RECOVERY IN TRACHEAL ORGAN CULTURES OF NOVEL VIRUSES FROM PATIENTS WITH RESPIRATORY DISEASE

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Despite recent advances in tissue culture methodology, only 20 to 35 per cent of adults with acute upper respiratory tract illness yield viruses which can be cultivated by the standard virus recovery techniques.[3, 4, 12] Recently, an increase in virus isolation from patients with common colds was achieved by Tyrrell and Bynoe through the use of human embryonic tracheal and nasal organ cultures.[19, 20] Viruses were detected in harvests from organ cultures either by the induction of hemadsorption or cytopathic effect (CPE) in subinoculated tissue culture or by the production of colds in volunteers. One virus, strain B814, which was recovered by these techniques and produced colds in volunteers, was of particular interest since it was ether-labile and grew only in human ciliated epithelium. Subsequently, Tyrrell found that this virus resembled in morphology the avian infectious bronchitis virus (IBV), a medium-sized virus which appears to be distinct from the myxoviruses.[2, 18] Another newly recognized, medium-sized, ether-labile virus, strain 229E, was recovered from students with colds by Hamre and Procknow using standard tissue culture techniques.[5]

During a survey of acute upper respiratory illness which was carried out in our laboratory, and in which the standard tissue culture techniques were used, we noted a sharp decrease in the rate of virus isolation during the winter of 1965–66, similar to that reported by others.[3, 4, 12] Stimulated by the findings of Tyrrell and Bynoe, we used the organ culture technique to study specimens, from this and a previous survey,[12] which failed to yield agents in standard tissue cultures. From 23 such specimens 8 agents were recovered, including 6 with an unusual morphology which resembled that of IBV. The recovery and properties of these 6 viruses form the basis of this report.

Materials and Methods.—Collection of specimens and virus isolation attempts in tissue culture: Specimens were obtained from employees of the National Institutes of Health with acute upper respiratory illness on or before the fourth day of illness.[12] Two-tenths ml of freshly collected nasopharyngeal wash fluid was inoculated into each of two tubes of the following tissue cultures: HEp-2, primary human embryonic kidney, rhesus monkey kidney, and human diploid cell strains (HDCS) WI-26, WI-38, and AT-39.[13] The remaining fluid was stored at −60°C. Tissue cultures were obtained from commercial sources, maintained as previously described,[11] and were incubated at 33°C on roller drums. The tubes were observed for cytopathic effect twice weekly. Monkey kidney cultures were tested for hemadsorption at 5- to 7-day intervals, and a single blind passage of most human embryonic kidney cultures was made at 21 days. Only those nasopharyngeal washings in which virus could not be detected by these methods were examined by organ culture techniques. Acute phase sera were drawn at the time the washings were collected and convalescent phase sera approximately 3 weeks thereafter.

Organ cultures: Human embryonic tracheal organ cultures were prepared and maintained by a modification of the method of Hoorn and Tyrrell.[6, 7] Tracheas were obtained from fetuses spontaneously aborted at 5–9 months' gestational age. The tissue was excised *en bloc* by sterile technique and immediately stored in cold Hanks' balanced salt solution with 10% fetal calf serum,

250 u/ml penicillin, and 250 μg/ml streptomycin. Within 2 to 48 hr the tracheas were trimmed, washed, and cut into fragments 2–3 mm square. A set of four fragments was placed mucosal side up in a plastic Petri dish previously scratched lightly with the point of a scalpel. The fragments were partially covered with 1.25 ml of Leibovitz medium,[9] supplemented with 0.2% bovine albumin, 0.1 mM glutamine, 250 u/ml penicillin, and 250 μg/ml streptomycin. The pH of the medium was adjusted to 7.4 with a few drops of 1 N NaOH. The beating of cilia was observed through a dissecting microscope by incident illumination. Only those fragments initially showing strong ciliary beating were used.

After inoculation organ culture plates were incubated at 33°C on a rocker platform moving at one full cycle every 2–3 min. Daily or every other day, medium was removed from the plates and stored at −60°C, and the cultures were refed. Control organ cultures retained full ciliary activity for a variable period up to 3 weeks, the longest interval tested. To perform subpassages, stored harvests from the second through the tenth day of incubation were thawed and combined, and 0.2–0.5 ml of this pool was inoculated into fresh organ culture plates.

