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CLINICAL VIROLOGY OF RHINOVIRUSES

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I. Introduction

Rhinoviruses are responsible for more episodes of human illness than any other infectious agent. Both children and adults have one illness due to rhinovirus every 1 to 2 years (0.5–1 illness per person-year). Rhinoviruses are the predominant cause of the common cold, a complex of subjective symptoms well known to everyone. The clinical illness is characterized by sore/scratchy throat, nasal obstruction, rhinorrhea, and malaise. Coughing and/or sneezing may be present. Young children may have fever early in the illness, but adults usually do not. Although symptoms and signs may persist for 10–14 days in children, in adults the illness subsides by 5–7 days after onset. Rhinovirus infections commonly precipitate exacerbations of asthma or chronic bronchitis and may predispose to secondary bacterial infections of the paranasal sinuses and middle ear in healthy children and adults.

This article is not intended to review all aspects of the virus and disease. Instead, three areas that may be of interest to the clinical virologist will be discussed. First, some attributes of the virus and epidemiology of disease that seem to have clinical relevance will be highlighted. Second, the efficiency of methods for detecting the presence of the virus in the human respiratory tract will be analyzed. Third, the means by which symptomatic illness is produced by rhinovirus infection of the respiratory tract will be discussed.

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II. Attributes of Virus and Epidemiology of Disease

Rhinovirus is a member of the picornavirus family. The virus contains single-stranded RNA within a capsid with icosahedral symmetry that is composed of 60 copies of each of four polypeptides (VP1–VP4). The viral particle is 30 nm in diameter and appears as a nondescript dot by electron microscopy. Infectivity of the virus is destroyed by acid treatment (pH 3–5), which differentiates it from the enteroviruses. The surface of rhinovirus is notable for the presence of depressions (canyons) at the base of which are the sites for attachment to receptors on the surface of susceptible target cells. The majority of the rhinovirus immunotypes (91 of the recognized 100 types) bind to the intercellular adhesion molecule-1 (ICAM-1) receptors on host cells; the receptor for the remaining immunotypes is not known. Neutralization testing with animal hyperimmune sera has been used to identify 100 different immunotypes, which have been given numbers.

Neutralization of virus infectivity by antibody occurs when IgG binds to the viral surface so that access of the host cell receptor to the canyon is blocked (1). The receptor binding site in the base of the canyon is inaccessible to antibody and is highly conserved across immunotypes (2).

Rhinovirus infects human respiratory epithelium. There is no evidence that cells in the submucosa of the respiratory tract are infected; virus has not been detected in blood. The optimal temperature range for growth of virus *in vitro* is 33°–35°C, which is the temperature range of the normal nasal mucosa. The nose and nasopharynx are the primary sites for viral replication in humans. Whether virus replicates in lung/bronchial epithelium during rhinovirus infection has not been definitively settled due to the difficulty of obtaining material from the lower airway uncontaminated by nasal secretions (3). During experimental infection, rhinovirus has been detected most frequently in the nasopharynx (Fig. 1). In one study (4), rhinovirus was inoculated onto the conjunctival surface of susceptible volunteers and was delivered to the nose below the inferior turbinate at the point where the nasolacrimal duct enters. Four sites in the nose (anterior and posterior parts of both inferior turbinates) and one site on the nasopharyngeal wall were sampled daily with a small cytology brush. The brush samples were cultured for rhinovirus. The viral recovery rate from the nasopharynx was consistently higher than that from the combined intranasal sites (Fig. 1). Virus was detected earlier in the nasopharynx and persisted until rapid decline occurred 16 days after inoculation. The authors (4) suggested that viral replication was occurring in cells at each virus-

