCl) [2]. The densities of the two CF antigens, A and B,

were found to be in the range of 1.28–1.32 g/cm^3 and 1.23–1.37 g/cm^3, respectively [54].

B. Chemical Properties

1. *Composition*

   a. *Nucleic acid type.* RNA, as demonstrated by lack of inhibition by 5-fluorodeoxyuridine (FUDR) [28]. *Nucleic Acid*

   b. *Proteins.* The nucleocapsid is surrounded by a lipoprotein envelope from which there are nonhemagglutinating spikes. The peptides associated with these structures have not yet been characterized. *Proteins*

   c. *Enzymes.* None characterized. *Sensitivity to Chemicals*

2. *Sensitivity to chemicals* *Sensitivity to Chemicals*

   a. *Organic solvents.* Diethyl ether (20%) at 4 C for 16 hr destroys the infectivity of the RS virus [2].

   b. *Inorganic chemicals.* Dextran sulfate and heparin (5 μg/ml) inhibited growth [89].

   c. *Enzymes.* Infectivity is retained after trypsinization of infected cell cultures.

   d. *Drugs.* Although the growth of RS virus was inhibited by the presence of interferon, attempts to demonstrate induction of interferon by RS virus failed [108]. Actinomycin D was not inhibitory [94].

C. Biophysical Properties *Biophysical Properties*

1. *General.* A soluble CF antigen can be separated from infected tissue culture harvest by centrifugation. This CF antigen has a smaller sedimentation constant than the infectious particle [2]. Smaller antigenic fragments can be produced by treatment with ether or Tween 80.

   No hemolysin has been demonstrated.

2. *Hemagglutinin.* None demonstrated.

III. ANTIGENIC COMPOSITION

A. Group Antigen *Antigens*

CF antigens of all variants of RS are reactive.

B. Specific Antigens

CF and neutralizing antibody have not shown cross-reactions with non-RS viruses. A CF antigen was demonstrable only when virus was grown in medium containing heat-inactivated serum [2]. When grown in tissue culture and concentrated, two or three precipitating antigens were demonstrable [55, 107]. The neutralizing capacity of RS immune serum of guinea pigs and ferrets was enhanced by the addition of 10 hemolytic units of complement [81].

Differences among strains of RS isolates have been found by neutralization tests with specific hyperimmune ferret sera but not with human sera [26, 53]. Of nine strains of RS virus tested by cross-neutralization, one strain, 8/60, was shown to be distinct from all others. Minor antigenic variation was detected with the Gwilliam, Randall, and 159/59 strains [46].

C. Antigenicity

1. *Animal.* Specific RS antiserum was produced by inoculation into guinea pigs and rabbits. After immunization, titers were in the range of 1:128 and 1:256 [1, 2]. Immunization of guinea pigs resulted in the production of serum that showed three precipitation lines when tested against concentrated RS virus [77]. An intracardiac immunization program, using the guinea pig, has been described [66].

   All infected chimpanzees had an antibody rise demonstrable by CF and neutralization tests [1].

   Ferrets inoculated intranasally with the prototype or CH 18537 strains of RS developed neutralizing antibody. On rechallenge with the homologous strain, there was a broadening of the antibody spectrum with regard to the heterologous variants but without a further rise in titer against the homologous virus [17, 26, 43].

2. *Human*

   a. *Primary.* In children under six months of age from whom RS virus was recovered, as many as 75% responded with a CF rise, and 43% had an increase in titer of neutralizing antibody. In children over six months of age, nearly all virus-positive cases developed rises in CF antibody and 90%–100% in titer of neutralizing antibody [4, 7, 16, 35].

      CF antibody formed by infants during an RS infection has a higher antigen requirement than antibody present in sera of adults or than that found as maternal antibody [113]. Neutralizing secretory antibody occurs in one-half or more of patients with lower respiratory disease and in 10%–20% of those with milder infections [97, 116].

   b. *Secondary.* A rise in CF and neutralizing antibody was observed after reinfection in the presence of pre-existing serum antibody [18]. The CF antibody response in adults was always weak compared with that in children [65].

Sera collected in 1956 reacted with the CH 18537 variant isolated in 1962; therefore, it was assumed that the latter was not a distinct antigenic variant, or that the two, somewhat different strains circulated simultaneously in the population [26].

IV. PROPAGATION OF VIRUS

A. Specimens

Isolation

1. *Source.* Virus has been recovered from throat and nasal swabs for as long as two days before and 10–11 days after the onset of illness; the optimal time for recovery is one to three days after symptoms appear [4, 61, 68].

2. *Handling.* Respiratory syncytial virus is labile when frozen and thawed. Virus can be preserved if specimens are collected in veal infusion broth and stored in sealed ampules at temperatures of − 70 C or lower. Addition of 5% chicken serum to the harvest improves the preservation of RS virus.

