STUDIES ON THE COMMON COLD

VI. Cultivation of the Virus in Tissue Medium

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The studies of Kruse (1) in 1914 and of Foster (2) in 1916 indicated that the common cold is caused by a filtrable virus. Subsequent investigations by later workers, although confirmatory in part, developed a doubt concerning the validity of Kruse's hypothesis. After a preliminary study of the relationship of the ordinary bacteria of the respiratory tract to common cold we have come to the conclusion that a filtrable virus is in fact the principal causative agent of this disease. This conclusion is based upon the observation that it is possible to produce experimentally typical colds in anthropoid apes and in human volunteers by intranasal inoculations of bacteria-free filtrates of nasopharyngeal washings derived from individuals in the acute stage of a common cold (3). The experimental production of colds in human beings by the methods described has been confirmed by other investigators (4).

In previously published studies we have reported the successful cultivation of the virus of the common cold in tissue medium containing living chick embryo (5). Foster (6) using media containing rabbit kidney, had previously reported a similar result and it has lately been confirmed by Powell and Clowes (7). Since the publication of these early studies our experience has been considerably extended both as to the methods of cultivation of this virus and as to the character of its activity. The details of this experience are set forth in the present paper.

Procedure

1. Method of Obtaining Virus Containing Material from Patients.—The following criteria are used in the selection of a suitable individual, judged to be suffering

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from common cold: the presence of an acute infection of the upper respiratory tract characterized by profuse catarrhal symptoms in an individual not subject to allergic rhinitis or recurrent sinus disease; the infection should run a course typical of the common cold; the absence of fever, great prostration, and marked inflammation of the pharynx and tonsils. The patient selected must have been free from symptoms referable to the upper respiratory tract for a considerable period of time before the onset of the current infection and preferably give a history of exposure to an infection of similar nature. Nasopharyngeal washings from such a patient are collected 18 to 30 hours after the onset of the cold by instilling into the nares in small amounts about 30 cc. of NaCl-free bouillon made from non-toxic casein peptone and containing 0.1 per cent gelatin. The patient also gargles an additional 30 cc. in divided portions. The products of the nasopharyngeal washing and the gargling are combined, shaken thoroughly with glass beads and passed through a Seitz filter at 20 pounds pressure. With a few exceptions noted in the ten protocols below, the filtrate is concentrated by vacuum distillation at 38°C. to approximately one-sixth its original volume. In order to test for the presence of visible, cultivable bacteria, the filtrate is cultured on blood plates which are incubated both aerobically and anaerobically.

2. Technique of in Vitro Cultivation of the Virus.—The medium is prepared under a dust-proof hood; fertile eggs incubated from 9 to 11 days are opened after preliminary cleansing with alcohol, and the embryo removed to a watch-glass, where, after washing with broth, it is minced with a scissors. The minced tissue is pipetted into culture tubes having a diameter of 2 cm., approximately one-half of a 10 day embryo being placed in each tube. 10 cc. of the special peptone broth described are then added, together with enough cysteine hydrochloride[1] to bring the final concentration to 1:2,000. The tubes are then covered with a heavy vaseline seal and kept in the ice box at 4°C. until used.

Studies of the time of survival of embryonic tissue cells under the conditions of the experiment have been made. Explants[2] upon media suitable for the *in vitro* cultivation of living tissue have been prepared. After 9 days storage of the medium at 4°C. 88 per cent of the explants grew actively. The percentage of positive explants after 9 days storage was exactly the same as that observed when the explants were made from fresh chick embryo, indicating a satisfactory survival of the embryonic cells of the medium under the conditions of storage. At incubator temperature of 37°C. the death of the embryonic cells of the medium is more rapid. After 24 hours incubation satisfactory viability of the cells is still observed, but after 48 hours at 37°C. viability can no longer be demonstrated. A culture is initiated by planting 1 cc. of virus containing material through the vaseline seal, and anaerobic conditions are restored by remelting the vaseline seal. Transfers of the culture are made after incubation at 37°C. by pipetting 1 cc. to

[1] Made up in 1 per cent solution, neutralized with sodium hydroxide, sterilized by autoclaving, and kept under vaseline seal.

[2] These explants were prepared through the courtesy of Dr. Thomas M. Rivers.

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a new culture tube. As will be seen from the following protocols, in the early stages of the work transfers were made at intervals of 5 to 9 days. As time has gone on, however, the importance of maintaining the virulence of the culture by more frequent transfer has been made manifest to us, so that cultures are now transferred every 48 hours. Reference to this point will again be made later on.