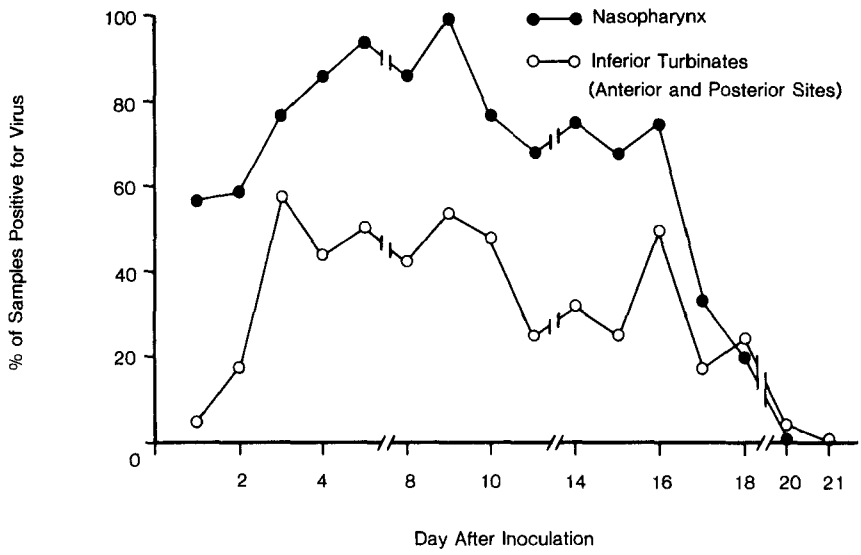

FIG 1. Rhinovirus recovery rates from brush biopsy specimens from nasopharynx and inferior turbinates of infected volunteers inoculated by way of the eye. Reprinted with permission from Ref. 4.

positive site, but the recovered virus may have been present only in the mucus overlying the site. The higher viral recovery rate in the nasopharynx may simply reflect the fact that this site is the endpoint of the mucociliary clearance system of the nasal cavity, paranasal sinuses, and middle ear (5).

Rhinovirus colds occur year round. However, a sharp rise in rhinovirus infections occurs every September. This rhinovirus peak initiates the yearly epidemic of colds in temperate climates that results from successive waves of different viruses moving through the population (6). Colds during the summertime are infrequent, but the proportion of summer and early fall colds that are due to rhinovirus is very high. Illness appears 1 to 2 days after rhinovirus is inoculated onto the nasal mucosa; oral inoculation of virus is an ineffective means of initiating infection. The home is the environment most conducive to transmission of rhinovirus infection (7). Rhinovirus may be transferred from an ill person to the nasal mucosa of a susceptible person by way of one (or more) transmission route(s): small-particle aerosol, large-particle aerosol, or direct contact of virus-contaminated hands with conjunctival or nasal mucosa (hand/self-inoculation route). The routes of transmission by which rhinovirus spreads under natural conditions have not

been established (8), but frequent hand washing to remove virus may reduce the likelihood of infection acquired by self-inoculation.

Immunity to reinfection with each immunotype of rhinovirus correlates with, but is not necessarily mediated by, neutralizing antibody in serum. Serum antibody appears to persist for years, but immunity to one immunotype does not provide protection against the other 99 types. As a consequence, a rhinovirus vaccine is not a practical prospect.

III. Detection of Rhinovirus

Rhinoviruses have long been known to be the major etiologic agent in common colds. In studies by several investigators using samples of nasal secretions inoculated prior to freezing into either HeLa cells or human embryonic lung fibroblasts (WI-38, MRC-5), 25–33% of secretions were shown to be rhinovirus positive. Sampling of nasopharyngeal secretions by a swab or nasal wash, use of cells with optimal sensitivity for rhinovirus, and incubation of cell cultures at 33°–35°C under conditions of motion were important requisites to achieve these results. More recently, with refinements in culture diagnosis and the use of polymerase chain reaction (PCR) technology, about 50% of common colds can be shown to be due to rhinovirus (9). In this section, information on the most efficient means to detect rhinovirus in the upper respiratory tract that may be helpful to the clinical virologist will be reviewed.

