which is capable of transferring PCA to guinea pigs, but rather their γ_2 antibody globulin.

Since the capacity to transfer anaphylactic reactions depends upon the presence on the sensitizing antibodies of receptors able to bind to specific tissue sites(15), the observations imply that the anaphylactic antibodies of different species differ in the structure of their tissue receptors. It is therefore a matter of chance, probably due to the fact that all mammalian γ-globulins are built according to similar patterns, that the γ_2 antibodies of some foreign species, man, monkey, dog, and mouse, possess the structure able to bind to guinea pig tissues, which characterizes guinea pig γ_1 antibodies. Such receptors are not found, however, in all γ_2 mammalian globulins since neither horse, cow, or sheep antibodies can sensitize guinea pigs for PCA(16). The fact that γ_2 antibodies of several mammalian species, their largest antibody type, are capable of sensitizing guinea pigs for PCA explains the great usefulness of this technique for detection of antibodies produced by these various species.

Summary. Guinea pigs may be sensitized for PCA reactions by guinea pig 7S gamma 1 antibodies but not by guinea pig 7S gamma 2 antibodies. However, with all other species capable of sensitizing the guinea pig, it is always the 7S gamma 2 antibody type which mediates sensitization of guinea pigs for PCA.

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A Description of Two Newly-Recognized Rhinoviruses of Human Origin. (29389)

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As presently constituted, the human picornaviruses are divided into 3 groups; (a) the enteroviruses, subgrouped as polioviruses, Coxsackie viruses and echoviruses (b) the rhinoviruses and (c) an unclassified group (1,2). Rhinoviruses, like the enteroviruses are small, ether-resistant viruses containing an RNA core. However, they can be distinguished from enteroviruses by their property of acid lability(3,4).

To date, 6 prototype rhinoviruses(5,6) have been sufficiently characterized to meet

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the criteria for inclusion in the picornavirus group established by the Panel for Picornaviruses(1). Numerous other strains have also been characterized(3,7,8). This paper describes 2 new candidate prototype strains, designated 363 and 1200, which are of human origin and antigenically distinct from all previously established rhinoviruses and from many of the candidate agents. Epidemiological observations based on isolation of several other agents related to the candidate strains also are presented.

Materials and methods. Specimens for virus isolation. Oropharyngeal swabs were obtained from male marine recruits at Parris Island, S. C., as previously described(6). The specimens were stored at —60°C until tested.

Tissue cultures. Roller tube cell cultures obtained from Microbiological Associates and Flow Laboratories Inc. were maintained as previously described(6). These included primary human embryonic renal epithelium (HEK), 2 strains of semicontinuous human diploid embryonic fibroblasts from embryonic lung (WI-26, WI-38), primary African green monkey kidney (AGMK), primary rhesus monkey kidney (RMK) and 2 continuous cell lines of human epithelial cells of malignant origin (KB, HEp-2). Petri dish cultures of WI-26 cells were prepared according to the method described by Kisch *et al* from a strain of cells maintained in this laboratory (9).

Preparation of antisera. Adult guinea pigs (500-700 g, both sexes) were inoculated intramuscularly with virus-infected KB fluids extracted with ½ volume of Fluorocarbon 113 (Genetron). Initial inoculation of virus (0.5 ml) plus complete Freund's adjuvant (0.5 ml) was followed in 2 weeks by inoculation of virus (0.5 ml) plus incomplete adjuvant (0.5 ml) and 3 weeks later by inoculation of virus alone. Animals were bled 5-7 days thereafter. In an attempt to find a small laboratory animal from which higher titered and larger volumes of antiserum might be obtained, a rooster was inoculated with a Genetron treated rhinovirus 1200 pool. Three intramuscular injections of 2 cc of virus were given at weekly intervals and the rooster bled one week after the third injection.

Infectivity titrations. Infectivity titrations were carried out by inoculation of 0.2 ml amounts of virus pool per tissue culture tube in serial 3.2 or 10-fold dilution in Hanks' balanced salt solution (BSS)-0.5% gelatin medium. Two to four cultures were used per dilution. End points based on observation of cytopathic effect (CPE) were calculated by the method of Reed and Muench(10).