B. Growth in Tissue Culture

Propagation

1. *Susceptible cell lines.* Chang liver, WI38, KB, HEp-2, HeLa, human kidney and amnion cells are susceptible to infection [1, 2, 5, 29, 68, 72, 105]. Human conjunctiva (Chang), intestine (Henle), and embryo fibroblasts are less susceptible. RS has been recovered in primary monkey and human kidney cell cultures [8–10]. Differences in sensitivity to RS virus have been found among various sublines of HeLa and HEp-2 cultures [34]. If Eagle's medium is used for maintenance, the development of CPE is dependent on the presence of glutamine [78]. The addition of proflavine [57, 94] or paraphenylenediamine [58] may promote growth of RS virus.

   a. *Infection of tissue culture.* Isolates were made from 0.1–0.2 ml of specimen inoculated onto a monolayer consisting of 100,000–300,000 HEp-2 cells. Eagle's basal medium, containing 5% inactivated chicken serum, is a suitable maintenance medium that should be changed every three to four days. Stationary incubation at 36 C is satisfactory.

   Inoculation of a culture of Chang liver cells, four to six days old, maintained with a medium of eight parts Eagle's basal medium, two parts inactivated horse serum, and 0.2 parts L-glutamine is satisfactory. The medium is changed every four days.

   Virus can be propagated in KB cells in a medium consisting of Eagle's basal medium with 2% chicken serum.

      i. *Preferred cell line.* The most sensitive cell cultures are HEp-2, monkey kidney, human amnion, and human kidney, in decreasing order [5, 44].

Preferred Cell Types

ii. *Growth cycle.* After a 2-hr adsorption period, there is an eclipse phase of about 12 hr [20, 79, 99]. Fifteen percent of the virus remains unadsorbed after incubation for 5 hr [79], except in cell suspensions [124]. New virus has been found at 14 hr, after which log-phase replication lasted for 10 hr. Intracytoplasmic viral antigen is detected by fluorescent antibody 10 hr after infection of the cell cultures [20]. On the third and fourth days after inoculation, about one-half of the virus is cell-associated, located on the cell surface [19, 99]. Addition of actinomycin D is not inhibitory [94]. The mode of spread from cell to cell may be via intracytoplasmic bridges [33, 90]. Maximal titers of virus in HEp-2 cells, grown in suspension, are attained 48 hr after infection and somewhat later (up to five days on monolayers [124].

iii. *Harvest of virus.* Addition of 50% glycerine to the harvest fluid has resulted in longer preservation of RS virus [44]. Addition of DEAE (30 μg/ml) to RS-infected HEp-2 cells resulted in a two- to five-fold increase in the infectivity of the harvest. At low concentrations ($< 50\mu$ g/ml) the same effect was observed with protamine sulfate [89].

b. *Recognition of virus.* *Virus Recognition*

i. *CPE.* The cytopathic effect begins with small syncytial areas randomly distributed early in infection. Within one to four days, the entire cell sheet may be involved, with syncytial areas enlarging and becoming more numerous [2].

The time of appearance of CPE depends, within limits, on the number of serial passages of the virus [2] but is nearly always demonstrable within five days.

ii. *Cytology.* Eosinophilic cytoplasmic inclusions are commonly found in infected cells, especially in the syncytia [15]. These inclusions are devoid of DNA, RNA, virions, and demonstrated specific antigens. Chromosomal abnormalities are not observed. In infected Vero cells, dense intracytoplasmic inclusions have diameters ranging from 90 to 130 nm [115].

iii. *Plaque formation.* Small macroscopic plaques developed after incubation for seven to nine days in HEp-2 cells overlayed with agar [30]. Four conditional-lethal, temperature-sensitive mutants of RS have been isolated and shown to be genetically stable. One of the mutants produced a typical, nonsyncytial plaques [93].

iv. *Hemadsorption.* None demonstrated.

2. *Nonsusceptible cells.* No data available. *Nonsusceptible Cells*

C. Growth in Eggs

Inoculation of RS virus onto the chorioallantoic membrane (CAM) and into the amniotic and allantoic cavities or yolk sac has not resulted in demonstrable virus multiplication [1, 2, 4].

