ISOLATION OF CYTOPATHOGENIC AGENTS FROM THE RESPIRATORY TRACT IN ACUTE LARYNGOTRACHEOBRONCHITIS

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[WITH SPECIAL PLATE]

Acute laryngotracheobronchitis is a common syndrome in North America, which is often called croup after the most prominent symptom. It is one of the leading causes for admission to the Hospital for Sick Children, Toronto. Modern methods of treatment have greatly lowered the mortality, which is now negligible, but the aetiology remains obscure. Bacteriological studies have not been very fruitful (Neffson, 1949 ; Rabe, 1948 ; Morgan *et al.*, 1956), but both *Corynebacterium diphtheriae* and *Haemophilus influenzae* Type B have been shown to cause a few cases. The lack of a bacteriological cause for most cases has led to increasing support for the idea that a virus may be responsible. Rabe (1948) has discussed the indirect evidence favouring this hypothesis.

During extensive studies of the aetiology of the condition undertaken in Toronto in recent years cytopathogenic agents have been isolated from the tracheal secretion of several patients (Morgan *et al.*, 1956 ; Beale, McLeod *et al.*, 1957). Chanock (1956) has recently described the isolation of two similar agents from cases of croup. In this paper an account of the investigations in Toronto during the winter of 1955–6 is presented.

Methods

The patients were admitted to the Hospital for Sick Children during October or November, 1955. The disease was sufficiently severe in all cases to raise the possibility of tracheotomy. Tracheal secretion was obtained as soon as possible after operation from all children undergoing tracheotomy. Nasopharyngeal secretion was obtained from the remaining patients by the method devised by Auger (1939). Serum was collected from all the patients soon after admission, and wherever possible a convalescent sample was obtained two to three weeks later. All specimens were stored at −40° C. A full clinical history was taken in each case, and all patients were followed up during convalescence in order to confirm the diagnosis.

Tissue Cultures for Virus Studies

HeLa and human amnion cells were used. HeLa cells were grown in Hanks's solution with 0.5% lactalbumin hydrolysate and 20% human serum. Culture tubes were washed three times in Hanks's solution before use, and then maintained in Earle's solution with 0.5% lactalbumin hydrolysate, 0.1% yeast extract, and 5% horse or rabbit serum. In virus isolation experiments culture fluid was changed about twice a week, in order to keep the cells healthy as long as possible. The human amnion cells were obtained by trypsinization by the method described elsewhere (Beale, Doane, and Ormsby, 1957). They were grown in the same medium as HeLa cells and the same maintenance fluid was used, but, unlike with HeLa cells, no fluid change was required during long periods of maintenance. Specimens were inoculated in 0.1-ml. amounts into two or three tubes containing 1 ml. of maintenance fluid. Cultures were examined daily under the low power of the microscope until control uninoculated cultures started to degenerate.

Serology

Neutralization tests were performed by adding undiluted tissue-culture-fluid virus to serial twofold dilutions of unheated patients' serum. The mixtures were allowed to stand for one hour at room temperature and then inoculated into culture tubes, employing four tubes per dilution. The results were read 48 hours after cytopathogenic effects were noted in virus control cultures. If the cultures were left for longer periods virus tended to break through the sera. Complement-fixation tests were performed by the micromethod devised by Fulton and Dumbell (1949). In order to prevent drying of the drops during performance of the tests a humidified cabinet was used. Haemagglutination tests were made in tubes as described by Chanock (1956), an equal quantity of virus and 0.25% newborn chick cells being used. Chick cells were stored at −20° C. in glycerol citrate as described by Mollison (1954). The tests were read by observing the patterns of sedimented cells after two hours at 4° C.

Results

Fifteen patients (Table I), all between the ages of 6 months and 3 years, were studied. There were 12 males and 3 females, which shows the usual predominance of males (Morgan *et al.*, 1956). Seven patients required tracheotomy and eight recovered without operation. In all cases recovery and convalescence were uneventful. Ten of the 15 patients yielded cytopathogenic agents in tissue cultures, five among the tracheotomized and five among the unoperated patients. In Table II the results of attempts to isolate virus in HeLa and human amnion cells are compared. It will be seen that of the seven tracheotomized patients five yielded a virus, all except for one (which was not tested in human amnion cells) in both cell types. The superiority of human amnion cells for the isolation of these agents is clearly shown by