A. Sampling and Cell Culture

During infection, rhinovirus is present most frequently in the nasopharynx (4). Secretions from the nasopharynx can be obtained by one of three methods: an intranasal swab inserted far enough in the nose to reach the posterior nasopharyngeal wall; aspiration of nasopharyngeal mucus by a thin tube inserted through the nose; or a nasal wash with 10 ml of physiologic salt solution. If subjects will tolerate aspiration and if mucus is present in the nasopharynx, this method is expected to be efficient. The relative efficiency of the nasal swab and the nasal wash has been compared in two studies. In 1964, Cate and co-workers (10) compared detection rates of rhinovirus with nasal wash, nasal swab, and pharyngeal swab in experimentally infected volunteers during the 7 days after inoculation. Virus was detected in 76% of nasal washes, 36% of nasal swabs, and 35% of pharyngeal swabs. Virus was

detected in 50% of samples if results of nasal and pharyngeal swabs were combined. Arruda *et al.* (11) compared the isolation rate of rhinovirus from a nasal wash to a "vigorous deep nasal swab sample through both nostrils" combined with a pharyngeal swab in 100 subjects with naturally acquired common colds in the fall. Virus was grown in WI-38 cells from one supplier in 53% of nasal washes compared to 40% of the nose and throat swab samples. The findings in these two studies suggest that the nasal wash is more efficient than the nasal swab for detection of rhinovirus.

For transport, nasopharyngeal secretions are dispersed in medium containing protein. If immediate inoculation into cell culture is not convenient, the samples can be stored frozen at −70°C. One freeze-thaw cycle prior to inoculation does not appear to reduce the recovery rate of rhinovirus (11). Arruda *et al.* (11) emphasized that the efficiency of detection of rhinovirus in cell culture is increased by the use of more than one sensitive cell type. They compared recovery rates of rhinovirus from nasal washings from subjects with natural colds in several cell types. The WI-38 strain of human embryonic lung fibroblasts and a rhinovirus-susceptible clone of HeLa cells were the most sensitive types used. However, neither cell type detected all the rhinovirus isolates from the subjects; each type missed 20–35% of the positive samples. The authors concluded that samples should be inoculated into both WI-38 cells and susceptible HeLa cells in order to have maximum sensitivity for culture detection of rhinovirus. This strategy of inoculating samples into both fibroblasts and HeLa cells rather than into a single type was also used by Mäkelä *et al.* (9).

In summary, the most efficient method for diagnosis of rhinovirus infection by culture would include sampling nasopharyngeal secretions by nasal wash or nasopharyngeal aspiration, storing the sample at −70°C dispersed in protein-containing medium, and inoculating the sample into both fibroblasts and susceptible HeLa cells.

B. Polymerase Chain Reaction

The RNA of the picornavirus genome contains 7.2–7.5 kilobases. There is a single open reading frame preceded by a 5′ noncoding region containing short, highly conserved sequences (12). The nucleotide sequences of a number of human picornaviruses have been delineated. Synthetic oligonucleotide probes have been used in hybridization assays to detect picornaviruses. However, the usefulness of direct hybridization for detection of virus in respiratory samples is limited be-

cause $\geq 10^2$ copies of the viral genome are required for a positive result. Beginning in the late 1980s, conserved sequences were employed to prime reverse transcription (RT) of segments of viral RNA to produce cDNA for amplification in PCR (RT-PCR). Refinement of RT-PCR has resulted in formats that allow detection of one viral genome in samples from the upper respiratory tract.

For detection of rhinovirus in respiratory secretions with RT-PCR, several things should be noted. Nasal mucus may contain RNases or inhibitors of transcriptase/polymerase enzymatic activity. In order to have an assay sensitive enough to detect one genome, either nested PCR with detection of amplimers on a gel with ethidium bromide staining (13) or standard PCR with detection of amplimers by hybridization with labeled oligonucleotide probes must be used. Finally, identification of picornavirus genomic material in respiratory samples may indicate infection with either rhinovirus or enterovirus. The two viruses have been differentiated by the use of primers that amplify a region of picornavirus genome in which the number of nucleotide bases in rhinovirus RNA differs from that in enterovirus RNA so that amplimers can be distinguished on gel electrophoresis (13,14). Alternatively, the product of PCR amplification of a conserved sequence may be detected with an oligonucleotide probe that is specific for either rhinovirus or enterovirus genomes (9,15,16).

