Studies on the Inability of Rhinovirus to Survive and Replicate in The Intestinal Tract of Volunteers. (31990)

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A previous report from this laboratory indicated that common cold syndromes could be reproduced in volunteers by intranasal inoculation with 3 rhinovirus serotypes(1). Although virus was readily detected in nasal and pharyngeal secretion specimens obtained from these men, 0 of 120 rectal swab specimens and only 1 of 75 stool specimens yielded virus. These results are similar to those reported by others with rhinoviruses(2,3).

In an attempt to determine if the failure to recover acid labile rhinoviruses from the lower intestinal tract was due to inactivation by gastric secretions, Mascoli *et al*(4) fed rhinovirus to volunteers in enteric-coated capsules. Although no attempts were made to recover virus from the subjects, the absence of illness and failure of serum antibody titers to increase suggested that infection did not occur.

In the present study rhinovirus NIH 1059 was shown to produce colds in volunteers following intranasal inoculation, and only one of many stool and rectal swab specimens from infected volunteers yielded virus. Additional *in vivo* and *in vitro* studies were performed using NIH 1059 as a model to evaluate factors which might account for the failure of rhinoviruses to multiply or survive in the gastrointestinal tract.

Materials and methods. Volunteers. Subjects were 17 healthy adult male volunteers from several federal correctional institutions. Procedures for their selection and supervision have been previously described(5). None of the volunteers possessed serum neutralizing antibody to rhinovirus NIH 1059.

Inoculum. Rhinovirus NIH 1059 was isolated from the oropharynx of a Marine recruit with a mild upper respiratory illness (6,7). The harvest from an initial isolation in human embryonic kidney (HEK) tissue culture was passaged twice in human embryonic fibroblast (HEF) tissue culture to prepare a virus pool which contained 5.0 $\log_{10}$ 50% tissue culture infectious doses ($TCID_{50}$) per ml. This NIH 1959 inoculum pool was safety tested to exclude the presence of detectable extraneous agents.

Procedures for inoculation and dose of virus administered. Intranasal inoculation consisted of spraying 1 ml of diluted§ inoculum into the nose with a DeVilbis 127® hand atomizer, and instilling 1 ml intranasally by pipette with the subject in a supine position. The total dose of NIH 1059 was 3200 $TCID_{50}$ as calculated from titration of the diluted inoculum in HEF tissue culture.

Enteric-coated capsules which release their contents within the intestinal tract distal to the stomach were prepared as in previous studies with adenoviruses(8), with minor modification. Two-tenths ml of the NIH 1059 virus pool (20,000 $TCID_{50}$) was placed in gelatin capsules which were then coated with cellulose acetate hydrogen phthallate. Capsules were administered to each of three volunteers within 15 minutes of preparation. A duplicate capsule was incubated 1 hour at 37°C, then cut and dissolved in 5 ml of tissue culture medium. Only 1 of 7 HEF tissue culture tubes inoculated with 0.2 ml (4 tubes) or 0.5 ml (3 tubes) of the latter fluid yielded virus. The maximal dose of infective NIH

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§ Diluent was tissue culture medium consisting of equal parts of medium no. 199 and Eagles minimum essential medium containing 2% inactivated (56°C, 30 min.) calf serum, 0.04% saturated solution of sodium Bicarbonate, and supplemental antibiotics.

1059 which could have been released in the intestine was estimated to be between 8 and 40 $TCID_{50}$. Virus inactivation was subsequently shown to be caused by the gelatin capsule and its cellulose acetate hydrogen phthallate coat, perhaps due to the acid pH of these materials when solubilized.

Direct intestinal inoculation was performed using a Rehfuss tube inserted through mouth into the duodenum. Samples of gastric and duodenal secretions were withdrawn before inoculation. The tube was assumed to be in the duodenum when bile appeared in the aspirates after the tube had been inserted an appropriate distance. Bile was never obtained from one volunteer (Vol. 17). A dose of 10,000-16,000 $TCID_{50}$ of NIH 1059 contained in 20 ml of tissue culture medium was injected through the Rhefuss tube, and was followed by an additional 50 ml of medium as a rinse. The tube was then withdrawn.