D. Infection of Animals

Infection of Animals

1. *Susceptible animals.* Baboons, chimpanzees, guinea pigs, ferrets, and suckling hamsters have been infected. RS virus has been adapted to grow in seven- to nine-day-old mice [1, 26, 52, 56, 70, 101].

   a. *Conditions of infection.* Infection can be induced by intranasal or intraperitoneal inoculation.

   b. *Signs of infection.* Virus can be recovered from the nasal turbinates and lungs of infected hamsters. A serologic response also occurs [26, 56, 101]. After inoculation of guinea pigs via the middle ear, virus was recovered from the middle-ear cleft seven days later in 41% of the ears studied, suggesting multiplication of the virus at that site [48]. An afebrile coryzal illness was produced in chimpanzees [1]. In mice an encephalitis was produced by a neuropathic strain of RS virus [70].

   c. *Immunity.* Chimpanzees challenged 55 days after original exposure had no clinical illness or change in the titer of antibody in serum [1].

2. *Nonsusceptible animals.* One-day-old mice inoculated intracerebrally or intraperitoneally [1, 2], weanling hamsters, rabbits, guinea pigs, mice, weighing 10 g, and young adult rats inoculated via the previously mentioned routes were refractory to infection [1].

V. ASSOCIATION WITH HUMAN ILLNESS

A. Natural Infection

Infection in Man

1. *Clinical.* Infection with RS virus has been observed every year since its recognition. It occurs in rather sharp epidemics, recurring at intervals of nine to 14 months, usually in the fall or spring.

   Illness may be gradual or abrupt after an incubation period of three to five days. The symptoms associated with infection in children are cough (97%), fever (93%), rhinitis (57%), pharyngitis (47%), malaise (38%), vomiting (30%), anorexia (27%), lymphadenopathy (22%), otitis media (17%), conjunctivitis (13%), and abdominal pain (7%) [13, 29, 49]. In an analysis of symptoms produced by infections with RS and by influenza A viruses, the two diseases could not be differentiated on a clinical basis [112].

   In infants, as many as 60% of cases of acute bronchiolitis have been caused by RS virus. Infection is especially prevalent during the first six months of life, but throughout the childhood years, 12%–27% of cases of bronchitis were associated with RS infection [22, 29, 35, 36]. RS virus has been causally implicated in 10%–40% of cases of bronchopneumonia in infants and children. In a roentgenographic analysis of RS infection in infants, 34 of 38 had pneumonia, which involved both lungs in two-thirds

of the cases. Lobar consolidation occurred in six. The pulmonary consolidation lasted longer than the symptoms [59].

RS virus has been isolated from middle-ear aspirates from children with lower respiratory disease [50, 51, 64, 67].

Croup was a manifestation of infection in 7% of patients under six months of age, and 15% had undifferentiated upper respiratory disease. Serologic evidence of RS infection without clinical illness occurred in 3% of children [4, 6, 7, 11, 13, 18].

Levels of total complement and $B_{1c/1a}$ globulin were not depressed in the acute phase of bronchiolitis [106]. Autopsies on infants with fatal RS virus infection showed severe necrotizing lesions of the epithelium of the bronchi and bronchioles and interstitial pneumonia [109, 117]. Specimens of bronchi from eight children with bronchiolitis contained intracellular RS antigen, as determined by immunofluorescence [47].

Infection of adults often causes coryzal illness [9, 18, 63], but it is also an important cause of pneumonia, bronchopneumonia, and bronchitis [74]. Among hospitalized adults with serologically demonstrated RS virus infections, about half had exacerbations of chronic bronchitis, one-quarter had bronchopneumonia, and one-quarter had the "flu" syndrome [32, 37]. Of 18 patients, 55–88 years of age, with serologically proven RS infection, six had bronchitis, and 12 had bronchopneumonia [73]. Double infections with RS and influenza A_2 viruses have produced unusual symptomology [111].

2. *Prevalence.* *Prevalence*

a. *Virologic.* Among infants under six months of age hospitalized for respiratory diseases, RS virus accounted for 50% with bronchiolitis, 25% with pneumonia, and 13% with upper respiratory infection. Between the ages of six months and two years, isolations were about half as frequent as from younger infants with bronchiolitis or pneumonia, but the rate of recovery from upper respiratory illness was the same [3, 4, 6, 7, 10, 17].

Recovery of virus from adults has been sporadic and has usually been from persons with upper respiratory infections.

b. *Serologic.* Serologic investigations of patients with respiratory disease suggest an infection rate double that indicated by recovery of virus [31, 38, 75]. In one epidemic, the CF test showed that 42% of all children hospitalized for respiratory illness had RS infection [12].

In a longitudinal study in a small open community, respiratory illness occurred during periods when antibody rises were observed. The largest number of rises in titer occurred in the group aged five to nine years [123].

Maternal antibody is present in the sera of 75% of infants, but it does not prevent illness caused by RS virus [120]; between three and 12 months of age, 35% possess RS antibody. By the age of 5 years, 95% of children have specific serum antibody [3, 11, 12, 27, 31, 40, 41].

Specific secretory (neutralizing) antibody occurs in 40%–70% of infected persons with lower respiratory disease [97, 116].