Viruses: Dr. Dorothy Hamre kindly supplied strain 229E,[5] which had been purified by the terminal dilution technique in HDCS (WI-38). Chick-embryo-grown avian infectious bronchitis virus, Beaudette strain (66579), was kindly supplied by Dr. Harold DeVolt.

Electron microscopy: Pooled fluids from inoculated or control organ cultures were clarified by low-speed centrifugation at 2000 rpm for 10 min in the PR-2 International centrifuge, and centrifuged onto a cushion of 60% sucrose at 111,000 × *g* for 90 min in the SW-39 rotor of a Spinco model L ultracentrifuge. A drop of material from the sucrose-medium interface was prepared by the pseudoreplication technique of Sharp[16] as modified by Smith and Melnick,[17] negatively stained with 2% phosphotungstic acid (PTA) at pH 5.0 or 7.0 and mounted on copper grids.

Suspensions of virus 229E grown in HDCS (WI-38) were clarified as above and concentrated by centrifugation at 18,500 rpm (54,000 × *g*) in the SW-39 rotor for 90 min. The pellet was resuspended in a small volume of 1% ammonium acetate, stained with PTA at pH 5.0, and spread on formvar coated grids.

Allantoic fluid suspensions of IBV were clarified by centrifugation as above and then dialyzed against distilled water at 4°C for 5 hr. A drop of the unconcentrated sample was then negatively stained with PTA at pH 5.0 or 7.0 and spread on a coated grid.

All electron micrographs were taken with a Siemens Elmiskop 1A at magnifications of 40,000–80,000×.

Ether sensitivity: Undiluted virus suspension was mixed with one-fourth volume of diethyl ether in a vial, shaken well several times, and allowed to stand at 4°C for 18 hr. Ether was allowed to evaporate, and 0.4 ml of the treated sample was inoculated into each of two organ culture plates. An equal volume of virus suspension to which ether had not been added was treated in the same fashion and inoculated into two organ culture plates. Two uninoculated plates served as additional controls. All cultures were washed after 24 hr. Every other day the medium was harvested and replaced and the cultures were observed for ciliary action. These harvests were then pooled and prepared as described above for electron microscopy. Under code the grids were examined for the presence of morphologically characteristic particles.

Serologic tests: A neutralization test in organ cultures was performed as follows. Equal volumes of inactivated (56°C for 30 min) acute or convalescent serum, diluted in Leibovitz medium supplemented as above, and undiluted virus were mixed and incubated for 2 hr at room temperature. Four-tenths ml of this mixture was inoculated onto each of two organ culture plates which were incubated at 33°. At 24 hr the cultures were washed, and daily thereafter the medium was harvested and prepared for electron microscopy. These fluids were centrifuged at 100,000 × *g* for 1 hr. The pellets were resuspended to 1/10 the original volume in ammonium acetate and stained with PTA.

Neutralizing antibody for virus strain 229E was assayed by the plaque reduction technique using HDCS (WI-38) Petri dish cultures. Complement fixation (CF) tests were done by the microtiter technique using overnight fixation at 4°C and 1.7 to 1.8 units of complement as previously described.[11, 15]

Results.—Recovery of agents from organ culture: Table 1 presents a summary of our experience with common cold patients whose nasopharyngeal washings were

TABLE 1

RECOVERY IN TRACHEAL ORGAN CULTURES OF AGENTS FROM PATIENTS WITH UPPER
RESPIRATORY TRACT ILLNESS

	Specimens Tested in Tissue Culture		Tissue Culture Negative Specimens Studied in Tracheal Organ Culture		
Months covered by survey	No. tested	No. yielding virus	No. tested	No. of Agents Isolated "IBV-like"	Ether-stable
Sept.–Nov. '63	39	9	2	0	0
Feb.–March '64	24	3	2	1	0
Sept.–Nov. '65	50	21	10	0	2
Dec. '65–Feb. '66	60	4	9	5	0

studied in organ culture. Two methods were used to detect the presence of virus in these specimens. First, inoculated organ cultures were observed for an effect on ciliary activity. Second, harvests of third or fourth passage culture medium were examined by electron microscopy for virus particles.