Four epidemiologic studies published since 1992 have utilized both RT-PCR and cell culture for detection of rhinoviruses in respiratory tract samples from adults or children with naturally acquired colds (9,14,16,17). These studies illustrate the variation in RT-PCR formats developed for detection of rhinovirus in clinical samples. In addition, they allow comparison of the efficiency of RT-PCR and cell culture for rhinovirus diagnosis. Highlights of the features of the RT-PCR protocols are shown in abbreviated, telegraphic form in Table I; full descriptions of the protocols are provided in the referenced publications. In all four studies, samples of respiratory secretions to be tested with RT-PCR (and to be inoculated into cell cultures) were stored frozen at $-70°C$ without additives. RNA was extracted from thawed samples in three of the four with proteinase K treatment followed by phenol extraction and ethanol precipitation. In the fourth sample the RNA was recovered with the use of a commercially available matrix affinity chromatography column. The primers used for RT and PCR in all four studies were from the 5′ noncoding region of the picornavirus genome except for one primer from the VP2 coding region in Method 1 (18) used by Mäkelä *et al.* (9). In three of the studies, the PCR product was detected with the use of labeled oligonucleotide probes; in the fourth, the amplimers

TABLE I

ATTRIBUTES OF RT-PCR ASSAYS FOR RHINOVIRUS EMPLOYED IN EPIDEMIOLOGIC STUDIES OF RESPIRATORY ILLNESS

RNA extraction	RT method	PCR design	Detection	Ref.
Proteinase K–phenol/ chloroform extraction–ethanol precipitation	Primer complementary to sense RNA sequence between 548 and 563 of HRV-14	"Touchdown" primary PCR with addition of primer of nucleotides between 183 and 198 of HRV-14. Seminested secondary PCR with 370–384 primer in addition to 548–563 primer (HRV-14)	Agarose/ethidium bromide detection of 202-bp product of seminested PCR	13, 14
Proteinase K–phenol/ chloroform/isoamyl alcohol extraction–ethanol precipitation	Primer complementary to sense cDNA sequence 547–562 on HRV-14	Second primer complementary to antisense sequence between 182 and 197 of HRV-14	PCR product applied to nitrocellulose membranes by slot blot manifold. Hybridized with ^{32}P-labeled probe complementary to 452–468 nucleotide sequence of HRV-14	16
Matrix affinity chromatography of diluted sample	Primer complementary to sense RNA sequence between nucleotides 540 and 555 of HRV-89	HotStart-5′ biotinylated primer complementary to antisense cDNA between base pairs 168 and 183 of HRV-89; 35 cycles	Amplimers separated from unreacted reagents; hybridization with digoxigenin dUTP-labeled probe complementary to sense viral RNA between nucleotides 451 and 467 of HRV-14; capture on streptavidin-coated plate for detection of labeled amplimers	17
Proteinase K–phenol extraction–ethanol precipitation	Two methods for RT-PCR *Method 1:* primers from 5′ noncoding region and from VP2 capsid protein coding region of enterovirus genome *Method 2:* both primers from 5′ noncoding region		Detection method not discussed	9

from nested PCR were detected by ethidium bromide staining after electrophoretic separation on an agarose gel. Differentiation of rhinovirus from enterovirus was based on the size of the amplimers (13) or on hybridization with a rhinovirus-specific probe (16); differentiation was not done in the study of samples collected in the fall (17).