B. Studies in Volunteers

Studies in Volunteers

1. *Challenge.* A dose of 160–640 $TCID_{50}$ of the second passage in monkey-kidney tissue culture of a strain of RS virus sprayed into the nose and throat of 41 adult male volunteers produced typical colds in 20 of them. Thirty-three subjects of this group were infected with RS virus, as shown by recovery of virus and/or serologic response. There were no febrile responses. The incubation period was four days, and illnesses lasted four to 10 days [8, 10].

   A temperature-sensitive mutant of RS virus, grown at permissive temperatures of 34 C or 28 C, infected the majority of adult volunteers and produced an acute but mild upper respiratory illness. Growth at 26 C resulted in a decrease in infectivity and a loss of virulence [84].

2. *Recovery of virus and serologic response.* The first isolations of virus were made one to two days before the onset of symptoms. Isolates were more frequent and positive over a longer period from subjects with illness than from those who were infected but without symptoms.

3. *Immunity.* All adults tested possessed detectable levels of neutralizing antibody to RS virus before challenge, but the titer of naturally acquired antibody had no significant effect on subsequent RS infection of volunteers and was poorly correlated with development of mild clinical illnesses. Resistance to infection and illness appeared to be related to the level of nasal antibody but not to the level of serum antibody [121]. Infection elicited an increase in the titer of serum antibody by CF and neutralization. Immunity upon rechallenge was not tested. Illnesses from infection are more severe in children than in adults [8, 10].

C. Prevention

Prevention

1. *Vaccine.* Several experimental vaccines have been developed from monkey kidney harvests of RS virus. Inoculation of guinea pigs with aqueous or alum-precipitated vaccine induced seroconversion with neutralizing antibody and also a delayed dermal hypersensitivity to both viral and cellular components of the vaccine. The purified, soluble CF antigens, A and B, did not induce either neutralizing antibody or a hypersensitivity reaction [83].

   Inoculation of humans with alum-precipitated, formalin-inactivated RS vaccine resulted in a 90%–100% seroconversion rate, as detected by CF, and a 43%–47% rate, as detected by neutralization [62, 76]. However, in all trials, subsequent natural infections in those receiving RS vaccine were more severe than in those given a non-RS virus vaccine or placebo. In one study, 70% of vaccinees who later became infected during an RS epidemic developed pneumonia, as compared with only 10% among those given a control inoculation [91, 92, 96, 98].

   A strain of RS virus, propagated at 26 C, is as antigenic as live virus, but its infectivity is low. Although other new procedures for production of vaccine

[71] or the use of new adjuvants [103] are under investigation, none has been proved to be effective in preventing infection, and without such protection, sensitization resulting from parenteral vaccination augments the naturally acquired disease.

2. *Chemotheraphy.* None available.

VI. LABORATORY DIAGNOSIS

Laboratory Diagnosis

A. Virology

Isolation of this virus is most successful in HEp-2 cells. A rise in titer of serum antibody can be shown by CF or neutralization. The use of immunofluorescence to recognize viral antigens has been extensively studied as a diagnostic tool [86, 88, 110, 118, 119]. Nasopharyngeal secretions proved more satisfactory than nose or throat swabs [85, 88, 114]. Electron microscopic diagnosis also is possible; nasopharyngeal secretions are used [100]. A and B antigens have been distinguished by CF, especially in primary infections.

B. Serology

The CF test, with 8 units of antigen, will detect 90% of infections among individuals older than six months and is as sensitive as neutralization. Below six months, the CF test detects only 20% of infections. The neutralization test is more sensitive than CF when serum from infants is used, but rises in neutralizing antibody have been detected in only half of the virus-positive infections in this age group. The use of unheated serum or the addition of antibody-free fresh serum increases the sensitivity of tests for neutralizing antibody [42, 60].

C. Reagents

Antiserum was produced in guinea pigs by three weekly ip inoculations of 1 ml of infected tissue culture harvest [2]. Rabbits given three weekly iv inoculations of 1 ml each followed by two weekly im inoculations of 1 ml of infected tissue fluid combined with 2 ml of a mixture of *Mycobacterium butyricum*, paraffin oil, and arlacel, produced CF and neutralizing antibody [2]. In adult rabbits, an alternative procedure is to give three injections at two-week intervals, the first two consisting of 8 ml of virus and adjuvant given im, and the third injection of virus alone administered iv [5]. A single injection of virus may elicit antibody if a sensitive test is used [47].

Standard virus may be obtained from the American Type Culture Collection. Specific antiserum is available commercially.