By these means 8 agents were detected in tracheal organ cultures inoculated with specimens from 23 patients. The presence of two of these could be detected only by their ciliary immobilizing effect (CIE); virus particles were not visualized in organ culture harvests, and both were ether-resistant. Possibly these agents are fastidious rhinoviruses which grow only in organ culture.[8] Six other agents were detected by electron microscopy of organ culture harvests. These viruses possessed a similar and characteristic morphology; because of their resemblance to IBV,[2] we have tentatively designated them "IBV-like" viruses. Four of these agents produced CIE.

Characteristics of "IBV-like" viruses in organ and tissue culture: (i) *Ciliary immobilizing effect:* Table 2 summarizes the behavior of the six agents in organ culture. Except for isolate 664, CIE was never observed before the third passage, but once detected was usually seen during later passages of the virus. Specimen 664 produced CIE on the second passage, but this effect was not observed again during repetition of the second passage or during two subsequent passages.

Following inoculation of cultures, the time at which CIE was first observed (hereafter referred to as the CIE interval) was never less than four days and never more than ten days. The usual CIE interval was eight days, and did not vary by more than two days among different dishes derived from the same embryo. However, when a virus suspension was inoculated onto cultures derived from different embryos, a difference in CIE interval of as much as four days was observed. Because of this variation each comparative test was carried out with tissue derived from a single embryo.

TABLE 2

DEVELOPMENT OF CILIARY IMMOBILIZING EFFECT (CIE) AND "IBV-LIKE" PARTICLES
IN HUMAN EMBRYONIC TRACHEAL ORGAN CULTURE

Specimen no.	Date collected	No. of passages performed	Passage at which CIE first observed	Electron Microscopy Passages examined for particles	Passages at which particles seen
501	Mar. '64	10	4	1, 2, 3, 5, 6, 7, 8	3, 5, 6, 7, 8
663	Dec. '65	7	3	4, 5, 7	4, 5, 7
703	Feb. '66	7	3	3, 5	3, 5
690	Jan. '66	5	No CIE*	3, 5	3, 5
691	Jan. '66	4	No CIE	3	3
664	Dec. '65	4	2†	3, 4	3, 4

* No CIE observed during any of the passages made.
† CIE not observed during repetition of the second passage, nor during subsequent third or fourth passages.

(ii) *Detection of "IBV-like" viruses by electron microscopy:* As is shown in Table 2, characteristic particles were not seen in the first and second passages of specimen 501 but appeared in moderate numbers in the third. Once observed during the third or fourth passage, particles were consistently seen during later passages of each isolate so tested. Control harvests from uninoculated organ cultures were frequently examined, and in no instance were "IBV-like" particles seen.

(iii) *Ether sensitivity:* The results of ether sensitivity tests of five of the agents are shown in Table 3. Of the five agents tested, four were clearly inactivated by ether. Isolate 663 still produced CIE after ether treatment although this effect occurred four days later than that observed with untreated virus; characteristic particles were not found in harvests from cultures inoculated with treated virus. These findings suggest either that ether reduced somewhat the titer of isolate 663, or that two agents were present in the specimen. The latter possibility is currently under study.

TABLE 3

EFFECT OF ETHER ON INFECTIVITY OF "IBV-LIKE" VIRUSES

Isolate no.	No. days observed	Day CIE First Seen in Organ Cultures Inoculated with		Particles Detected by Electron Microscopy of Harvests of Organ Cultures Inoculated with	
		Ether-treated virus	Untreated virus	Ether-treated virus	Untreated virus
501	12	CIE not seen	6	None seen	+
663	14	10	6	None seen	+
703	10	CIE not seen	8	None seen	+
664	12	CIE not seen	CIE not seen	None seen	+
690	12	CIE not seen	CIE not seen	None seen	+

Note: Uninoculated organ cultures maintained ciliary action throughout period of test.

Attempts to grow "IBV-like" viruses in tissue cultures, eggs, and ferret organ culture: Attempts to grow these viruses in tissue culture were unsuccessful. These tests included (*a*) observation for CPE and hemadsorption in numerous cell lines and strains of human and nonhuman origin, (*b*) attempts to visualize immunofluorescent antigen in HDCS cells, (*c*) search for evidence of interference with ECHO 11 virus, (*d*) electron microscopic examination of tissue culture harvests, and (*e*) back passage of tissue culture harvests into tracheal organ culture with examination for CIE and virus particles. Replication of "IBV-like" viruses was likewise not detected in embryonated eggs or in ferret tracheal organ culture.