Size determination and ether sensitivity. Procedures described previously were followed throughout(6). Gradocol filtration was employed for estimation of virus size. Fresh diethyl ether was used for ether sensitivity studies.

Neutralization tests. All sera tested were heat inactivated at 56°C for ½ hour. Equal volumes of virus-serum mixtures were incubated at room temperature (25°C) for one hour, 0.2 ml amounts of the mixture were inoculated into 2 tissue culture tubes, which were incubated at 33°C on a rotating drum, and read daily for evidence of CPE. Neutralization tests using the reagents described previously(6) were carried out in a similar manner. However, in view of the consistent results and reliability of the acid stability test, it was not deemed necessary to carry out reciprocal neutralization tests against prototype polioviruses, Coxsackie viruses or echoviruses 1-27, and 29. Thus tests differed from those previously described in that the candidate virus strains were tested against antisera to the acid stable picornaviruses but antisera for the candidate strains were not assayed against these viruses. Reciprocal neutralization tests were carried out with the "newer" echoviruses, Echo 30 (Bastianni), Echo 31 (Caldwell) and Echo 32 (PR-10), with the prototype rhinoviruses, and candidate rhinovirus strains listed in Table I. In addition, 3 other candidate rhinovirus strains (127-1, 164-A, 137-3) obtained from Dr. Dorothy Hamre, University of Chicago, were tested against 363 and 1200 antisera; however, the reciprocal test was not carried out since antisera for 127-1, 164-A and 137-3 were not available(11). Strain, source and dilution of sera used to contain approximate-

ly 20 antibody units also are given in Table I. Several of the sera used were contributed by Dr. Herbert Wenner, University of Kansas, Dr. Jacob Holper, Abbott Laboratories, and Microbiological Associates, Bethesda, Md.

Tests for neutralizing antibodies in human serum were carried out in WI-26 tissue culture tubes. Convalescent sera were screened at a 1:4 dilution against 3-32 $TCID_{50}$ of both virus strains and end points were read 2 days after appearance of CPE in the highest positive dilution of the simultaneous virus titration. Positive sera were retested with matching acute sera using 4-fold dilutions of serum, and read in like manner.

Mouse pathogenicity. Tissue culture fluids containing approximately 100-320 $TCID_{50}$ of virus were inoculated into litters of newborn white mice (NIH general purpose). Three routes of inoculation were used; intracerebral (0.015 ml), intraperitoneal (0.03 ml) and subcutaneous (0.03 ml) using 2 litters of 8 mice per route. Mice were observed for evidence of abnormality. Sick or dead animals were harvested, brain and muscle emulsified and a 10% suspension made in BSS. Each suspension was then inoculated into 2 other litters of newborn mice, and serial 10-fold dilutions (10^0-10^{-3}) were inoculated into WI-26 tissue to test for presence of virus.

A further experiment was performed in which litters were inoculated as described above. One mouse from each litter was harvested on the third day after inoculation and every second day thereafter through the fifteenth post-inoculation day, the remaining mouse being harvested on the twenty-first day. A 20% suspension of brain and muscle was made from each mouse, centrifuged at 1500 rpm for 30′ and inoculated into two WI-26 tissue culture tubes in 0.2 ml amounts. Cultures were washed and fresh media was added after 4 hours' incubation at 33°C.

Acid lability. The method of Ketler *et al* was employed(3). Virus pools were diluted 1:10 in Eagle's minimum essential medium (MEM) without bicarbonate, pH 2.7, and in MEM with tris buffer, pH 7.2, and incubated 3 hours at room temperature. Infectivity titrations were carried out in WI-26 tissue culture tubes.

Determination of nucleic acid type. Five iodo-deoxyuridine (IUDR) obtained from the California Biochemical Corp. was used for these tests. For convenience the IUDR was stored frozen at —20°C as a $10^{-3.3}$ M solution, and thawed just prior to use; no apparent loss of potency resulted from this method of storage. WI-26 tissue culture tubes were fed with 1 ml of a medium composed of 50% Eagle's basal medium-50% medium 199 supplemented with 2% inactivated horse serum, rather than calf serum which is known to contain thymidine. A final concentration of $10^{-4.3}$ M was achieved by adding 0.1 ml of the stock IUDR to each tube. WI-26 tubes maintained in a similar manner without IUDR were used for simultaneous control titrations. Polio Lsc-1, a known RNA-containing virus, Vaccinia (known DNA-containing virus), and the candidate strains 363 and 1200 were titrated in WI-26 cultures with and without IUDR. All tubes were incubated on a rotary drum at a temperature of 33°C and observed daily for a period of 10 days for appearance of CPE. It was not necessary to refeed the tubes during the 10-day period.