VII. COMMENT

Comment

Respiratory syncytial virus is considered to be a paramyxovirus on the basis of its size, appearance by electron microscopy, and sensitivity to ether; it differs from other paramyxoviruses in that it has no known hemagglutinin. Although minor antigenic variants have been found, they are not well discriminated by antibody in human sera. Because there has been no sequential drift in the antigenic character of the prevalent strain, RS virus is considered to be a single type.

Epidemiologically, respiratory syncytial virus is very important, because it causes annual epidemics of acute respiratory diseases affecting infants, children, and adults. Infection spreads rapidly from person to person and characteristically occurs as a discrete outbreak of acute respiratory illness in the winter or early spring. In infants and children, especially during the first six months of life, respiratory syncytial virus is the most important cause of bronchiolitis and a major cause of pneumonia.

Serum antibody acquired by transplacental passage does not provide immunity against infection and might possibly augment the local respiratory disease by an immunopathologic process. The virus may replicate in the middle ear, but its role in otitis is unproven. Croup is an infrequent manifestation. Pneumonia, as determined radiographically, is frequent, usually bilateral, and multilobar; it may be lobar with secondary bacterial infection. The pathologic lesion is one of necrosis of the epithelial mucosa of the trachea and bronchi and an interstitial inflammation.

In adults, infection with RS virus usually causes upper respiratory symptoms; however, because of the prevalence of infection, it is an important cause of exacerbations of bronchitis, pneumonia, and "flu," requiring the hospitalization of adults. In some years, virus infection has given rise to an increase in secondary pneumonia due to *Diplococcus pneumoniae*. Infection is followed by an increase in serum CF and neutralizing antibody; also by secretory neutralizing antibody in the nasopharyngeal and tracheal secretions. Primary infection does not establish complete immunity, and reinfection is common at all ages.

Inactivated vaccines of the type and potency previously produced have been detrimental because they have failed to prevent infections and they have induced a more severe disease with exaggerated pneumonia. Attenuated live virus vaccines have not yet been successful, nor has any effective chemotherapy been developed.

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Addendum—Respiratory Syncytial Viruses of Non-Primate Origin

A virus isolated from wild cottontail rabbits was shown to possess chemical, physical, and biologic characteristics of the paramyxovirus family. Although formation of syncytia was characteristic of its growth in tissue cultures, no antigenic relationship was detected by CF or neutralization tests with any known member of the paramyxovirus family [1].

Isolates shown to be antigenically related to human RS virus were recovered from cattle with bronchopneumonia [2]. Cytologic examination of BHK_{21} cells infected with bovine RS virus revealed intranuclear and intracytoplasmic viral components [4]. An A type particle with a diameter of 65 nm has been described in the cytoplasm of such cells [3]. The viral envelope was added as the virion passed through the cytoplasmic membrane in a budding process [4].

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I. IDENTITY

A. Name

Isolates

Coronavirus

B. Initial Isolate

1. *Clinical specimen.* Strain 229E from nasal washings, Chicago, Illinois, 1962 [2].

2. *Clinical illness.* Four isolates from subjects with minor upper respiratory illness and one isolate from a healthy subject.

3. *Isolation system.* Human embryonic kidney cell culture after blind serial passage.

4. *Serologic confirmation.* Rises were detected by neutralization in five of five subjects and by CF in four of five.

C. Other Isolates

Viruses with similar biologic characteristics have been isolated in other localities of the United States [10] and England [1, 25].

D. Classification

1. *Virus group.* Coronavirus.

2. *Related but distinct viruses.* The viruses are biologically similar, in size, architecture, internal structure, and sensitivity to ether, to infectious bronchitis virus of chickens (IBV), mouse hepatitis virus (MHV), rat pneumotropic virus, and swine viruses causing gastroenteritis and encephalomyelitis [5, 12, 24].

Related Viruses

3. *Identification and differentiation.* No antigenic relationship was demonstrated between these agents and influenza A, B, C, parainfluenza types 1, 2, 3, 4, SV_5, respiratory syncytial, measles, mumps, or Newcastle disease virus [2].

4. *Natural host.* Data are incomplete, but man is a known host. Morphologically similar viruses infect chickens, mice, and pigs [37].

II. CHARACTERIZATION

A. Physical Properties

1. *Size.* By gradocol filtration, the size of the virion was calculated to be 89 nm [2]. By electron microscopy, the usual diameter was measured as 80–160 nm [5]. *Size*

2. *Structure.* The virions are pleomorphic. Most particles are covered with projections (spikes) more densely packed than those seen on influenza viruses. These spikes are attached to the virion by narrow stalks with a thickening (90–110 A) at the distal end [5, 11]. The internal component is a hollow, threadlike structure with a diameter of 70 nm and a definite structural pattern [21]. *Structure*

3. *Heat stability.* Infectivity was destroyed at 56 C within 10 min. There was no loss of titer after 2 hr at 37 C or 10 days at 4 C. Rates of thermal inactivation are dependent on the amount of particle aggregation. Aggregation is dependent on the concentration of serum in the medium [31]. *Stability*

4. *pH stability.* Coronaviruses are acid-labile [3].