TABLE I.—*Isolation of Virus from Patients With Acute Laryngotracheobronchitis*

Case No.	Age (Months)	Sex	Tracheotomy	Virus Isolation
1	8	M	Yes	+
2	12	M	Yes	−
3	24	M	Yes	+
4	18	M	Yes	+
5	27	F	Yes	−
6	26	F	No	+
7	13	M	No	+
8	8	M	No	−
9	24	M	Yes	+
10	32	M	Yes	+
11	39	F	No	+
12	17	M	No	−
13	26	M	No	+
14	6	M	No	−
15	8	M	No	+

TABLE II.—*Virus Isolation in HeLa and Human Amnion Cells*

	From Tracheal Secretion			From Nasopharyngeal Secretion		
Case No.:	9	1, 3, 4, 10	2, 5	6, 13	7, 11, 15	8, 12, 14
HeLa cells	+	+	−	+	−	−
Human amnion cells	Not tested	+	−	+	+	−

*On leave from the Public Health Laboratory Service. Now at the Public Health Laboratory, Newcastle upon Tyne.

LORD ROTHSCHILD: THE HUMAN SPERMATOZOON

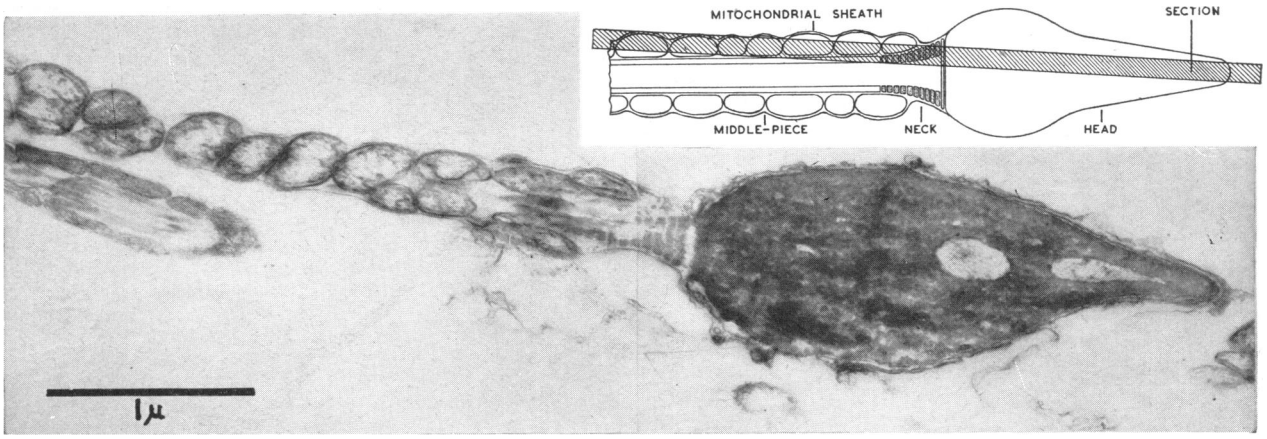

FIG. 1.—Longitudinal section through head and middle-piece of human spermatozoon. (Osmium tetroxide/methacrylate. ×27,500.) Small diagram shows approximate plane of section.

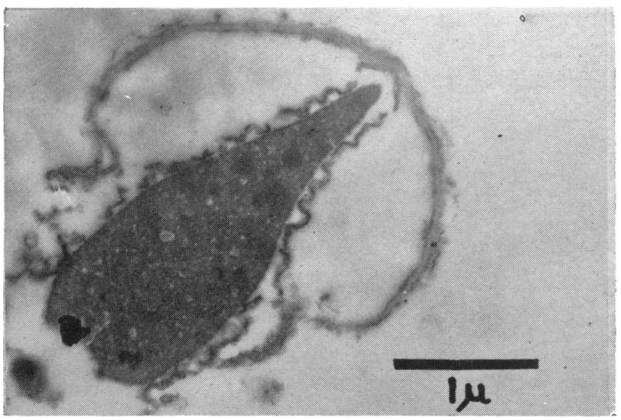

FIG. 2.—Longitudinal section through head of human spermatozoon. Acrosome and enveloping membranes have separated from surface of sperm head. (Osmium tetroxide/methacrylate. ×20,000.)