Elaborate precautions against loss of the culture through contamination must be observed. Our routine procedure is as follows: Embryos are minced one at a time, fresh glassware and instruments (sterilized by dry heat) being used for each. After two tubes have been prepared from an embryo, a little of the embryonic residue is put in a separate smaller tube of culture medium and incubated for 24 hours. Three successive aerobic blood plate cultures are made from this at intervals of 24 hours, and should a contaminant appear the two corresponding tubes of culture medium are discarded. Each strain of virus under cultivation is carried in eight tubes of culture medium. As each tube is opened for transfer a smear is made for microscopic examination, together with an aerobic and an anaerobic blood plate. Should a contaminant appear, the appropriate tube is discarded. These precautions are deemed necessary because of the occasional appearance of a slow growing diphtheroid, an anaerobe which is capable of becoming by adaptation a facultative aerobe. This organism appears to come from the egg, and cannot always be detected amidst the debris of the minced tissue in a stained smear made from a contaminated culture. Staphylococci, streptococci, and *B. subtilis* are infrequent contaminants and are easily detected. Before use for experimental purposes, each culture strain is injected intracerebrally into a rabbit to rule out the presence of herpes virus.[3]

3. Selection of Volunteers for Transmission Experiments.—Males between the ages of 21 and 60 who give no history of sinus infection, rheumatic fever, or tuberculosis are selected. The nose and throat must show no evidence of recent infection and the general physical examination must be negative. At least 6 weeks freedom from symptoms of upper respiratory infection is required. By means of preliminary nose and throat cultures and mouse inoculation of sputum, all carriers of Pneumococcus Types I, II, or III, or hemolytic streptococcus are excluded from the tests.

4. Technique of Isolation.—All experiments are conducted in a private room in Harkness Pavilion. No visitors are permitted except the special nurse in charge of the patients and the physician in charge of the experiment. Such individuals when in the room wear sterile masks, operating gowns, and rubber gloves. The food is cleanly prepared by the nurse in charge, but not sterilized. No attempt is made to sterilize the floor. A minimum of 48 hours preliminary observation is carried out in all experiments. This isolation technique appears adequate since no spontaneous infection has ever occurred.

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[3] It is possible that the anaerobic technique facilitates the inauguration of growth by slight injury to the embryonic cells, thus diminishing their resistance to invasion by the virus.

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5. Inoculation of the Culture to Be Tested.—The volunteer lies on his back, and between 1.0 and 1.5 cc. of the inoculum is permitted to run slowly up each nostril. The volunteer then turns on his face for a period of 1 minute. Two inoculations of this type are made at intervals of about 5 hours. As a rule, each culture to be tested is tried simultaneously on 3 volunteers. No experiments have been performed in order to determine the minimal infective dose of a culture of virus of common cold.

6. Clinical Observation of the Inoculated Individual.—The physician in charge of the experiment observes and questions the subject frequently during the study and a trained nurse notes the objective manifestations of respiratory infection which occur during the day. Her notes are made when the volunteer is off guard and are helpful in evaluating the symptoms of the individual who overstates or understates his complaints. At times it has been found helpful to convince the volunteer that the inoculated material is expected to be inert.

7. Bacteriological Observations.—Daily cultures of the nose and throat are usually made throughout the volunteer's stay in the hospital.

8. Preparation of the Virus Growing in Tissue Culture for Intranasal Inoculation of Volunteers.—The contents of a tube is transferred to a sterile mortar, and the minced embryonic tissue is ground up without sand. The supernatant fluid is used for inoculation after centrifuging 3 minutes at 1,000 R.P.M.

Description of the Experimental Cold

The experimental cold of average severity has an incubation period of about 10 hours. A volunteer who receives his first inoculation at noon usually notes some dryness of the throat and heaviness of the head before going to bed; symptoms of coryza, in individuals successfully inoculated, regularly appear the following morning. This incubation period is remarkably constant except in the case of cultures which have been frequently transferred and are falling off in potency, when it is likely to be prolonged. Symptoms usually increase in severity for 48 hours, and then begin to subside, so that the individual is nearly restored to normal at the end of 4 or 5 days. Recrudescences after discharge from the hospital are not uncommon, however.

As a rule, symptoms conform to the familiar pattern of the common cold—*i.e.* sneezing and congestion of the nasal passages at onset, with a discharge which thickens later so as to cause obstructed breathing. There is usually a productive cough. Constitutional symptoms are not infrequently noted but fever has never been observed after inoculation of virus of common cold. The throat shows increased redness and the solitary lymph follicles are enlarged, but severe sore

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throat is very rarely encountered. Variations of the typical picture occur from time to time; nasal symptoms may be almost entirely absent, and cough the predominant feature, or there may be a moderate degree of malaise. Occasionally secondary sinusitis develops which may prolong the duration of symptoms from the usual 5 days to as much as 2 weeks. When marked cough is present this symptom frequently persists for a number of days after other manifestations have subsided.