Magnesium chloride stabilization, 2 (α hydroxybenzyl) benzimidazole (HBB) inhibition and plaque assay procedures were done by methods previously described(6,9).

Results. Isolation and tissue culture properties of virus strains. Virus strains 363 and 1200 were isolated in primary HEK cell cultures from throat swab specimens obtained from 2 marine recruits at Parris Island, S. C. Both recruits had an afebrile upper respiratory illness at the time of virus sampling. Strain 363 was recovered from a specimen obtained on 24 July 1959 from a 17-year-old male who had been in camp less than one week at the time of his illness; the donor of strain 1200 was an 18-year-old male swabbed on 17 Nov. 1959 in his tenth week of training. Both viruses were reisolated in WI-26 tissue from original throat specimens after storage at —60°C for 38 months (strain 363) and 34 months (strain 1200). Only strain 363 could be reisolated in HEK.

TABLE I. Source of Picornavirus Antisera Used in Reciprocal Neutralization Tests of Candidate Rhinoviruses 363 and 1200.

Virus type and reference		Source	Animal of inoc	Dilution used
"Newer echoviruses"				
Echo 30 (Bastianni)	(1)	Wenner	Monkey NFIP	1:100
Echo 31 (Caldwell)		"	"	1:100
Echo 32 (PR-10)		"	"	1:100
Prototype rhinoviruses†				
Echo 28	(5)	MBA	Monkey	1:10
353	(6)	Johnson	Guinea pig	1:5 *
1059		"	"	1:100
1734		NIH	Goat	1:8
11757		Johnson	Guinea pig	1:100
33342		"	"	1:100
British rhinoviruses				
Sal/1/57M (HGP)	(7)	MBA	Monkey	1:10
Sal/1/60M (B632)		Holper	Calf	1:50
Sal/1/58H (FEB)		Johnson	Guinea pig	1:100
Sal/1/51H (No)		NIH	Goat	1:100
Sal/1/59H (Th)		"	"	1:8
Sheffield/1/60H (16/60)		Johnson	Guinea pig	1:5 *
Candidate rhinoviruses				
179-E	(8)	Holper	Calf	1:10
140-F		"	"	1:20
106-F		"	"	1:50
363		Johnson	Guinea pig	1:10*
1200		"	"	1:10

* Containing approximately 16 antibody units. All other sera contained $\geqq$20 antibody units *vs* 100-320 $TCID_{50}$ homologous virus.

† Accepted by Panel on Picornaviruses, U. S. A.

First passage HEK fluids of both strains induced cytopathic changes characteristic of picornaviruses in WI-26 and KB cell cultures. After 3 blind passages at 7-8 day intervals, each strain also produced CPE in HEp-2 cultures. Repeated attempts to produce CPE in primary rhesus and green monkey cultures were unsuccessful. After several serial passages the behavior of both agents was stabilized fairly well in WI and KB cells. In either WI-26 or WI-38 cultures characteristic foci of irregular ovoid cells appeared first at the outer margins of the cell sheet in from 2-4 days, then spread and coalesced resulting in 75-100% destruction of the cell sheet by 5-7 days. CPE in KB tissue appeared at about 72 hours and an end point was reached by 7-8 days. Titers achieved in each type of culture ranged from $10^{3.2}$ to $10^{4.7}$ $TCID_{50}$ per 1.0 ml. Reproducibility and clarity of end points in WI-26 or WI-38 cultures were definitely superior to those obtained in KB cultures.