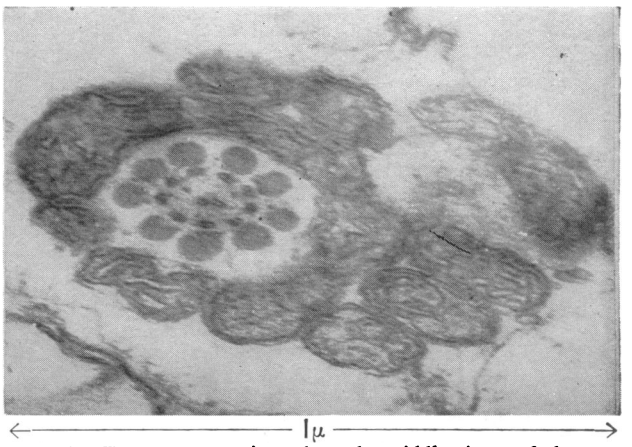

FIG. 3.—Transverse section through middle-piece of human spermatozoon which may be immature. (Osmium tetroxide/methacrylate. ×80,000.)

A. J. BEALE *ET AL.*: ACUTE LARYNGOTRACHEOBRONCHITIS

FIG. 1.—Normal human amnion cells. (Unstained. ×70.)

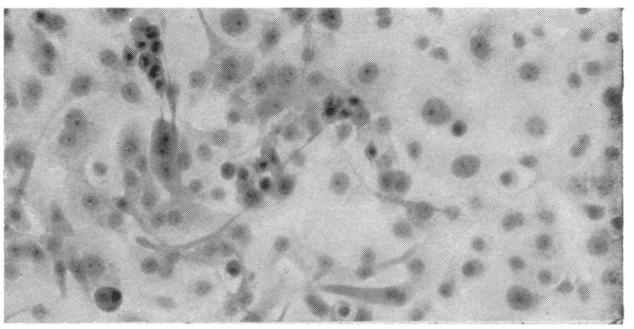

FIG. 2.—Normal human amnion cells. (Haematoxylin and eosin. ×175.)

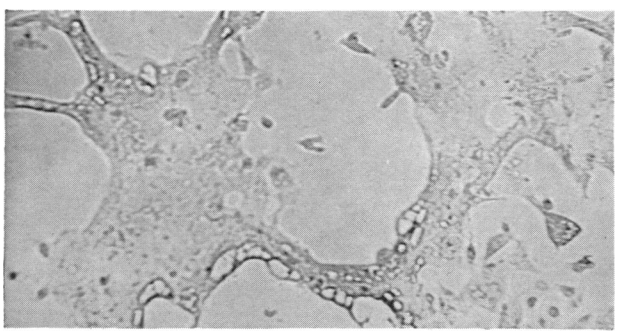

FIG. 3.—Effect of virus from case of laryngotracheobronchitis on human amnion cells. (Unstained. ×70.)

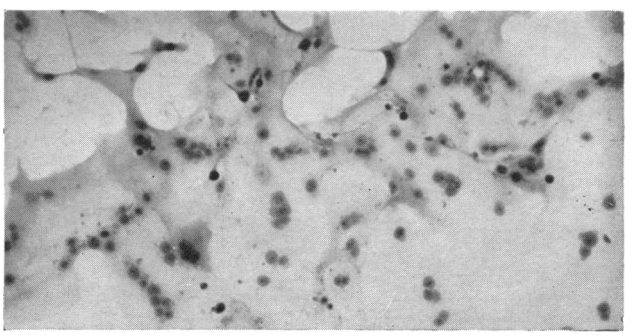

FIG. 4.—Effect of virus from case of laryngotracheobronchitis on human amnion cells. (Haematoxylin and eosin. ×175.)

the results for nasopharyngeal secretion. Two patients yielded agents in both types of cells, and three only in human amnion cells. Two did not yield virus. Amnion cells were superior partly because of the greater ease of maintenance of healthy cultures. The agents are slow to produce cytopathogenic effects, taking 17 or more days on primary isolation.

The results of the serum neutralization tests performed on the paired sera from six patients are shown in Table III. It will be seen that all six showed a rise in antibody level during convalescence.

TABLE III.—*Virus Neutralization Tests: Acute and Convalescent Sera Titrated Against Virus Isolation from Patients*

Case No.	Acute Sera		Convalescent Sera	
	Date of Collection	Neutralization Titre	Date of Collection	Neutralization Titre
1	4/10/55	4	14/10/55	64
3	24/10/55	4	12/11/55	64
4	29/10/55	4	5/11/55	32
9	11/11/55	4	19/11/55	32
10	11/11/55	64	21/11/55	1,024
13	14/11/55	4	24/11/55	256