Controls

In order to gauge the effect of simple irritation that is caused by letting fluid run up the nose of an individual who thinks he is being given a cold, on 31 occasions we have inoculated volunteers with control material 3 to 5 days before testing them with active virus culture. These control materials consisted of concentrated broth with cysteine added (3), uninoculated tissue culture medium (10), heated culture virus (1), nasal washings taken from normal individuals (2), and cultures of virus inactivated by various means (15). In 23 instances no effect whatsoever was produced. In 8 instances evidence of minor nasal irritation resulted, but such irritation always disappeared after the first day and never progressed to the development of a typical experimental cold.

RESULTS

Before an effort was made to cultivate the virus of common cold in tissue medium, a preliminary set of experiments was performed during October, 1930, designed to determine the period of survival of the virus outside the human body.

Nasopharyngeal washings were obtained from a patient with common cold and filtered in the manner previously described. Cysteine hydrochloride was added to the filtrate to a final dilution of 1:2,000 and the material sealed with vaseline. Strictly anaerobic conditions are always maintained in order to protect the virus from the possible injurious action of any organic peroxides that may be present in the virus containing material. In the first test the filtrates were allowed to stand for 2 weeks at room temperature and, in all, four different filtrates were tested each on a single volunteer. The results are shown in Experiment 1.

As will be seen from Experiment 1, 2 of the volunteers contracted colds as a result of the inoculation and 2 remained without symptoms. The colds in both instances were mild.

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Experiment 1

October, 1930. Unconcentrated Virus Containing Washings Preserved 2 Weeks at Room Temperature

Volunteer No.	Filtrate	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
2	S	±* 3 days	0	Nasal obstruction, productive cough, red throat
3	B	+ 4 "	0	Nasal obstruction, productive cough, red throat
4	C	0	0	
5	D	0	0	

* ± indicates mild symptoms; + denotes a definite cold; ++ indicates a cold of average severity; +++ of more than average severity; ++++ indicates an extremely severe cold.

In the next series of tests the virus containing filtrates were preserved at ice box temperature for from 4 to 13 days. Five filtrates from different individuals were preserved in this manner and were inoculated intranasally into 5 separate volunteers. In all but one instance (filtrate H) the filtrates were concentrated by vacuum distillation at 38°C. Volunteers 6, 8, and 11 were used for the passage of the virus in series; volunteer 6 received filtered nasopharyngeal washings from a spontaneous human cold, volunteer 8 the filtered washings from volunteer 6 at the height of his symptoms, and volunteer 11, those from volunteer 8 taken under similar conditions. The results of these tests are shown in Experiment 2.

Experiment 2

Concentrated Virus Containing Washings Preserved 4 to 13 Days at Ice Box Temperature

Volunteer No.	Filtrate	Duration and intensity of symptoms		Remarks
		Respiratory	Constitutional	
6	H	++ 3 days	0	Nasal obstruction and discharge, productive cough, red throat, headache
8	6	++ 4 "	0	Marked coryza, red throat
9	DP	++ 4 "	0	Coryza, productive cough, red throat, headache
10	I	+++ 4 "	+ 2 days	Coryza, productive cough, red sore throat, headache, anorexia, malaise, sweating
11	8	++ 4 "	+ 1 day	Coryza, cough, anorexia, malaise, sweating, red throat

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The above experiment indicates that the common cold virus when stored at 4°C. retains its activity for at least 14 days, for in every instance of inoculation a cold was produced. Four of these colds were of average severity, and one was of more than average severity. Furthermore, the experiment indicates that cold virus can be passed successfully from one individual to another in series, the virus used for infection of volunteer 11 having been passed serially and produced experimental infections in volunteers 6 and 8.

Strain W, the first successful culture of the virus of common cold in tissue medium, was obtained in November, 1930, from volunteer 11, whose record appears in Experiment 2. The inoculation of volunteer 11 represented the 3rd serial passage in human beings of a strain of virus obtained from a spontaneous human cold. Nasopharyngeal washings were prepared from volunteer 11, 18 hours after the onset of symptoms. The washings were filtered though a Seitz filter concentrated 7 times by vacuum distillation and preserved for 5 days at