In all four studies, 200 or more respiratory tract samples from people with colds were tested for rhinovirus by both cell culture inoculation and RT-PCR (Table II). Samples were obtained year round in three studies; in the fourth, they were obtained in the fall at the time of highest rhinovirus prevalence. Appropriate samples of nasopharyngeal mucus were obtained in three of the four studies. Anterior nasal swab culture in the fourth study (14) would not be expected to provide maximum sensitivity because nasopharyngeal secretions were not sampled. In accord with this expectation, the detection rate for rhinovirus in this study was only 33%. Rhinoviruses were detected in 50% of colds during year-round surveillance when RT-PCR results were combined with cell culture detection (9,16); 80% of illnesses in the fall were associated with rhinovirus (17).

The relative sensitivity of detection of rhinovirus with cell culture inoculation could be compared to that of RT-PCR in the three studies in which nasopharyngeal secretions were obtained. Only one sensitive cell line was inoculated in one study (16); only 32% of the rhinovirus positives were detected with cell culture, whereas RT-PCR detected 99%. In contrast, in the two studies in which two sensitive cell lines were inoculated (9,17), rhinovirus was grown in cell culture in 76–82% of the positive samples and RT-PCR was positive in 98–100%. Although RT-PCR was more sensitive, properly done cell culture inoculation using two cell systems detected three of every four positive samples.

Appropriate precautions to prevent false-positive results were employed in the PCR protocols in these studies. The consistency of the results and the cell culture detection of virus in at least three quarters indicate that the RT-PCR results were true positives. It is reasonable to conclude that (1) nasopharyngeal mucus must be sampled for reliable detection of rhinovirus; (2) RT-PCR is the most sensitive way to detect virus; (3) cell culture with two sensitive cell lines is 75–80% sensitive; and (4) fully 50% of colds during a year in children and adults are due to rhinovirus.

IV. Pathogenesis of Symptoms

In three reports, the published information on symptom pathogenesis in rhinovirus infection has been reviewed and referenced (5,6,19).

TABLE II

SENSITIVITY OF CELL CULTURE COMPARED TO RT-PCR FOR DETECTION OF RHINOVIRUS IN EPIDEMIOLOGIC STUDIES OF RESPIRATORY ILLNESS[a]

					Rhinovirus			
Population	Season	Sample	RV Infections/no. of samples tested	Cells used for culture	Culture no. positive	PCR no. positive	Culture no. positive/sample no. positive	Ref.
Adults with asthma	2 years	Anterior nasal swab plus throat swab	76/229 (33%)	Ohio HeLa and MRC-5	14	76	14/76 (18%)	14
Children	1 year	NPA	147/292 (50%)	Ohio HeLa	47	146	47/147 (32%)	16
Adults	Fall	NW	283/346 (82%)	HeLa I and WI-38	231	283 (PCR not done on all culture-positive samples)	231/283 (82%)	17
Adults	1 year	NPA	105/200 (53%)	Ohio HeLa and foreskin fibroblasts	80	103	80/105 (76%)	9

[a] NPA, Nasopharyngeal aspirate; NW, nasal wash.

In this article, selected features of current knowledge of symptom pathogenesis will be emphasized as a background for speculation on how the viral infection, the host response to the viral infection, and the symptoms perceived by the host may fit together.

Five features of rhinovirus infections of the human nose deserve emphasis: (1) There is no discernible destruction of the nasal epithelium by the virus during rhinovirus infection either *in vivo* or *in vitro*. This feature was illustrated in a study in which nasal epithelium in culture was infected with four different respiratory viruses (20). Rhinovirus and coronavirus produced no apparent cytopathic effect in the epithelial monolayer, whereas adenovirus and influenza virus had a profound effect (Fig. 2). This picture was obviously different from that produced by rhinovirus infection of susceptible fibroblasts in culture, in which