Serological characterization. An immune guinea pig serum prepared with strain 363 neutralized 100 $TCID_{50}$ of this agent to a dilution of 1:160. Homologous titer of a strain 1200 serum was 1:320 *vs* 100 $TCID_{50}$. There was no evidence of reciprocal inhibition when these sera were used at a dilution of 1:10. Similarly there was no evidence of neutralization in reciprocal tests with the 15 rhinovirus strains listed (Table I). The candidate strains were also tested against antisera for all other known picornaviruses. Neither agent was neutralized by any of these sera.

To ascertain purity of the candidate strains, they were carried through 3 terminal dilution passages (not more than 2 of 10 cultures positive at the end point dilution of each titration) and new antisera were then prepared in guinea pigs. These sera were found to have similar titers when tested against homologous unpurified and purified virus pools.

A rooster serum prepared against strain 1200 titered 1:320 *vs* 500 $TCID_{50}$ of ho-

TABLE II. Biophysical Properties of Rhinovirus Candidate Strains 363 and 1200.

Test	Conditions of test		Infectivity titer expressed as $\log_{10}$ $TCID_{50}$/ml: Rhinovirus 363	Rhinovirus 1200	Control virus: Known similar properties	Control virus: Known dissimilar properties	
Ether sensitivity	20% Ether	16 hr	5.2	4.4	N.T.	N.T.	
	20% Isotonic NaCl	16 hr	5.7	3.2			
					Echo 28	Echo 22	Echo 1
Acid lability	pH 3.0		<1.7	<1.7	<1.7	3.5	7.2
	pH 7.2		4.5	4.9	5.7	3.8	7.5
					Polio-1	Vaccinia	
Nucleic acid type	IUDR $10^{-4.3}$ M		4.9	3.2	5.9	<1.5	
	No IUDR		4.2	3.7	5.9	4.9	
Thermal stability with $MgCl_2$	$MgCl_2$	R.T.	5.5	4.0	N.T.	N.T.	
		50°C	4.5	3.7			
	Isotonic NaCl	R.T.	4.7	4.0	N.T.	N.T.	
		50°C	1.7	1.0			
					Echo 28	Echo 11	
HBB inhibition	HBB 200 μM		4.2	4.2	5.1	<1.7	
	No HBB		4.2	4.2	4.9	4.2	

R.T. = Room temp. (23-25°C). N.T. = Not tested.

mologous virus. When used at a dilution of 1:15, this serum effectively neutralized several other 1200-like viruses and failed to inhibit several known heterologous rhinoviruses.

Mouse pathogenicity. Suckling mice inoculated by 3 different routes showed no evidence of illness during a 21-day period of observation. Although a few sporadic deaths occurred in mice inoculated by IP or SC route, subsequent mouse passage of a 10% brain and muscle suspension from each harvest produced no deaths. No CPE was noted upon inoculation of these suspensions into WI-26 tissue culture tubes. No evidence was obtained to indicate that virus multiplication occurred without producing recognizable illness in mice. Brain and muscle suspensions from mice sacrificed on specified days after inoculation failed to induce CPE in WI-26 cell cultures.

Size determination. Both viruses passed gradocol membranes of 25 mμ and 20 mμ APD. Virus positive filtrates from these membranes were successfully reidentified in neutralization tests. Estimation of size, using Black's formula(12), would indicate that the agents are from 12-15 mμ.

Ether sensitivity, acid lability, nucleic acid type, thermo-resistance to $MgCl_2$ and inhibition by HBB. Results of these tests are summarized in Table II. No loss of infectivity was found after overnight incubation at 4°C with diethyl ether or upon incubation with 200 μM HBB. Exposure of virus pools to pH 3.0-3.5 for 3 hours at 23-25°C resulted in at least a 1000-fold loss of infectivity. Preservation of infectivity at a temperature of 50°C in the presence of molar magnesium chloride was clearly demonstrable. Lack of inhibition of virus multiplication by IUDR, an inhibitor of DNA synthesis, inferentially supports the conclusion that these viruses possess a ribonucleic acid core.