Experiment 3

Strain W Transfers Made at Intervals of 5 to 9 Days

Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
12	1st	0	0	
14	1st	± 3 days	0	
16	6th	++ 5 "	0	Coryza
17	6th	0	0	
18	6th	++ 5 days	0	Nasal obstruction, nasal discharge, cough
19	10th	0	0	
20	10th	0	0	
21	10th	0	0	
22	12th	± 3 days	0	Slight coryza
23	12th	0	0	
24	12th	0	0	
27	15th	++++ 9 days	+ 4 days	Nasal obstruction, purulent bloody discharge, productive cough, sinusitis
28	15th	+++ 9 "	+ 3 "	Nasal obstruction, nasal discharge, productive cough
29	15th	0	0	
32	25th	0	0	
33	25th	+ 7 days	0	Nasal obstruction, nasal discharge, productive cough, headache
34	25th	0	0	

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ice box temperature in order to permit the testing of the filtrate for the presence of visible bacteria. After the lapse of this interval 0.25 cc. of the concentrated, bacteria-free material was inoculated into tissue medium of the nature described. The results of the inoculation of human volunteers with the various transfers of this culture are shown in Experiment 3.

In order to test the communicability of the experimental colds produced by culture virus, nasopharyngeal washings were obtained from volunteers 27 and 28 infected with the 15th transfer of strain W. These washings were prepared on the day of infection when the symptoms were well developed and Seitz filtrates were inoculated intranasally into 2 volunteers who were in waiting for the occasion. The results are shown in the following protocol.

Volunteer No.	Passage virus	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
30	From volunteer 27	± 2 days	0	Slight coryza
31	From volunteer 28	+ 3 days	0	Nasal obstruction, slight bloody discharge, productive cough, headache

Experiment 3 indicates that the common cold virus has been propagated outside the human body by cultivation in chick embyo medium for at least 25 successive transfers involving a total period of 107 days. Volunteers were successfully inoculated with transfers 1, 6, 12, 15, and 25.

The experimental colds varied in intensity from a mild symptomatology to one of great severity. The severest colds were produced with the 15th transfer and both showed complications—one sinusitis and the other bronchitis. The reason for the variability in severity of colds developing from inoculation of the different transfers is obscure and may be due either to varying susceptibility to infection of the volunteers or to changes in infectivity of the virus. In all, six cultures of strain W were inoculated into 17 volunteers, of whom 7 manifested symptoms of the common cold and 10 remained free from infection. From volunteers 27 and 28, who received the 15th transfer of strain W, filtered nasopharyngeal washings were obtained and 2 additional volunteers were infected by the intranasal instillation of these filtrates, indicating that an experimental cold induced by culture virus can be passed in series to susceptible human beings.

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In April, 1931, strain M was cultivated in a manner similar to strain W from a patient suffering from a typical common cold. The infectivity of this strain (culture transfer 17) was tested on 3 human volunteers. The results are shown in Experiment 4.

Experiment 4

Strain M Transfers Made at Intervals of 4 to 5 Days

Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
35	17th	0	±	Headache, anorexia, vomiting, red edematous throat, postnasal discharge
36	17th	0	0	
37	17th	++ 5 days	+	Nasal obstruction, nasal discharge, productive cough, headache, malaise, anorexia, vomiting, red edematous throat, postnasal discharge

As a consequence of the above series of inoculations volunteer 37 experienced an acute head cold of average severity associated with a moderate degree of constitutional reaction. Volunteer 35 developed no respiratory symptoms and showed constitutional reaction only consisting of listlessness, malaise, and vomiting. Volunteer 36 remained free from symptoms. The above experiment indicates the successful cultivation of the virus of the common cold in chick embryo medium for 17 transfers, a total period of 76 days.

Cultures of strain J when inoculated into human volunteers produced experimental common colds by the use of transfers 2, 19, and 50. Of a total number of 11 tests, 5 gave positive and 6 negative results. In this culture cold virus was propagated in the tissue medium for at least 50 transfers and for a total period of time of 159 days.

In Experiment 6 an effort was made to determine whether the virus of common cold could be successfully cultivated under aerobic conditions.

The medium used was in every way similar to that used in the anaerobic technique with the exception that no cysteine hydrochloride was added and the cul-

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Experiment 5

Culture strain J was isolated in September, 1931, in a manner similar to that of the two preceding cultures. Transfers were made at intervals of 3 to 5 days.

Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
38	2nd	0	0	
39	2nd	+ 5 days	0	Moderate coryza, headache
40	2nd	0	0	
41	15th	0	0	
42	15th	0	0	
43	15th	0	0	
44	19th	++ 4 days	+ 1 day	Nasal obstruction, nasal discharge, productive cough, red edematous throat, headache, malaise
45	19th	0	0	
46	19th	+ 4 days	0	Moderate coryza, productive cough, red throat
47	50th	± 4 "	0	Slight coryza productive cough
48	50th	+++ 4 "	+ 1 day	Nasal obstruction and discharge, productive cough, red edematous throat, headache, malaise

tures were not sealed with vaseline. Strain J was used for the purpose and anaerobic and aerobic cultures derived from transfer 29 were carried along under exactly similar conditions. The 50th transfer of the anaerobic culture was tested and the results are shown in Experiment 5. The 49th and 50th transfers of the culture, representing the 20th and 21st aerobic transfer, were tested in human volunteers and the results are shown in Experiment 6.