The cytopathogenic effect of these agents is distinctive. The appearance of normal human amnion cells is illustrated in the Special Plate, Figs. 1 and 2. In the unstained preparation (Fig. 1) a clear, almost invisible sheet of cells is shown, in which an impression of the cell outline can be made. In the stained preparation (Fig. 2) the nuclei are prominent, each containing one or more nucleoli. The nuclei are uniformly dispersed in finely granular cytoplasm, but the cell boundaries are not clearly defined. The appearance of infected cultures after one week is depicted in Special Plate Figs. 3 and 4 (strain Manganero from Case 1). There is a loss of cells with vacuolation of the cytoplasm. In unstained preparations the rest of the culture has an amorphous appearance (Fig. 3); Chanock (1956) has likened these changes to a sponge or Swiss cheese. In stained preparations more or less normal-looking nuclei are lined up in columns or aggregated in amorphous cytoplasm (Fig. 4). No intranuclear inclusion bodies have been seen in preparations stained with haematoxylin and eosin after fixation in either Bouin's fluid or a methyl-alcohol–acetic-acid mixture (Doane *et al.*, 1955). The changes in HeLa cells consisted in clumping of cells with loss of the cell walls to give multinucleated giant cells. The changes progressed in both cell types so that finally most of the cells fell off the glass.

These changes are unlike those produced by other viruses studied in this laboratory (poliomyelitis, Echo, Coxsackie, adenoviruses, herpes simplex, and vaccinia). The changes are somewhat similar to those described for measles virus, although no intranuclear inclusions were seen (Enders and Peebles, 1954). They are also similar to those seen in some of the viruses found in normal monkey kidney cells (Hull *et al.*, 1956). However, none of these agents was passed in monkey kidney cells. As an additional control in passage experiments fluids from previous control cultures were also passed in parallel into fresh cultures.

One strain (Manganero) was inoculated into suckling and adult mice, hamsters, and a rabbit without producing any pathogenic effects. The virus did not agglutinate human Group O red cells at room temperature, but, following advice from Dr. Chanock, it was found that newborn chick cells, and to a lesser extent human cells, were agglutinated at 4° C. The characteristics of this haemagglutination are illustrated by the experiment in Table IV. It will be seen

TABLE IV.—*Haemagglutination*

Treatment	Dilutions of Virus					
	5	10	20	40	80	160
4° C. for 2 hours	+	+	+	+	−	−
37° C.	−	−	−	−	−	−
4° C. for 2 hours	+	+	+	+	−	−

that the cells are agglutinated at 4° C., but settle normally again if they are shaken up and allowed to settle at 37° C. This is similar to the behaviour of the influenza group of viruses (myxovirus); however, unlike members of this group, the agents from laryngotracheobronchitis will reagglutinate the cells if these are placed at 4° C. again. During the process of agglutination virus is absorbed from the fluid and subsequently released at 37° C. Although Chanock (1956) has described the use of haemagglutination inhibition titrations for serum antibody, this technique was not used because of the widespread occurrence of non-specific inhibitors.

One strain (Manganero) was sent to Dr. Chanock, who found that it was similar in haemagglutination inhibition tests to the two agents he isolated from cases of croup. He kindly provided monkey antiserum to his Greer strain, and all six of the strains (Nos. 1, 3, 9, 10 and 13, and Greer) subjected to complement-fixation tests gave a titre of 1:40 with this serum.

It was found that both the infectivity and haemagglutinating power of the virus was destroyed by ether.

Discussion

The relationship of these agents isolated from patients to the aetiology of laryngotracheobronchitis remains to be determined by future work. Although they appear to be present in the respiratory tract in a high proportion of cases, and to be associated with an antibody rise during convalescence, no studies were undertaken in Toronto into the incidence of the virus in normal children. Chanock (1956) has presented evidence of widespread past infection with these agents in young adults. It is clear that the agents isolated in Toronto are similar to those isolated by Chanock. Chanock, on the basis of their size, ether sensitivity, and haemagglutination, has provisionally assigned them to the myxovirus group (Andrewes *et al.*, 1955). He has also noted a serological relationship to mumps virus, a virus which also produces giant cells in HeLa cells.

Summary

The isolation of 10 agents in tissue culture from 15 patients with acute laryngotracheobronchitis is described.

Human amnion cells proved superior to HeLa cells for the isolation of these agents.

All six patients tested developed neutralizing antibodies in convalescence.

The cytopathogenic effects are illustrated and some of the properties described. These are similar to those of the influenza–mumps–N.D.V. group of viruses.

There is suggestive evidence that the agents actually caused disease in the patients studied, but more work is required to establish this beyond doubt.

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