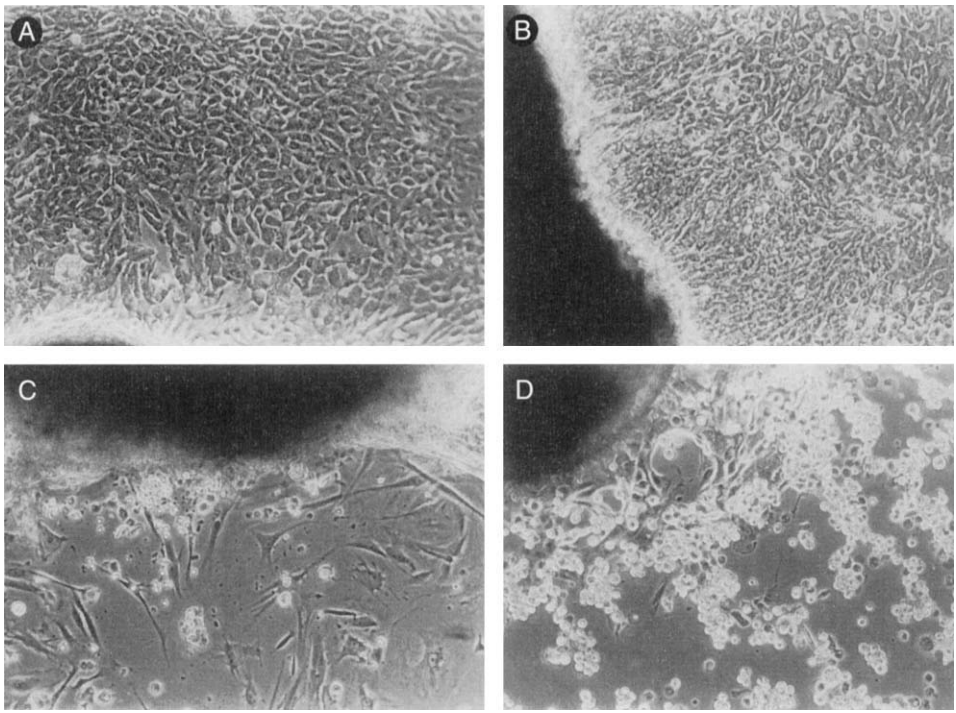

FIG 2. Outgrowth of epithelial cell monolayer from fragments of nasal mucosa 96 hours after exposure to virus (phase contrast; magnification: × 100). (A) Rhinovirus, (B) Coronavirus. (C) Influenza type A. (D) Adenovirus. Reprinted with permission from Ref. 20.

distinct cytopathic changes were apparent (20). (2) Very few cells in the nasal epithelium of volunteers are infected at any point during symptomatic rhinovirus infection. Two different groups of investigators have demonstrated this using *in situ* hybridization. (3) An epithelial cell can elaborate cytokines in response to infection with rhinovirus. This important observation may be central to symptom pathogenesis. (4) Symptoms in volunteers with experimental rhinovirus infections parallel the presence of markers of inflammation and the concentration of virus in nasal secretions (Fig. 3). Volunteers who are infected but not ill do not have elevated levels of inflammatory markers in their nasal washes. In the volunteer model, symptom severity, viral titers, and concentrations of serum albumin, polymorphonuclear neutrophils (PMNs), interleukin-8 (IL-8), and kinins all peak 48 hours after inoculation, and rise and fall in parallel. (5) Symptomatic illness subsides in adults after 5–7 days in spite of the fact that virus does not disappear from the nasopharynx until 16 to 18 days after inoculation (Fig. 1). Persistence of virus in the absence of symptoms was seen in the study from which Fig. 1 was taken (4).

With the above features in mind, one can speculate on the mechanism by which symptoms are produced during rhinovirus infection of nasal