Experiment 6

Strain J. Cultivated under Aerobic Conditions

Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
49	49th + 50th (mixed)	0	0	
50	49th + 50th (mixed)	? 0	0	Very slight coryzal symptoms
51	49th + 50th (mixed)	0	0	
52	50th	0	0	
53	50th	0	0	
54	50th	0	0	

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Strain J used for the inoculation of 6 volunteers in Experiment 6 had been cultivated for 21 transfers under aerobic conditions. None of the volunteers inoculated developed respiratory symptoms of sufficient definiteness to justify the conclusion that an experimental cold had been produced by inoculation of an aerobic culture of strain J. The question mark opposite volunteer 50 indicates that he developed slight symptoms for 2 days following inoculation which were not, however, of sufficient magnitude to designate them as indicating a positive result. Experiment 5 shows that the 50th transfer of strain J cultivated under anaerobic conditions was capable of producing a severe upper respiratory infection in one volunteer and a mild infection in another. The above experiment would seem therefore to show that aerobic conditions of cultivation are not favorable to the maintenance of infectivity by the virus of common cold, the virus either failing to grow under these conditions or else falling off in virulence to such a degree that it is no longer capable of producing experimental infection.

Experiment 7

Strain K. The culture was isolated in October, 1932, from a patient on the 2nd day of a typical cold. Transfers were made at intervals of 3 to 4 days.

Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
71	34th	0	0	
72	34th	++ 5 days	0	Moderate coryza, productive cough

The 34th transfer of strain K when inoculated into 2 human volunteers produced in one of them a typical cold of moderate severity. The virus was active after 124 days of cultivation.

Inoculation of the 19th transfer of strain P produced no experimental infection in any of 3 volunteers. The technique of isolation and cultivation of the virus was the same as that used in previous experiments. Although a single series of inoculations is not sufficient proof of the activity or inactivity of a culture, Experiment 8 must be regarded as illustrating a failure to cultivate the virus of common cold.

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Experiment 8

Strain P. The culture was isolated in November, 1933, from a common cold of uncertain duration and was transferred at 3 to 4 day intervals.

Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
85	19th	0	0	
86	19th	0	0	
87	19th	0	0	

Experiment 9

Strain T. The culture was isolated in February, 1934, from a typical common cold on the first day and was transferred at 2 to 4 day intervals. The period of cultivation extended from April to November, 1934.

Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
91	17th	0	0	
92	17th	++ 4 days	+ 1 day	Coryza, productive cough, headache, anorexia, malaise, red throat
101	81st		0	
102	81st		0	
103	81st		0	

Inoculation of the 17th transfer of culture strain T produced an experimental cold of average severity in one of 2 volunteers. The 81st transfer of this culture was inoculated into 3 volunteers but no experimental infections resulted from the inoculations, indicating that the virus at the end of 81 transfers was either dead or had lost its infectivity. The volunteers used for the inoculation of transfer 81 of strain T were shown 72 hours later to be susceptible to an experimental cold by the inoculation of a recently isolated strain R of cold virus following which all 3 developed typical colds. Our experience leads us to believe that the cultivated virus of common cold loses its infectivity for human beings after a certain period of cultivation *in vitro* but that in all probability the virus continues to grow outside the body for an indefinite length of time.

The two cultures of cold virus described in the following experiments have been transferred at 48 hour intervals. As has been

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previously stated chick embryo tissue kept under strictly anaerobic conditions at incubator temperature is probably dead after the lapse of about 36 hours. This is indicated by the fact that after this length of time no positive explants on medium suitable for the cultivation of tissue can be obtained. We have believed that dead degenerating tissue cells would exert an injurious influence on the growing virus and that if it were protected as far as possible against such effect the virulence might be maintained for longer periods of time. That this assumption seems to be true is shown in the following experiments.

Experiment 10

Strain Ro. The culture was isolated in October, 1934, from a typical cold on the 2nd day of symptoms. The transfers were made at intervals of 48 and 72 hours. Total duration of cultivation 343 days.

Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
101	13th	+ 4 days	0	Moderate coryza, headache
102	13th	+++ 7 "	+ 3 days	Severe coryza, productive cough, headache, anorexia, malaise
103	13th	+++ 7 "	+ 3 "	Nasal discharge, productive cough, malaise, sore throat
107	75th	++ 7 "	+ 5 "	Marked coryza, cough, sore red throat, headache, anorexia, malaise
108	75th	+++ 6 "	0	Marked coryza, slight cough, sore red throat, headache
109	75th	0	0	
110	88th	+++ 7 days	0	Marked coryza, moderate cough, sore red throat, headache
111	88th	++ 6 "	0	Nasal obstruction and discharge, productive cough
112	88th	+++ 7 "	+ 6 days	Marked coryza, productive cough, sore red throat, severe headache
113	135th	0	0	
114	135th	±	0	Sore throat, headache
115	144th	±	0	Nasal obstruction, slight sore throat, cough
116	144th	±	0	Slight coryza, cough

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Study of the above table indicates that when a culture of common cold virus is transferred at 48 and 72 hour intervals the virulence and infectivity seem to be well maintained for a considerable period of time and a high proportion of relatively severe infections result from the inoculation of human volunteers. Strain Ro maintained a high degree of virulence for 88 transfers, a total period of 219 days.

Experiment 11

Strain Wh. This culture was isolated in September, 1935, from a typical common cold on the first day of symptoms. Transfers were made at 48 and 72 hour intervals. The period of cultivation has been during October, 1935.

Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
117	14th	0	+ 2 days	Sore throat, headache, malaise
118	14th	+++ 5 days	+ 2 "	Severe coryza, productive cough, red sore throat, anorexia, malaise
119	14th	+++ 5 "	+ 2 "	Nasal obstruction and discharge, productive cough, sore red edematous throat, headache, anorexia, malaise

The 14th transfer of culture strain Wh produced severe colds in 2 inoculated individuals and moderate constitutional reaction without respiratory manifestations in a 3rd.

In order to illustrate the apparently greater virulence and infectivity of the cultivated virus of common cold when transfers are made in chick embryo medium at intervals of 48 and 72 hours instead of the longer intervals of 3 to 5 to 9 days, Table I is presented. The comparison is made between cultures that have received 100 transfers or less.

Although the total number of inoculations of volunteers with the cultures transferred at 48 and 72 hour intervals is small, the increased percentage of colds produced and the increase in severity and duration of symptoms is obvious. All cultures of cold virus are now transferred routinely at 2 and 3 day intervals, 2 transfers at 48 hours and 1 at 72 hours each week.

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For purposes of illustration the protocol of a typical positive infection with culture virus of common cold is inserted at this point.

Protocol 1.—Volunteer 118. Nov. 7, 1935. This is volunteer 118's sixth admission to the isolation quarters. His previous record appears under Nos. 82, 103, 105, 110, and 113 in this and a later paper on the cultivation of a filtrable virus from examples of human influenza. He ordinarily experiences from two to three colds a year. He has had no recent colds and in September, 1935, experienced no infection following inoculation with strain Ro 135 dried with gum acacia. On examination he showed no signs of recent infection of the respiratory tract. He was first inoculated with strain Ce 126, a tissue medium culture from a patient with influenza. This inoculation proved negative. He was later inocu-

TABLE I

Strains W, M, J, K, and T. Transfers at 3 to 9 day intervals		
		per cent
Total No. of inoculations	38	
No. of negative results	23	60
Infections indicated as ± to ++	12	32
Infections indicated as +++ or more	3	8
Total No. of positive infections	15	40
Strains Ro and Wh. Transfers at 48 and 72 hr. intervals		
Total No. of inoculations	12	
No. of negative results	2	17
Infections indicated as ± to ++	3	25
Infections indicated as +++ or more	7	58
Total No. of positive infections	10	83

lated with strain Wh 14, a freshly isolated culture from a patient with common cold. The culture had been transferred at 2 day intervals.

Nov. 7, 1935. Admitted to isolation quarters. Nov. 8. Complains of no symptoms. Nov. 9. Complains of no symptoms. At 12 noon received 1.5 cc. Ce 126 in each naris. 5 p.m. received 1.5 cc. Ce 126 in each naris. Nov. 10–14. No signs or symptoms whatever have resulted from this inoculation. Nov. 14. Throat appears as on admission. 12 noon received 1.5 cc. Wh 14 in each naris, 5 p.m. received 1.5 cc. Wh 14 in each naris. Nov. 15. His first symptom, cough, began at 9:30 p.m. on the evening of inoculation. This morning he has nasal obstruction with a watery coryza. Symptoms became increasingly severe during the day. Nov. 16. He has nasal obstruction and a mucoid discharge from the nose. A severe productive cough is present. In the afternoon some malaise and anorexia appeared. He was given an ephedrin spray and a mixture contain-