5. *Other physical properties.* The mean buoyant density in CsCl was calculated to be 1.18–1.20 g/cm^3.

B. Chemical Properties

1. *Composition.*

   a. *Nucleic acid type.* RNA, as indicated by lack of interference of replication by addition of iododeoxyuridine (IUDR) or FUDR [2]. *Nucleic Acid*

   b. *Proteins.* The envelope proteins have not been characterized. Six polypeptides have been identified from a related coronavirus [35]. *Proteins*

   c. *Enzymes.* None characterized.

2. *Sensitivity to chemicals.* *Sensitivity to Chemicals*

   a. *Organic solvents.* Coronaviruses are sensitive to ether, which decreases or abolishes infectivity and hemagglutination [2].

   b. *Inorganic chemicals.* No data available.

   c. *Enzymes.* No data available.

   d. *Drugs.* Tetracycline had no effect on virus replication [2].

C. Biophysical Properties

Biophysical Properties

1. *General.* Strains have both structural and soluble specific components and common functions as noted below.

2. *Hemagglutination.* With strain 229E, no hemagglutination was observed with guinea pig or chicken erythrocytes at 4 C, 22 C, or 37 C [2]. Coronavirus strains OC 38-43, propagated in mouse brain, caused hemagglutination of rat, human type O, and chicken erythrocytes at 4 C–37 C. There was no hemagglutination of sheep, rhesus monkey, or guinea pig erythrocytes. The HA reaction was not affected by neuraminidase or by sialic acid-containing inhibitors [19].

III. ANTIGENIC COMPOSITION

A. Group Antigen

Antigens

Common CF antigens have been observed in known members of the coronavirus family, except in avian bronchitis virus. Serologic data are still incomplete, but some observed interrelations are shown in table 1.

Table 1. Antigenic mosaic of coronavirus as determined by neutralization (N) and CF tests with animal serum [20, 25].

Hyperimmune serum to strain	Antigens produced in tissue culture or mouse brain											
	229E		OC43		B814		LP		MHV		AIB	
	N	CF	N	CF	N	CF	N	CF	N	CF	N	CF
229E	+	+	0	+	0	ND‡	+	+	0	0	0	0
OC43	+	+	+	+	ND	+	0	+	0	+	0	0
B814	0	ND	0	+	+	+	0	ND	0	0	0	0
LP	+	+	0	+	0	ND	+	+	0	0	0	0
MHV*	0	+	0	+	ND	+	0	0	+	+	0	0
AIB†	0	0	0	0	ND	0	0	0	0	0	+	+

*Mouse hepatitis virus.
†Avian infectious bronchitis virus.
‡Not done.

CF antigens have been prepared from harvests of infected tissue culture and mouse brain, but attempts to prepare antigens from organ cultures have not been successful [2].

B. Specific Antigens

Strains OC38 and OC43 cross-react, as shown by neutralization tests in mice or monkey-kidney cell culture. Strains 229E and LP cross-react in neutralization tests but not to identical titers, indicating a close but not

completely similar antigenic mosaic. A one-way cross exists between 229E and OC32; antisera against the latter and B814 do not neutralize other coronaviruses. Avian bronchitis virus reacts only with homologous virus (see table 1).

In immunodiffusion tests, the number of detectable lines of precipitation varies from one (strain B814) to four (strain OC43). This may be a result of the procedure used for production of antibody [25].

C. Antigenicity

Antigenicity

1. *Animals.* In animals, specific antibody is elicited by the initial series of inoculations of cell-culture harvests. In neutralization tests with animal serum, no antigenic relationship has been detected between strains 229E and OC38-43 [20].

2. *Human.*

   a. *Primary response.* Initial infection results in an increase of specific neutralizing antibody. About 50% of volunteers challenged with the OC strains of coronaviruses developed CF rises to MHV [20].

   b. *Secondary Response.* The antigenic primacy of the initially infecting strain, heterologous interrelationships, and anamnestic responses are still to be worked out.

IV. PROPAGATION OF VIRUS

A. Specimen

Isolation

1. *Source.* Nasal secretion, swabs, or washings.

2. *Handling.* Virus is relatively heat-stable. No extraordinary precautions are necessary.