Reisolation of "IBV-like" viruses: Three attempts were made to reisolate "IBV-like" agents from the original nasopharyngeal washings, and all were successful. In each of the three reisolation series "IBV-like" particles were seen by electron microscopy in third passage harvests. With each reisolation attempt a parallel passage series was initiated using 0.2 ml of medium as inoculum instead of nasopharyngeal washing. This control series and the reisolation series itself were carried out by identical techniques in organ cultures derived from the same embryos. Concentrated harvests from infected and control cultures were coded and examined "blind." In no instance were "IBV-like" agents seen in control material.

Electron microscopic appearance: Figures 1 and 2 show the appearance of an "IBV-like" agent, isolate 501, after six passages in organ culture. Comparison with 229E (Fig. 3) and IBV (Fig. 4) shows their remarkable similarity. An electron micrograph of influenza A2 is also shown for comparison (Fig. 5).
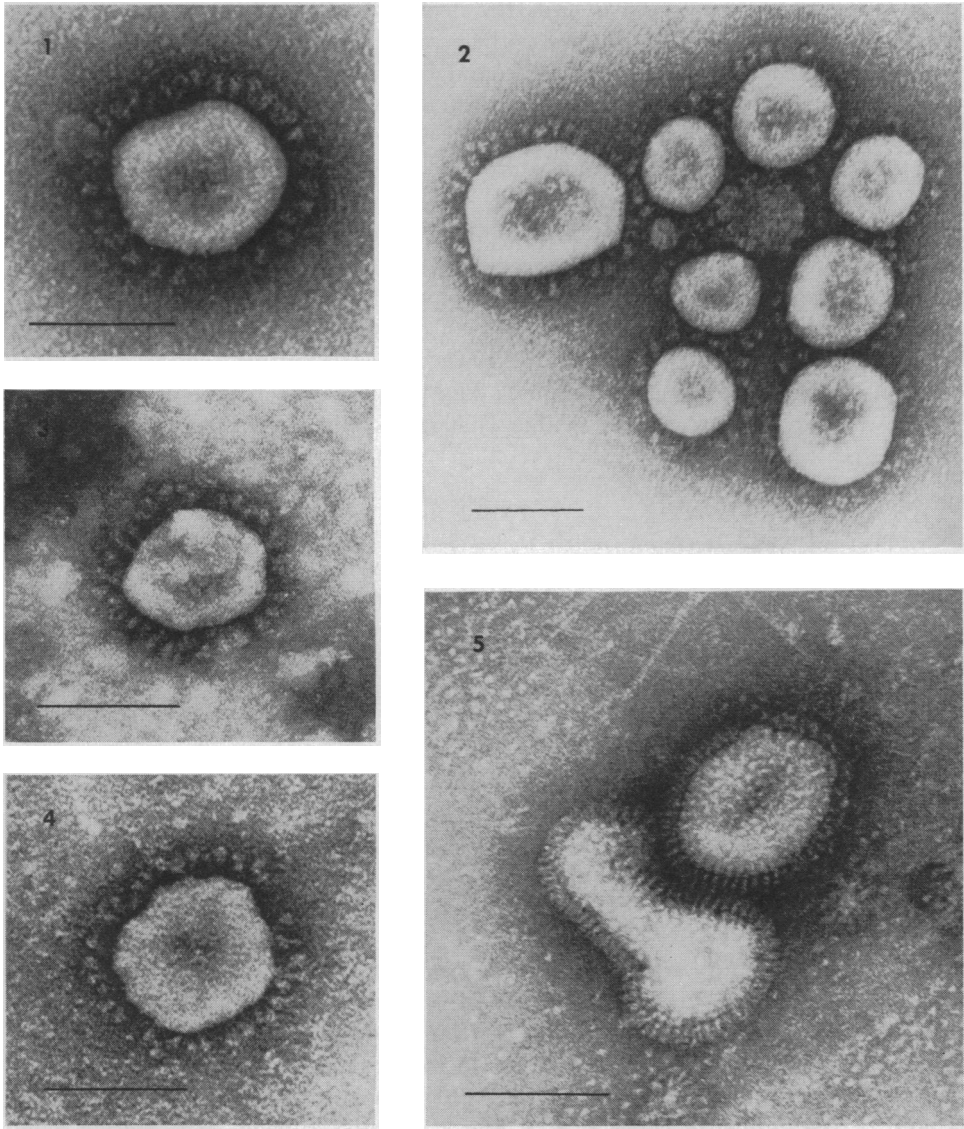
FIG. 1.—Isolate 501. Tracheal organ-culture-propagated "IBV-like" virus particle, negatively stained with phosphotungstic acid (PTA). The characteristic "club-shaped," widely spaced surface projections are evident. Magnification 192,000×. The marker in each figure represents 100 mμ.