Plaque formation. Both virus strains produced macroscopic plaques in WI-26 monolayers. Some variation in size and number of plaque forming units resulted from addition of one of two polysaccharides, either sodium dextran sulfate (NaDS) or DEAE-dextran, to the standard agar overlay. Preliminary studies indicated that strain 363 plaque size and number were enhanced by addition of 100γ/ml NaDS to the overlay medium whereas incorporation of 50 γ/ml DEAE-dextran increased the number of plaques produced by strain 1200 although the size remained unchanged. Seven to eight days were required for development of countable plaques and under optimum conditions infectivity titrations

TABLE III. Temporal Pattern of Recovery of Rhinovirus Strains Related to Strains 363 and 1200 from Marine Recruits, Parris Island, S. C. 1959-1960.

	1959								1960		
	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Total
363 isolates	2	0	6	1	0	0	0	0	1	0	10
1200 isolates	1	3	4	2	0	0	1	0	0	0	11
No. men tested	22	22	32	38	31	36	20	25	25	27	276

were comparable to those obtained in tube cultures. Although no difficulty was encountered in obtaining plaques with either virus strain, passage of virus from a single plaque "picked" by capillary pipette and inoculated into WI-26 roller tube cultures was not achieved readily. When CPE was obtained with a strain 1200 plaque, it was not noted until the seventh day after inoculation and progression to a 50% end-point took another 3 days. Reidentification of the virus by serum neutralization test was accomplished. Further study is required before purification of viruses by this method can be considered practical.

Epidemiological observations. Recovery of virus strains related to 363 and 1200. Nineteen strains of rhinoviruses immunologically similar to the candidate viruses were recovered in HEK cultures from throat swabs obtained from marine recruits at Parris Island. These isolations were made from 278 persons sampled between May 5, 1959 and March 31, 1960. Nine of these agents were related to strain 363 and 10 to strain 1200. The temporal distribution of these agents, including the prototype strains, is shown in Table III. The month of peak incidence was July; 19 of 21 isolations were made during the interval May-August. Seventeen of the 21 strains were recovered from persons in the first half of their 12-week training period. Five of ten 363 strains were obtained during a 4-week interval from members of a single training platoon of some 70 men. Although sample size was small, (not more than 12 men) no other platoon yielded more than a single isolate of either type.

Relationship of virus isolation to acute mild upper respiratory illness. Of 11 strains of 1200 virus, 9 were isolated from men suffering from acute upper respiratory illnesses. Fever of 100°F or greater was present at time of sampling in 3 instances. Similarly 8 of 10 recoveries of 363 virus were temporally associated with respiratory illness. Neither virus was recovered from a group of undiagnosed cases of pneumonia studied during the months of Nov. 1959 through Feb. 1960.

Persons sampled only during weeks when homologous virus strains were isolated were used in a test of the statistical relationship between virus isolation and occurrence of respiratory illness. In the case of 363 strains, 8 of 39 ill persons and 2 of 25 control subjects were positive for virus. Nine of 44 ill persons and 2 of 35 control individuals shed detectable virus during weeks when 1200 strains were recovered. Applying the chi-square test with Yates' correction for small numbers, there was no significant correlation between recovery of 363 virus and occurrence of illness. A positive correlation was found for 1200 virus, but this was significant only at the level of $p = 0.05$.

Neutralizing antibodies in human sera. Paired serums from the 21 virus-positive persons were tested for homologous neutralizing antibodies to the candidate viruses. All "acute" sera were obtained on the day of sampling for virus. In one instance a second serum was taken 8 days later; all other "convalescent" sera were obtained 14-22 days following the acute specimen. Six of 10 individuals positive for 363 virus had 4-fold or greater increases in neutralizing antibody titers, and such increases were found in 6 of the 11 cases from which 1200 strains were recovered. "Acute" phase antibodies were found in titer of 1 in 4 in 3 instances of 363 infections; all others were negative. "Convalescent" phase titers were generally low, exceeding 1:32 in 5 cases. There was no apparent relationship between presence, absence or severity of clinical illness and antibody response.

Similar studies were carried out with a group of 60 serum pairs obtained from virus negative men during the months of May-August 1959. Significant increases in neutralizing antibodies were detected in 8 instances, 4 with each virus. Seven of these cases were temporally associated with acute upper respiratory illness.

The incidence of acute phase antibodies at a dilution of 1:4 in these 60 sera was determined. Strain 363 was neutralized by 21 (35%) sera and strain 1200 by 15 (25%). Occurrence of these antibodies appeared to be random with respect to the 2 viruses since the number of sera positive to both agents was 5 and the expected number was 7.