B. Growth in Tissue Culture

Propagation

1. *Susceptible cells.* Coronaviruses have a restricted host cell range, and low virus yields are usually obtained in monolayers. Replication of strain 229E was detected by the appearance of CPE after blind passage in human diploid cells. Replication of certain strains can be detected only in organ cultures using explants from human embryonic trachea and nasal epithelium [1, 3, 10, 14]. Certain strains of coronavirus cause CPE in L132 cells, a line derived from a human embryonic lung [16]. Coronaviruses of avian origin have phenotypic differences in the susceptible avian host cell range [4] and grow to a limited degree in nonavian tissues [32]. Murine coronavirus has grown adequately only in tissues from mice [20]. Coronavirus of rats has

grown in only one of several cell lines studied [24]. Coronaviruses of swine grow only in tissues of porcine origin [37].

a. *Infection of tissue culture.* For growth of explants, a medium of 2 ml of Eagle's medium with 0.2% (wt/vol) bovine plasma albumin and incubation at 33 C in a humidified atmosphere containing 5% (vol/vol) CO_2 in air is satisfactory [5]. A *p*H of 7.0 is required for growth since inactivation is accelerated at *p*H 7.7 and 6.7 [25].

Preferred Cell Types

i. *Preferred cell line.* Human tracheal organ cultures.

ii. *Growth cycle.* After 1 hr at 33 C, only 18% of L132 cells were infected with strains 229E. Virus structures were detected 6–8 hr later [17]. Infection of WI-38 cells with strain 229E resulted in a reorganization of the cytoplasm, as determined by electron microscopy. New viral structures were observed 6–12 hr after infection. Clusters of virus were observed in intracytoplasmic vacuoles, called cisternae, 24–36 hr after challenge.

Strain OC43 in WI-38 matures in intracytoplasmic vesicles similar to those observed with strain 229E. Budding, such as that described for strain 229E was not observed [30], and the budding process described for coronavirus is into cytoplasmic vesicles rather than from the plasma membrane, as has been observed with myxoviruses [6, 8, 28].

iii. *Harvest of virus.* Neither CPE nor hemadsorption are reliable indices of viral growth. Generally low yields of virus are obtained.

b. *Recognition of virus.*

Virus Recognition

i. *CPE.* The CPE that gradually developed in human diploid cells gave them a stringy appearance. Some intracytoplasmic vacuoles were observed [2]. Strain B814 caused no CPE and could be detected only by electron microscopy or by interference with Echovirus type 11 [1].

ii. *Cytology.* No inclusions have been observed [2]. Fluorescent antibody has been used for identification of viral antigen [15]. Morphology, as observed by electron microscopy, is characteristic [5, 37]. The use of specific antisera, in combination with electron microscopy, can facilitate recognition of the virus in culture harvests [38].

iii. *Plaques.* At 33 C, with a methyl cellulose overlay, plaques were formed in WI-38 cell cultures [8]. Plaques can be produced in L132 cells infected with the 229E strain [17].

iv. *Hemadsorption.* Rat erythrocytes were bound to monolayers infected with strain OC43 to give a pseudohemadsorption reaction. Strain OC43 hemadsorbs rat and mouse erythrocytes at 4 C or 37 C

after replication in BS-C-1, rhesus monkey kidney, HEK, and WI-38 cell cultures.

2. *Nonsusceptible cells.* Hemadsorption and CPE were not observed in cultures of kidneys derived from patas or vervet monkeys. No growth was observed in HeLa or HEp-2 cultures [1, 2]. The B814 strain had no gross effect in HEK, WI-26, or WI-38 cells [1]. *Nonsusceptible Cells*

C. Growth in Eggs

No replication has been detected in embryonating hens' eggs [1, 2], except for strains of avian infectious bronchitis virus.

D. Infection of Animals *Infection of Animals*

1. *Susceptible animals.* Different members of the coronavirus group cause infectious bronchitis of chickens and birds, and sometimes nephrosis-nephritis, mouse hepatitis, rat pneumonitis, and swine gastroenteritis and/or encephalitis [4, 12, 24]. After four to five passages in tissue culture, strains OC38 and OC43 were administered intracranially to suckling mice. On the first passage in mice, illness, characterized by tremors, rigidity, and lethargy, was observed on days 11–15 after challenge. By the fourth passage in mice, these viruses were lethal for mice within 48–60 hr after challenge [9].

2. *Nonsusceptible animals.* There is marked host specificity of different strains.

V. ASSOCIATION WITH HUMAN ILLNESS

A. Natural Infection *Infection in Man*

1. *Clinical.* The use of explants of human embryonic nasal epithelium or trachea has resulted in the isolation of coronaviruses. Serology has shown them to be associated with acute respiratory diseases of man. The exact importance of these viruses with accompanying epidemiologic data is unavailable because of the variability of strains and the difficult techniques required to establish diagnosis of the infection.