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TABLE II

Date, *1935*	Strain Ce 126						Strain Wh 14									
	Nov. 7	8	Inoculation Nov. 9	10	11	12	13	Inoculation Nov. 14	15		16		17		18	19
									a.m.	*p.m.*	*a.m.*	*p.m.*	*a.m.*	*p.m.*		
Nasal obstruction	0	0	0	0	0	0	0	0	++	+	++	+	++	++	++	+
Sneezing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Coryza	0	0	0	0	0	0	0	0	++	+	0	0	0	0	0	0
Nasal discharge	0	0	0	0	0	0	0	0	++	++	++	++	++	++	+	+
Cough	0	0	0	0	0	0	0	0	++	+++	+++	+++	+++		++	+
Sputum	0	0	0	0	0	0	0	0	+	+	++	++	++	++	++	+
Sore throat	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+++	+
Headache	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Anorexia	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	0
Malaise	0	0	0	0	0	0	0	0	0	0	±	+	+	+	±	0
Fever	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Throat	0	0	0	0	0	0	0	0	Slight edema		Increased redness		Marked injection		Marked injection	
Postnasal discharge	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tonsils	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

± = mild; + = moderate; ++ = marked; +++ = severe.

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ing codein and aspirin. Nov. 17. His symptoms remain the same as on previous day with a severe productive cough still present. Nov. 18. The symptoms are abating somewhat but he now complains of marked sore throat. Nov. 19. Feels considerably better, appetite returning. Productive cough and nasal discharge still marked, however. Discharged from isolation with rather pronounced residual manifestations. Table II shows the daily record of symptoms.

The experiments detailed in Table I indicate beyond any question that the virus of common cold can be isolated from human beings suffering from acute colds and grown *in vitro* in tissue medium containing minced 10 day old chick embryo. Cultures prepared in the manner described retain their infectivity for long periods of time and under the most favorable conditions for as many as 88 transfers in tissue medium. The dilution of the original nasopharyngeal washings serving as the source of the culture is by the 10th transfer *in vitro* already so great that any possibility of its continued presence being responsible for the experimental infection of volunteers is entirely ruled out. Intranasal inoculation of culture medium and other types of control material, although it may produce symptoms of slight irritation for short periods of time, has never caused in any individual inoculated the typical picture even of a mild common cold. The colds produced in individuals inoculated with active culture vary in severity and duration of symptoms, which in some are entirely referable to the respiratory tract and in others are combined with varying degrees of constitutional reaction. Fever has never been produced by inoculation of common cold virus and there is no significant change in the leucocyte count. Complications such as sinusitis and tracheobronchitis are occasionally observed. Blood agar plates have generally been prepared daily for the study of the nasopharyngeal flora of inoculated volunteers. No important changes in the types or numbers of the common organisms of the upper respiratory tract have been observed and the presence in the nasopharynx of such pathogenic types as pneumococcus does not seem to influence the result of inoculation of virus of the common cold.

One of the objectives of the present study has been to develop methods for the practical use of the culture virus of common cold for purposes of active immunization of chimpanzees and human beings against spontaneous colds. In view of the fact that for such an effort to be successful, living active virus would have to be used for inocula-

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tions, a number of experiments have been performed which have been designed to test the viability of cold virus under different conditions of storage. In the prophylactic inoculation of human beings a minimal period of 5 days' storage is essential for the carrying out of the necessary tests of sterility of the material to be inoculated. The effect of freezing and drying was first explored.

The 18th transfer of culture strain Ro, a virus proved subsequently to be infective for human beings in the 88th transfer, was rapidly frozen, desiccated, and sealed *in vacuo*. This preparation was stored for 10 weeks at ice box temperature and then tested for activity on 3 human volunteers. Since this preparation proved inactive the same volunteers were inoculated a few days later with the original nasopharyngeal washings from which strain Ro had been cultivated and which had been dried and preserved in a similar manner for a period of 17 weeks. The results are shown in Experiment 12.

Experiment 12

Volunteer No.	Material inoculated	Intensity and duration of symptoms	Remarks
104	Culture Ro 18 dried	0	
	Original Ro virus dried	+++ 5 days	Nasal obstruction, nasal discharge, productive cough, sore throat, headache, malaise
105	Culture Ro 18 dried	0	
	Original Ro virus dried	++ 5 days	Nasal obstruction, nasal discharge, productive cough, sore throat, headache
106	Culture Ro 18 dried	0	
	Original Ro virus dried	0	

This experiment indicates that culture virus of common cold, frozen and dried under the conditions described, loses its infective power after 10 weeks storage. However, the original virus of the nasopharyngeal washings retained its activity under similar conditions for 17 weeks. It would seem, therefore, that chick embryo medium contains substances destructive to the virus, possibly disintegration products of dead tissue cells of the nature of soaps of the unsaturated fatty acid series. On the other hand, the virus of the original nasopharyngeal washings survives dried for long periods of time. It is possible that the presence of mucus in these washings exerts a protective action.