FIG. 2.—Isolate 501. Group of "IBV-like" virus particles, negatively stained with PTA. Some variation in size is seen, but shape is relatively uniform. Magnification 144,000×.

FIG. 3.—Strain 229E. Single virus particle, negatively stained with PTA. Magnification 192,-000×.

FIG. 4.—Avian infectious bronchitis virus (IBV), Beaudette strain. Single virus particle negatively stained with PTA. Magnification 192,000×.

FIG. 5.—Influenza A2. Two virus particles negatively stained with PTA. The closely spaced, "rod-like" surface projections contrast with those of IBV, strain 229E, and the "IBV-like" viruses in Figs. 1 and 2. Magnification 192,000×.

All "IBV-like" viruses, 229E, and IBV itself show the following characteristics: (1) an over-all diameter of 160 mμ with a variation of ± 40 mμ; (2) a moderate pleomorphism with resultant elliptical, round, or tear-drop shapes but no filamentous or "tailed" forms; (3) characteristic spikes 20 mμ long, usually club- or pear-shaped, narrow at the base and 10 mμ wide at the outer edge, spaced widely apart and distributed fairly uniformly about the circumference of the particle.

Neutralization tests in organ cultures: Two patients who yielded "IBV-like" viruses were tested for a neutralizing antibody response to the agent recovered. Undiluted virus suspension, which regularly induced the development of virus particles detectable by electron microscopy, was used as inoculum in these tests. As shown in Table 4, in both instances a 1:4 dilution of convalescent phase serum inhibited the development of virus particles in organ culture, whereas the same dilution of acute phase serum did not. The temporal effect of acute and convalescent sera upon virus growth (Table 4) suggests that neutralizing antibody was measured.

TABLE 4

TEST FOR NEUTRALIZING ANTIBODY USING GROWTH OF VIRUS IN TRACHEAL ORGAN CULTURES AS INDICATOR SYSTEM

Isolate	Serum of Person Yielding Isolate* Phase	Dilution	Detection of Particles by Electron Microscopy on Indicated Day after Inoculation						
			2	3	4	5	6	7	8
501	Acute	1:4	−	+	+	+			
		1:16	+	+	+				
	Convalescent	1:4	−	−	−	−	−	−	−
		1:16	−	+	+	+			
703	Acute	1:4	−	−	−	+	+		
		1:16	−	+	+	+			
	Convalescent	1:4	−	−	−	−	−	−	−
		1:16	−	−	+	+			

* Sera were inactivated at 56° for 30 min. Mixtures of serum and undiluted virus were incubated for 2 hr at room temperature and inoculated onto duplicate plates. All plates were washed 24 hr after inoculation. Cultures were harvested and refed daily. In other experiments involving organ cultures derived from a single embryo, virus reached a detectable level at the same time in each of a series of 6–8 replicate plates.

Tests for relationship with other agents: An attempt was made to determine the relationship between the "IBV-like" viruses and certain other agents. Acute and convalescent sera from the patients from whom the "IBV-like" agents were recovered were tested for a neutralizing antibody response to strain 229E by the plaque-reduction technique and for a CF antibody response to influenza types A, B, and C, parainfluenza types 1, 2, 3, and 4, mumps, rubella, *Mycoplasma pneumoniae*, adenovirus, and 229E. None of the six patients developed a significant antibody response to strain 229E as measured by either the plaque-reduction or the CF test. Similarly, these individuals failed to develop a rise in CF antibodies for the various other antigens described above.