Specimens for virus. Nasal, pharyngeal and anal swab specimens, 10 ml nasopharyngeal wash (NPW) specimens, 10 ml gargle specimens and stool specimens were collected, processed, and tested for virus in HEF cultures as described previously(1). Pre-inoculation specimens were additionally tested in HEK and monkey kidney cultures for adventitious viruses. Except as noted below, the first and last isolates from each volunteer were identified as NIH 1059 by neutralization of 32 or more $TCID_{50}$ of virus with not more than 20 antibody units of specific hyperimmune guinea pig serum.

Herpesvirus hominis was isolated from 2 volunteers (Vols. 6 and 8, Table I) during infection with NIH 1059. In addition, agents were recovered from volunteers 11 and 14 which produced enterovirus-like cytopathic effect, were ether stable (method of Johnson and Rosen(6)), and were acid labile (pH 4 citrate-citric acid buffer method(9)). These agents were not neutralized by NIH 1059 or *Herpesvirus hominis* antiserum, and are hereafter referred to as extraneous rhinoviruses. One of these extraneous rhinoviruses was detected in specimens collected both before and after inoculation (Vol. 11), and this volunteer developed a mild bronchitis. Volunteer 14 had been inadvertently exposed to a person with a similar extraneous infection the day before inoculation with NIH 1059, and the first specimen yielding an extraneous rhinovirus was collected the day following inoculation; he was never clinically ill. Both of these volunteers were inoculated with NIH 1059 by the intestinal route.

Antibody determinations. Neutralization tests versus 6-10 $TCID_{50}$ of NIH 1059 were performed on paired sera as previously described(1).

In vitro *tests.* Trypsin sensitivity tests were performed as described by Kisch *et al* (7). Acid lability tests were performed using the citrate-citric acid buffer method(9). Similar methods were adapted for testing gastric and duodenal secretions. The pH of samples of gastric and duodenal secretions was determined with a Beckman Zeromatic pH meter. NIH 1059 virus pool was mixed with equal portions of secretion, incubated at 37°C for 1 hour, and neutralized with equal volumes of 0.5 Molar phosphate buffer (final pH = 7.2). Mixtures and control virus suspensions were then analyzed for infectivity in HEF tissue culture. The influence of body temperature on rhinovirus survival was evaluated by storage of virus suspensions at 37°C with subsequent infectivity titer assay in HEF tissue culture incubated at 34°C. Effects of body temperature on rhinovirus growth and production of cytopathic effect (CPE) were tested by incubation at 37°C of HEF tissue cultures (WI 38 strain‖) inoculated with 10-fold dilutions of virus. Growth was assessed in the latter case by passage of fluid and cells to fresh cultures incubated at 34°C.

Results. Intranasal inoculation. The response of 9 volunteers to intranasal challenge with rhinovirus NIH 1059 is shown in Table I. Seven of the 9 developed an upper respiratory illness ranging in severity from a mild rhinitis (Vol. 7) to a febrile illness with nasal obstruction and discharge, cough, malaise, and myalgia (Vol. 3). Onset of illness was 1 to 2 days following inoculation, and symptoms persisted for 1 to 3 days. A slight increase in blood neutrophiles with a slight decrease in blood lymphocytes usually accompanied the acute illness as was previously reported(10).

‖Propagated in this laboratory from stock kindly supplied by Dr. L. Hayflick, Wistar Institute.

TABLE I. Response of Antibody-Free (<1:2) Volunteers After Intranasal Challenge with Rhinovirus NIH 1059.

Volunteer	Illness*	Virus shedding, nasopharynx, days 1-7	Antibody titer 3 weeks
1	0	4/7†	1:4
2	++	4/7	1:64
3	+++(38.8)	2/7	1:64
4	++	3/7	<1:2
5	0	3/7	<1:2
6	+++(37.9)	3/7	1:4
7	+	4/7	1:128
8	+++(37.7)	3/7	1:16
9	++	4/7	>1:128
Totals	7/9 Ill	30/63 (48%)	7/9 ≥4-fold rises

* 0 = No illness. + = Nasal obstruction and discharge. ++ = Nasal obstruction and discharge and systemic symptoms. +++ = Nasal obstruction and discharge, systemic symptoms and fever (≥37.5°C, oral). Numbers in parentheses are highest temperature.