Discussion. The properties described for these 2 viruses fulfill current criteria for their designation as prototype human rhinoviruses. Since neither was found capable of inducing cytopathic changes in primary monkey kidney cell cultures, they may be considered as "H" strains according to the terminology of Tyrrell and associates(13).

Both 363 and 1200 viruses were found to be more "indolent" than 5 other "H" type rhinoviruses previously described(6). They were less readily cultivated in KB cells, and multiplied to lower titer in WI-26 cells than the original serotypes. Production of progressive CPE by strains 363 and 1200 in WI-26 cultures was much more dependent upon incubation of tubes on a rotating drum than was the case for the first 5 agents studied. More difficulty was also experienced in obtaining satisfactory antisera for virus identification of the present serotypes. Other candidate rhinoviruses, even more difficult to handle, are under study in this laboratory. From these observations and others in the literature, it appears that the rhinovirus group is marked by wide variation in conditions necessary for successful *in vitro* cultivation. The original prototype, ECHO 28 virus, was cultivated in primary monkey cell cultures maintained at 36°C(5). Subsequent experience with other M strains indicates that they most readily propagate to good titer at 33-34°C in a near neutral environment. In contrast, English workers have reported a potentially new serotype that could not be grown under these conditions in either primary monkey or human fetal cells. This agent was finally isolated in semicontinuous diploid human embryonic fibroblasts(14).

Although the data are by no means convincing, there was a strong suggestion that 1200 virus was statistically associated with the occurrence of mild "cold-like" illnesses in a population of young males undergoing military training. Several studies have shown that infection by rhinoviruses as a group is related to such disease(15,16,17,18,19). ECHO-28 virus is the only other individual member of the group, however, for which the evidence is at all convincing(20,21).

The serological observations reported suggest that neutralizing antibody may provide protection against infection with 1200 virus. Thus, none of 11 persons shedding detectable virus were found to have pre-existing neutralizing antibodies whereas 25% of persons from whom the virus was not isolated were positive for antibodies. This pattern was qualitatively similar to that found for ECHO-28 and HGP (M strain rhinoviruses) in another military training base(22). In that study incidence of antibodies in the population was 62-65%. The lower incidence of antibodies to 363 and 1200 viruses may reflect differences in past net prevalence of these agents as compared to the 2 M strains. In addition, it may be the result of differences in current ability to measure antibodies in human sera following M and H strain rhinovirus infections, and also the antibody response following infection may be less(23).

In any case the tools necessary for dissection of the natural history of infection by this new group of human viral pathogens are rapidly becoming available. Major progress now depends upon careful longitudinal studies of human populations observed under "normal" conditions as in family units, working groups or school classrooms.

Summary. Properties of 2 new viruses of human origin recovered from the human oropharynx are described. These agents were found to be less than 30 mμ in size and ether resistant. By inference they were found to contain RNA. These properties are consistent with their classification as picornaviruses.

Both agents were unstable in acid pH; thus they were designated as rhinoviruses. The viruses were immunologically distinct from each other and from all previously characterized rhinoviruses. Nineteen other virus strains serologically related to the prototype viruses were recovered from recruits at a military training camp. Statistical analysis suggested that viruses similar to prototype strain 1200 were associated with occurrence of mild "cold-like" illnesses. These studies also indicated that homologous neutralizing antibodies protected against infection by these viruses.

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Ultrafiltration of Simian Viruses.* (29390)

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Most simian viruses (SV) have been isolated from monkey tissues taken from apparently healthy asymptomatic animals. The extensive use of primary monkey kidney cell cultures in research and for the commercial production of vaccines has resulted in recovery of large numbers of viruses from these animals, but classification of the simian viruses is a problem. Hull and coworkers(1-3) grouped these viruses into 8 categories based on cytopathic changes in infected tissue culture and on serological differences. Hsiung and Melnick(4) suggested that studies on host cell infectivity spectra and plaque morphology be used for grouping these viruses, and recently, Malherbe and Harwin(5) grouped viruses obtained from monkeys according to cytopathology as observed in stained cultures. Although there is some overlapping in the grouping of these viruses

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