   In most studies, coronaviruses usually caused infections in the period from January through March [18, 33, 34]. Only about one-half of naturally occurring infections cause clinical illness [26]. Spread appears to be preferentially within families. In one study, secondary cases occurred in 17 of 26 families [22]. Serum neutralizing antibody does not influence the occurrence of reinfection [33]. Acquisition of infection with avian bronchitis virus from chickens is suggested by studies in poultry handlers [13].

2. *Prevalence.* *Prevalence*

a. *Virologic.* In a survey of acute illnesses of the upper respiratory tract, nasopharyngeal washings from 23 subjects were inoculated into cultures of human embryonic trachea. Of these 23 specimens, six yielded coronaviruses, as determined by electron microscopic examination [10].

Of five subjects from whom coronaviruses were isolated, all developed a serologic response [3].

b. *Serologic.* Measurement of the CF antibody response was found to be twice as sensitive an index of infection as virus isolation. The CF antibody tends to be transitory, whereas titers of neutralizing antibody remain elevated for a longer period of time [13]. The observed prevalence of infection varies widely in different years [29, 33].

During a spring outbreak of respiratory disease in 1967, the infection rate in a community was 34% [22]. From observations in a children's home, it was estimated (based on serology) that 19% of respiratory illness in a single season was caused by coronavirus strain OC43 [26]. Among groups of adults during two of four winters when there were high rates of respiratory disease and infrequent virus recovery, infection with 229E occurred in 10%–24% of those with upper respiratory tract disease [18].

In three separate studies, each covering a seven- or eight-year period, coronavirus OC43 accounted for 3% of 1,328 illnesses observed in children [26]; coronaviruses 229E and OC43 accounted for 4% of colds in an adult industrial population [34], and 15%–35% of students were infected during three seasons of high prevalence [33].

A serologic survey of adults and children showed that infection in infants below one year of age was infrequent. Infection with OC38 and/or OC43 occurred principally in the preschool years, whereas infection with 229E occurred later [23]. Data from sera collected in 1966 indicate that 30% of adults and 15%–20% of children had neutralizing antibody specific for strain 229E [7]. In a study of sera collected since 1967, 50%–80% of the population were found to have neutralizing antibody against 229E at a dilution of 1:20, as measured by plaque reduction in L132 cells. In CF tests (serum diluted 1:20) between 50% and 98% of those tested had antibody [25]. Sera collected from 139 adults between January 1969 and October 1970 showed that 8.6% had a neutralizing antibody titer $\geq$ 1:8 against strain 229E [27].

B. Studies in Volunteers *Studies in Volunteers*

1. *Challenge.* In an early study, harvest medium from human embryonic trachea containing strain B814 was used as inoculum. Illness was observed in five of 11 volunteers [1]. After tissue and organ-culture passage, strain 229E was passed four times in volunteers and produced illness in each passage [7]. Later, six coronaviruses isolated from persons with illness were given to volunteers. All six produced colds. Heterologous antibody re-

sponses occurred in 10%–20% of these and other volunteers infected with coronaviruses [29].

2. *Recovery of virus and serologic response.* Strain 229E was recovered from sick as well as healthy volunteers in each of the four challenge passages [7]. Volunteers challenged with the OC or B816 strain often showed CF rises to strains 229E and LP [20].

3. *Immunity.* Incomplete.

C. Prevention

Prevention

1. *Vaccine.* None.

2. *Chemotherapy.* None.

VI. LABORATORY DIAGNOSIS

A. Virology

Laboratory Diagnosis

Diagnosis is based on electron microscopic examination of cell explants or, with some strains, detection of CPE in monolayers after serial blind passage.

B. Serology

Rises in the titer of CF antibody against strain 229E and HAI antibody against strain OC43 are the most practical tests. Neutralization is the serologic test of choice for specific identification.

C. Reagents

Commercially unavailable.

VII. COMMENT

Comment

Coronaviruses are a distinct group of viruses with common morphology and various degrees of antigenic similarity. The number of different coronaviruses that infect man and their exact interrelations are still unknown. Their etiologic role in respiratory diseases has been established. Also, they are associated with hepatitis in mice and avian bronchitis. Data suggest that coronaviruses cause

3%–4% of acute respiratory illnesses in humans. The clinical syndrome is usually that of a common cold. Asymptomatic infections also occur, possibly because of partial immunity to reinfection. Coronavirus infections are most prevalent in the winter months and may occur in epidemic fashion, with the same strain being geographically widespread. Recurrent epidemics of the same type do not seem to occur in sequential years. Preliminary serologic investigations indicate that certain strains may infect children preferentially, whereas others are prevalent in adults. An alternative explanation of the findings is the emergence of new epidemic strains with disappearance of older strains for a period. Transmission of the virus within families is frequent, and acquisition may be possible from poultry and other sources. No effective vaccines or chemotherapy have been developed.

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