† Number of specimens yielding virus/number tested.

Rhinovirus was detected in the NPW specimens collected from each volunteer on the first and second days following inoculation, but subsequent specimens yielded virus more sporadically. The number of NPW specimens yielding virus among those collected during the first week following inoculation is shown in Table I. Specimens collected on day 9 from 3 volunteers yielded virus, but no other specimens collected from each volunteer every 2 or 3 days during the 2nd and 3rd week following inoculation were positive for virus.

Nasal and pharyngeal swabs and gargle specimens were inferior to NPW specimens for recovery of this rhinovirus. During the first week following inoculation, 41%, 37%, and 19% respectively, of the former specimens yielded virus, as opposed to 48% for NPW specimens. The order of specimen collection was varied to exclude washing out of virus as a factor in these differences.

Only one of the 63 stool specimens collected from the group of volunteers within the first 11 days following inoculation contained detectable rhinovirus. This stool specimen was obtained from Volunteer 3 on the third day following inoculation during the period of maximal symptoms, and it contained approximately 100 $TCID_{50}$ per gram. No virus was detected in any of 104 rectal swab specimens, including one collected from Volunteer 3 on the same day that the virus positive stool specimen was obtained.

Neutralizing antibody titers of sera collected 3 weeks following inoculation are shown in the last column of Table I. A 4-fold or greater increase in antibody titer against rhinovirus NIH 1059 occurred in 7 of these 9 initially antibody-free volunteers.

Intestinal inoculation. Ingestion of rhinovirus NIH 1059 contained in an enteric-coated capsule did not result in gastrointestinal symptoms (Table II). Volunteer 11 developed mild bronchitis, but he was subsequently shown to be infected with an extraneous rhinovirus (see *Materials*). Volunteer 11 also developed a repeatedly demonstrable 4-fold increase in serum neutralizing antibody titer versus NIH 1059, perhaps an example of heterotypic antibody response(11). With this possible exception, there was no virological or serological evidence of infection with rhinovirus NIH 1059 following ingestion of the capsules.

The effect of intestinal inoculation was retested by depositing 10,000-16,000 $TCID_{50}$ of NIH 1059 directly into the duodenum of 5 volunteers by means of a Rehfuss tube (Table II). No symptomatic, cultural or serological evidence of infection of the gastrointestinal tract was detected, nor was virus detected in stools or rectal swab specimens collected during the days immediately following inoculation which might indicate passage of the virus inoculum through the intestinal tract. One volunteer developed an asymptomatic infection with an extraneous rhinovirus (see Materials) and 2 developed nasopharyngeal infection with NIH 1059. Virus shedding by the latter volunteers was first detected in NPW specimens collected 4 and 6 days following inoculation, and may have resulted from contamination of the pharynx with a small amount of NIH 1059 during the withdrawal of the Rehfuss tube. It is of interest that this presumed deposition of a small dose of virus in the pharynx resulted in a small number of positive NPW specimens, no significant increase in neutralizing antibody titers, and only a mild pharyngitis in one of the two individuals.

TABLE II. Response of Antibody-Free (≤1:2) Volunteers After Intestinal Challenge with Rhinovirus NIH 1059.

Method of inoculation	Volunteer	Illness	Virus shedding, days 1-25 Nasopharynx	Anal swab	Stool	Antibody titer 5-6 weeks
Capsule	10	0	0/10*	0/10	0/6	<1:2
	11	Mild bronchitis	†2/10	0/10	0/4	1:4
	12	0	0/10	0/10	0/4	<1:2
Rehfuss tube	13	0	0/11	0/11	0/3	<1:2
	14	0	†4/10	0/10	0/4	<1:2
	15	0	0/14	0/14	0/14	<1:2
	16	0	‡2/14	0/14	0/14	1:2
	17	Mild pharyngitis	‡4/14	0/14	0/14	<1:2

* Number of specimens yielding virus/number tested.
† Virus strains isolated were rhinoviruses other than NIH 1059.
‡ Identified as NIH 1059.