Repeated attempts to cultivate mycoplasmas from suspensions of "IBV-like" viruses were negative. In addition, aureomycin did not inhibit their growth.

Discussion.—Evidence from this study for the human origin of the "IBV-like" agents is provided by the successful reisolation of viruses with characteristic morphology in each of the three attempts made. This evidence is strengthened by our failure to detect such virus particles in control uninfected preparations which were passaged parallel to the infected material in cultures derived from the same

embryos. In addition, two of the individuals who yielded "IBV-like" viruses appeared to develop a rise in neutralizing antibodies during convalescence.

However, the data reported here furnish no evidence that the viruses recovered were the cause of illness, for individuals without respiratory disease were not studied during the same time period. In this regard it is of interest that strain 229E was recovered from five individuals all of whom developed fourfold or greater rises in neutralizing antibody and four of whom had an upper respiratory illness at the time the virus was isolated.[5] Moreover, strain B814 was shown to cause colds in volunteers who received organ-culture-grown virus.[19] It appears likely, therefore, that on further study the "IBV-like" viruses isolated in this study will be shown to cause illness in man.

Our experience with the recovery of "IBV-like" agents during the winter of 1965–66 provides grounds for speculation as to the possible role of this group in acute upper respiratory illness. In September, October, and November of 1965, 21 of 50 specimens tested by standard tissue culture techniques yielded recognizable agents. During this period ten specimens were studied in organ culture and no "IBV-like" viruses were recovered. During the subsequent three-month period, when only four agents were isolated by tissue culture techniques from 60 specimens tested, "IBV-like" viruses were recovered in organ culture from five of nine specimens studied. If these viruses are shown ultimately to cause respiratory tract disease in man, then it is possible that they are important pathogens during those periods in mid-winter when rhinovirus prevalence declines.[3, 4, 12]

The limited supply of human embryonic tracheas restricts rapid expansion of our understanding of the "IBV-like" viruses. To facilitate their investigation, it will be necessary to adapt them to growth in tissue culture. It is clear, however, that ciliated human embryonic organ culture represents a sensitive system for the recovery of agents from patients with respiratory disease.[20] With the addition of the electron microscope for examination of concentrated harvests, organ culture could prove to be a useful tool in the search for new agents in many differing diseases.

The causative organism of avian infectious bronchitis[14] is a filterable, ether-labile,[10] RNA-containing[1] and medium-sized[2] virus and was initially assigned to the myxovirus group. Berry *et al.*[2] examined IBV by the negative staining technique and pointed out the contrast between its morphology and that of the myxoviruses. A human counterpart of IBV was, however, not recognized until Tyrrell[18] drew attention to the resemblance between strain B814 and the avian pathogen. It appears now that a new group of viruses is emerging with members which infect the respiratory tract of birds and man. One member of the group, strain 229E, grows and produces CPE in tissue culture; strain B814 and the viruses reported here can only be propagated in the laboratory in human ciliated respiratory tract organ culture. The group resembles the myxo- and paramyxoviruses in size, ether lability, and, where tested, nucleic acid type; however, it differs in morphology. In place of the closely spaced, narrow surface projections carried by the myxo- and paramyxoviruses those of the "IBV-like" viruses are club-shaped and more widely spaced.

Summary.—In a study of acute upper respiratory tract disease in adults, nasopharyngeal washings which failed to yield viruses by standard tissue culture techniques were examined in human embryonic tracheal organ cultures. From 23

specimens, 8 agents were recovered, 2 of which appeared to be ether-stable and were detectable only by their ciliary immobilizing effect in organ culture. The remaining 6 were detected when organ culture harvests were examined by electron microscopy. These viruses exhibited an unusual morphology closely resembling that of avian infectious bronchitis virus (IBV) and two other ether-labile agents recovered from man: strain 229E, described by Hamre and Procknow, and strain B814, an organ-culture-propagated virus described by Tyrrell. Five of the six "IBV-like" viruses were examined and found to be inactivated by ether. This group, for which IBV is the morphologic prototype, appears to be distinct from the myxoviruses. The implications of these findings are discussed in relation to acute upper respiratory tract disease of undetermined etiology.

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