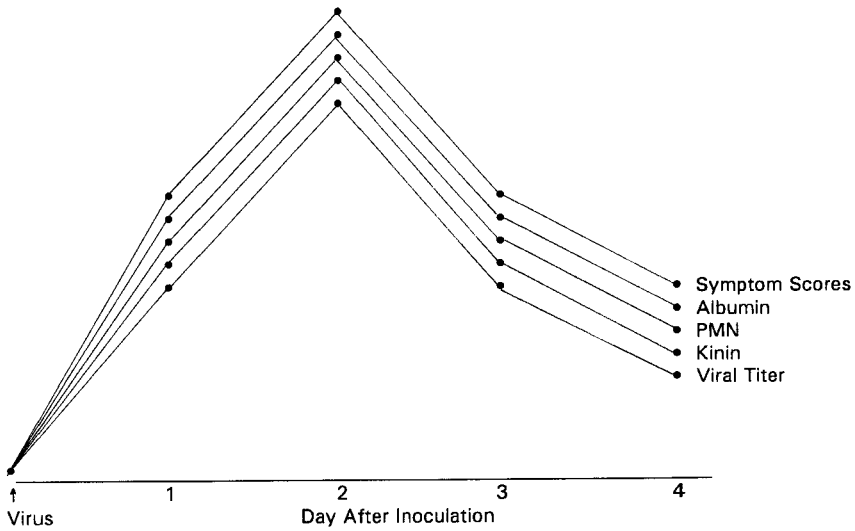

FIG 3. Schematic composite of average symptom scores compared to concentrations of markers of inflammation and virus in nasal washes following intranasal inoculation of rhinovirus.

mucosa. Following inoculation of virus into the nose, single epithelial cells that are infected elaborate cytokines (and chemokines) as "distress signals." Cytokine release results in a localized vascular leak with extravasation of albumin and other serum proteins and an influx of PMNs into mucosa from the IL-8 effect (21). Appearance of kinins in nasal secretions is a side effect of the vascular leakage of kininogen. If enough epithelial cells are infected and elaborate cytokines, the inflammatory response produces symptomatic illness. At the same time, the localized inflammatory response is effective in limiting viral replication, perhaps by washing extruded infected cells away from the epithelial surface before the virus spreads to neighboring cells. This results in a decline in viral titer after the peak at 48 hours postinoculation. As fewer cells are infected, the extent of the inflammatory response and the symptoms diminish. Finally, between the second and third weeks after inoculation, sufficient neutralizing antibody becomes available to shut down viral replication and the infection ends. The antibody may be secretory IgA or serum IgG that has leaked into the mucosa.

In brief, the symptoms during rhinovirus infection of the nose appear to be caused by the host response, not by the virus *per se*. This introduces the notion that the virus need not be killed to ameliorate the illness if the host response can be altered. However, it should be noted that the use of oral prednisone, a broad and potent anti-inflammatory agent, by infected volunteers resulted in increased titers of rhinovirus in nasal washings (22). This finding is consistent with the speculation that the localized inflammatory response to rhinovirus infection of epithelial cells is instrumental in limiting viral replication in the nasal mucosa. Although theoretically there is no need to kill the virus in order to reduce the symptoms, an effective antiviral may be required in conjunction with anti-inflammatory treatment in order to alter the illness in view of this demonstrated enhancement of viral replication by steroid.

V. Summary

Rhinoviruses cause more infections in humans than any other microorganism. These acid-sensitive picornaviruses infect epithelial cells following inoculation onto the nasal mucosa and are detected reliably in nasopharyngeal secretions. Rhinovirus colds occur year round, with a peak of illness in the fall. Type-specific serum antibody correlates with protection against infection. The fact that there are at least 100 different immunotypes makes development of an effective vaccine unlikely.

Nasopharyngeal secretions must be sampled for detection of rhinovirus by culture or RT-PCR. Efficient isolation of virus requires inoculation into two different types of sensitive cell cultures (i.e., fibroblasts and HeLa cells). RT of conserved sequences in the 5′ noncoding region of the viral RNA to produce cDNA for PCR amplification has been coupled with detection of amplimers either by gel electrophoresis after nested PCR or by hybridization with labeled oligonucleotide probes to detect one viral genome in samples. In two studies in which both RT-PCR and cell cultures were used, virtually all of the positives were identified with RT-PCR; culture in two cell lines identified 75–80% of the positives. In year-round surveillance, 50% of colds in adults and children were rhinovirus positive.

The symptoms occurring during rhinovirus colds are caused by the host's response to the virus, not by the virus itself. Elaboration of cytokines by infected epithelial cells is central to symptom pathogenesis.

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