Effect of physiologic conditions of gastrointestinal tract on survival and infectivity of rhinovirus NIH 1059. Samples of gastric fluid (pH 3.9, 4.2, 6.1, 6.6, and 8.0) were collected from volunteers 13-17 at the time of inoculation, and the effect of these secretions on virus survival was tested as described. An approximately 100-fold reduction in virus titer occurred with one specimen (pH 8.0), but no loss of virus infectivity titer occurred following incubation with the other four. Failure of the two gastric secretions with moderately low pH values to reduce virus titers may have been due to partial neutralization of the small amount of hydrochloric acid in those specimens by the tissue culture fluid containing the virus. Maintenance of secretion-virus mixtures at pH 4.0 using the citrate-citric acid buffer method(9) demonstrated continued acid lability of NIH 1059 in these mixtures.

Duodenal secretions (pH 4.6, 7.0, 7.4, and 8.3) were obtained from volunteers 13-16 and similarly tested. Reduction in virus titer occurred in all tests (10-60 fold), although duodenal secretions were toxic for HEF tissue culture and may have interfered in part with detection of virus in the mixture. The strain of NIH 1059 used in these studies was shown to be trypsin-resistant.

Storage of virus for 1 hour at 37°C resulted in a 2-fold loss in titer, and by 4 hours a 4-fold loss in titer had occurred. Incubation at 37°C completely inhibited CPE production in WI-38 HEF tissue culture tubes inoculated with up to 4.75 $\log_{10}$ $TCID_{50}$ of virus as determined by simultaneous assay in tubes incubated at 34°C. Fluid and cells from inoculated tubes which had been incubated at 37°C for 7-14 days without CPE production were subpassed to fresh cultures which were then incubated at 34°C for an additional 14 days; CPE occurred although final virus infectivity titers were only 2.0-1.75 $\log_{10}$ $TCID_{50}$, 500-1000-fold less than control titers.

Discussion. These studies demonstrate that rhinovirus NIH 1059 can reproduce natural common cold syndromes in volunteers following intranasal challenge. Patterns of illness, virus shedding, and antibody response were similar to those reported from this laboratory following intranasal inoculation with 3 other rhinoviruses(1). In keeping with the characteristic failure to detect rhinoviruses in stool or rectal swab specimens obtained from persons with rhinovirus common colds(2,3), only a single stool specimen from 1 of 9 volunteers infected by intranasal challenge yielded NIH 1059.

Factors which might prevent intestinal infection and excretion of rhinoviruses were evaluated. Since rhinoviruses are acid labile it seemed reasonable that inactivation of virus by acid contained in the stomach might result in failure of infectious virus to reach the intestine. Therefore, establishment of intestinal infection was attempted using direct inoculation of NIH 1059, first unsuccessfully in enteric-coated capsules, and subsequently with instillation of virus through a Rehfuss tube.

No rhinovirus was detected in stool specimens nor was there serological evidence of infection following placement of 10,000-16,000

$TCID_{50}$ of NIH 1059 in the duodenal lumen through a Rehfuss tube. Since the method of processing stool specimens results in inoculation of an HEF tissue culture tube with 0.4 ml of a 1:10 dilution (weight:volume) of stool, the amount of virus per gram of stool necessary for consistent detection is about 80 $TCID_{50}$. With an average adult stool wet-weight of 75-170 g per day (12) several virus positive stool specimens should have been obtained from the volunteers a day or two after NIH 1059 was placed in the duodenum if the 10,000-16,000 $TCID_{50}$ of virus were merely transported through the intestine. That this did not occur suggests that, in addition to failure to establish infection, inactivation or removal of rhinovirus took place in the intestine *distal to the stomach.*

Furthermore, *in vitro* tests indicated that gastric fluid would *not,* with known variability in pH(13), inactivate rhinovirus with any consistency. Thus, much of the rhinovirus contained in respiratory secretions which are ingested by ill persons probably passes through the stomach into the duodenum as active virus, and often in considerably larger quantities than were instilled in the present studies since nasal wash specimens from ill persons, which represent a dilution of their nasal secretions, frequently contain 5,000-160,000 $TCID_{50}$ of rhinovirus per ml(14).