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In the next experiment the survival of the virus under the technique of preservation employed for human vaccination was tested.

Active culture was ground up, centrifuged to remove large clumps of material, and the supernatant fluid was kept under vaseline seal at ice box temperature for 6 days. At the expiration of this time the material was inoculated into human volunteers. The results are shown in the following experiment.

Experiment 13

Volunteer No.	Material inoculated	Intensity and duration of symptoms	Remarks
107	Ro 71 preserved 6 days	0	
	Ro 75 fresh	++ 7 days	Marked coryza, cough, sore red throat, marked anorexia, malaise
108	Ro 71 preserved 6 days	0	
	Ro 75 fresh	+++ 6 days	Marked coryza, slight cough, sore red throat, headache
109	Ro 71 preserved 6 days	0	
	Ro 75 fresh	0	

The above experiment shows that storage of culture virus of common cold in the culture fluid for 6 days at ice box temperature results in complete loss of infectivity. Another transfer, No. 75, of the same strain was proven 4 days later to be active in the same volunteers when used in the fresh state. From these two experiments it is apparent that contact, even for a short period of time, with disintegrated chick embryo medium, either in the wet or dry state, completely inactivates the culture virus of common cold. For a number of practical reasons previously mentioned it has become important to be able to store the culture virus of common cold in an active state. The observation of Rivers and Ward (8) that the presence of small amounts of gum acacia exerts a protective action on the culture virus of vaccinia suggested the use of this substance for the above purpose.

To test the effectiveness of gum acacia the 88th transfer of strain Ro of cold virus was mixed with 3 per cent gum acacia, frozen and dried *in vacuo*, and stored at ice box temperature for 6 days. As a control the 88th transfer of strain Ro was preserved in the same way without the admixture of gum acacia. 3 volunteers were then inoculated, first with the virus dried without gum acacia and a few days later the same volunteers were inoculated with virus dried with gum acacia. The results are shown in the following experiment.

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Experiment 14

Volunteer No.	Material inoculated	Intensity and duration of symptoms	Remarks
110	Ro 87 without acacia	0	
	Ro 88 with acacia	+++ 7 days	Marked coryza, productive cough, sore red throat, headache
111	Ro 87 without acacia	0	
	Ro 88 with acacia	++ 6 days	Nasal obstruction and discharge, productive cough, sore red throat, headache
112	Ro 87 without acacia	0	
	Ro 88 with acacia	+++ 7 days	

This experiment shows very clearly that complete inactivation of strain Ro 87 had taken place in 5 days although the culture had been preserved in the dry state *in vacuo*; on the other hand Ro 88 similarly prepared and preserved except for the addition of 3 per cent gum acacia was highly active in the same volunteers 4 days after inoculation with Ro 87 without acacia. This result indicates that gum acacia exerts a protective action against constituents of tissue medium injurious to the cultivated virus.

From time to time during the course of this study attempts have been made to detect visible evidence of growth of the virus of common cold. None, however, has been obtained, no inclusion bodies are formed, no visible or chemical change in the media occurs which would indicate growth, nothing significant can be seen with the dark field microscope; nor do colonies form either aerobically or anaerobically on solid media.

SUMMARY AND CONCLUSIONS

1. Studies of the cultivation of the virus of common cold in tissue medium, and the capacity of the culture virus to induce infection in human volunteers are reported.

2. Detailed descriptions are given of the methods employed to isolate the virus, preserve and cultivate it, and to test its activity in human volunteers.

3. The virus of common cold can easily be isolated from properly selected patients and cultivated in tissue medium.

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4. When kept in the original nasopharyngeal washings, the virus will survive at ice box temperature under anaerobic conditions for at least 13 days.

5. If the nasopharyngeal washings are frozen and dried *in vacuo*, the virus retains its activity for at least 4 months.

6. The virus of common cold has been proven to multiply in medium containing chick embryo tissue. Such cultures retain their capacity to produce typical infections in human beings for many transfers involving a period of several months. Attempts to cultivate the virus have been successful in seven out of eight instances.

7. Prolonged cultivation of the virus in tissue medium eventually leads to a loss of activity.

8. Strains of virus under cultivation maintain their potency best when transfers are made at 2 and 3 day intervals.

9. After removal from the incubator a culture of virus rapidly becomes inactive whether it be kept under seal in the ice box or frozen and dried *in vacuo*.

10. The destructive action of the medium can be prevented if the culture is mixed with gum acacia before freezing and drying *in vacuo*.

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