Mechanisms responsible for the failure of rhinovirus to infect or survive once having reached the duodenum were next sought. Duodenal secretions appeared to cause a 10-60 fold reduction in infectivity. Reasons why duodenal secretions might affect NIH 1059 are not known; rhinoviruses do not contain essential lipid (e.g., are ether-stable(9)) and, although many rhinoviruses are apparently trypsin sensitive, this particular serotype is not(7). Nevertheless, a reduction in virus titers of the magnitude which occurred *in vitro* with duodenal secretions would be sufficient *in vivo* to frequently obscure virus excretion in the stool if no replication occurred within the intestine. This effect of duodenal secretions is not absolute, however, and would be unlikely to cause the essentially complete absence of intestinal infection with rhinoviruses which is observed. Failure of persistent virus excretion to occur despite known exposure of the intestinal mucosa to virus (as indicated by a positive stool specimen from Volunteer 3, this report) further suggests that mechanisms in addition to inactivation of rhinovirus can prevent intestinal infection.

The effects of internal body temperature (37°C) on growth, CPE production and survival of rhinovirus were evaluated because optimal conditions for isolating rhinoviruses have been shown to include incubation at 33°-34°C, a temperature resembling that of the nasal mucosa(15). Requirements for optimal rhinovirus growth *in vitro* may be dependent in part on the tissue culture system however; growth of certain rhinoviruses (including NIH 1059) has been reported to be inhibited in HEK incubated at 37°C, but to be equal at incubation temperatures of 33° and 37°C in the WI-26 strain of HEF(6). Nevertheless, in the present study, when growth and production of CPE by NIH 1059 was tested in the WI-38 strain of HEF incubated at 37°C, no CPE occurred and virus titers decreased. Persistence of a small part of the virus population in the tissue culture system for 14 days at 37°C was demonstrable on subpassage and incubation at 34°C. The role of such an effect in preventing rhinovirus infection of the intestinal mucosa *in vivo* is uncertain, but as demonstrated by the present *in vitro* tests, it could be decisive. Temperature may also be a factor in the failure of rhinovirus to survive transit through the gastrointestinal tract since it was shown in this study, as well as others(16), that rhinovirus is slowly inactivated during storage at 37°C.

In conclusion, these studies suggest that the combined effects of temperature, transit time, and gastrointestinal secretions could account for the failure of rhinovirus to infect or survive passage through the gastrointestinal tract, although participation of other unevaluated factors is not ruled out. The results further suggest that intestinal immunization, which has been successful in the immunoprophylaxis of respiratory infection with adenovirus type 4(17), would be unsuitable for the prevention of rhinovirus common colds. It remains possible, however, that rhinovirus strains adapted to grow at 37°C in the human intestine could be developed and might be suitable for this purpose.

Summary. Following intranasal inoculation with rhinovirus NIH 1059, 9 of 9 volunteers developed a respiratory infection and 7 of 9 had illness ranging from mild rhinitis to febrile upper respiratory tract disease. Only 1 of many stool and rectal swab specimens from these men yielded rhinovirus. No detectable intestinal infection occurred in 3 volunteers who received NIH 1059 in enteric-coated capsules, nor in 5 volunteers inoculated directly into the duodenal lumen *via* Rehfuss small intestinal tube. Effects of gastric and duodenal secretions, trypsin, and body temperature (37°C) on growth and survival of NIH 1059 were tested *in vitro*, and their role in preventing intestinal infection and excretion of rhinoviruses evaluated.

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Immunologic Responses in Hamsters to Homologous Tumor Antigens Measured *in vivo* and *in vitro*.* (31991)

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Efforts in our laboratories(1-6) to prepare and to assay vaccines against tumor led to the development of a model system in which oncogenic virus is given to hamsters when newborn, and homologous virus-free tumor antigen is given sometime later, prior to first appearance of virus-induced tumor. This is called the SV_{40} virus-newborn hamster system. Irradiated and idodeoxyuridine (IUDR) treated SV_{40} tumor cell antigens proved highly effective (as great as 100%) in preventing the appearance of tumor when given 34 to 76 days after homologous virus. Though very dependable for measurement of vaccine ef-

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