Bovine Amniotic Fluid as Tissue Culture Medium in Cultivation of Poliomyelitis and Other Viruses.* (20035)

JOHN F. ENDERS.

From the Research Division of Infectious Diseases, the Children's Medical Center and Department of Bacteriology and Immunology, Harvard Medical School, Boston, Mass.

Following the demonstration(1) that poliomyelitis virus could be propagated *in vitro* in cultures of human extraneural tissues a variety of cell types both of human and simian origin and of media for the support of these cells have been used by different investigators(2-8). In this laboratory human tissues have been largely employed and experiments have been continued with the purpose of developing more convenient and satisfactory procedures for the preparation and maintenance of large numbers of roller tube cultures. In particular we have been interested in tissues such as uterus that may be obtained in any large hospital as a by-product of surgical operation. Cultures of mature tissues have for some time been maintained with a medium consisting of balanced salt solution, ox serum ultrafiltrate, normal horse serum and beef embryo extract(9). In an attempt to reduce the number of constituents of this medium and with the hope of improving the growth of cells and of virus bovine amniotic fluid was substituted for the balanced salt solution and serum ultrafiltrate. The results of experiments will be briefly presented in which cell outgrowth and viral multiplication in these two kinds of medium were compared.

Materials and methods. The technics and materials adopted previously for the cultivation of mature human tissues in roller tubes have been described elsewhere(9). Bovine amniotic fluid was withdrawn from the amniotic sac in the following manner. The gravid uterus was removed *in toto* at the abbatoir and conveyed to the laboratory within 2 to 3 hours. It was suspended from a support by a cord. After cleansing and drying the entire surface of the organ, an appropriate site near the embryo and close to the dependent area was selected and lightly cauterized with a free flame. Taking care to avoid piercing the embryo, a special trochar (bore 1.5 mm, length 22.5 cm)† was then passed through this portion of the uterine wall into the amniotic cavity. The clear fluid which usually ran rapidly and freely was collected in sterile flasks. The yield of fluid varied from about 0.5 to 1.5 liters depending upon the size of the embryo. Occasionally fluids were contaminated with blood, presumably because of injury to the embryo by the trochar. These were not used for the preparation of culture medium. Embryos about 7.5 to 25 cm in length were selected. After withdrawal of the fluid the embryo was removed under aseptic precautions and its tissues employed for the preparation of embryonic extract(9). To the fluid withdrawn from each embryo sufficient penicillin and streptomycin were added to yield a final concentration of 50 units and 50 micrograms respectively per ml. Portions of the fluid (0.1 ml) were then added to thioglycollate broth and incubated for 48 hours to determine the presence or absence of bacteria insensitive to these antibiotics. In the meantime the fluids from each embryo were kept at about 6°C in tightly stoppered flasks. A pool was then made of the fluids from several embryos. Sufficient stock phenol red solution (0.4%) was added to the pooled fluid to yield a final concentration of 0.002%. The material was stored in the ice box. On storage the pH of certain batches was found to attain a high level (*e.g.*, 8.) This was reduced to 7.5-7.6 by "gasing" with a mixture of 5% CO_2 in air just before use.

Experimental. Effect of amniotic fluid on migration and growth of human cells deriving from mature uterine and renal tissues and from embryonic skin-muscle. In a number of experiments the growth of cells from human uterine tissue in roller cultures maintained with bovine amniotic fluid (BAF) alone or with the addition of beef embryonic extract

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† Macalaster Bicknell Co., Cambridge, Mass.

TABLE I. Outgrowth in Roller Tube Cultures of Human Uterine Tissue Maintained with Various Media Containing Bovine Amniotic Fluid (Exp. 1).

Medium	Cell outgrowth on indicated day of cultivation 5	8	11	13	15	Remarks
BAF–100*	1‖	2	3	4	3	Slight retraction of coagulum
80, HS–20†	<1	2	2	3	3	Moderate to marked retraction
90, BEE–10‡	2	3	4	>4	>4	No retraction, cells granular
70, HS–20, BEE–10	3	4	>4	>4	>4	Moderate retraction
Regular med. (no BAF) §	2	3	4	3	3	Marked "

* Bovine amniotic fluid–100%.
† Normal horse serum inactivated at 56°C for ½ hr–20%.
‡ Beef embryo extract–10%.
§ Regular medium: horse serum–20%, BEE–10%, Hanks-Simms' mixture–70%.
‖ Numerals indicate mean outgrowth of cells in 6 comparable cultures estimated by simple inspection and recorded at 1+, 2+, etc.

(BEE) has been compared with the cell outgrowth in the "regular" medium hitherto used as routine for uterine tissue prior to inoculation of virus. The latter consisted of 10% BEE, 20% horse serum and 70% of a mixture consisting of 3 parts Hanks' balanced salt solution and 1 part bovine serum ultrafiltrate. No attempt has been made to determine by quantitative methods the amount of cell growth. A rough estimate that we regarded as sufficient for our purposes was obtained, however, by comparing in the various cultures at 2-day intervals the extent of cell outgrowth, its thickness (*i.e.*, one or more layers of cells) and the frequency of mitotic figures. The growth was thus evaluated as 1+ . . . . 4+ and >4+. Mean estimates of the outgrowth in 4 or 6 comparable cultures were calculated. The results of two experiments of this sort are summarized in Tables I and II. In the first experiment the medium was removed and replaced at intervals of 2 or 3 days; in the second the medium in one set of 4 cultures was removed and replaced at these intervals while in another set of 4 comparable cultures it was changed every 4 days.

The data of the first experiment indicate that BAF alone provides a medium in which certain cells of a tissue derived from a mature human being (age 40 years) not only survive but migrate from the original fragment and appear to increase considerably in numbers. These observations strongly suggest that the amniotic fluid not only provides a physiologic medium but contains a growth-stimulating factor or factors. It is evident, however, that BAF alone is incapable of inducing optimal cell growth since the addition of BEE and horse serum leads to greatly increased proliferation as indicated not only by the greater area and thickness of the zone of migrating cells but by the presence of large numbers of mitotic figures. When either BEE or horse serum is added to BAF, cell growth is enhanced, although BEE appears to be somewhat more effective. If employed without horse serum BEE favors the formation of granules in the cytoplasm. Accordingly it has seemed desirable to include horse serum as well in a medium for use as routine, since horse serum inhibits granulation when both these constituents are present in BAF. The cell growth in this medium was definitely more luxuriant than that observed in the so-called "regular" medium. Moreover, the cells nourished with the mixture containing BAF appeared to be in a more active condition as indicated by their larger size and the greater number of mitotic figures.

Although some retraction of the coagulum was noted in cultures containing BAF when horse serum was included, this did not seem to be so extensive as in those maintained with the regular medium. In this respect also the BAF appears to be an advantageous substitute for the balanced salt-serum ultrafiltrate mixture. Another useful property of BAF consists in the efficiency of its buffering action which is capable of preventing the pH from falling below 6.9-7 during a period of several days in actively growing cultures containing large amounts of tissue. The buffering action of BAF, therefore, is definitely more effective than that of the balanced salt solution-serum

TABLE II. Outgrowth in Roller Tube Cultures of Human Uterine Tissue Maintained with Various Media Containing Bovine Amniotic Fluid (Exp. 2).

Medium*	Medium changed	Cell outgrowth on indicated day of cultivation 6	11	15	Remarks
BAF–100	2†	1§	3	3	pH remained >7.5
	4‡	<1	3	3	
80, HS–20	2	1	4	4	Cells free of granules, very transparent —pH not <7.5
	4	1	3	4	
95, 5	2	1	4	4	
	4	1	3	4	
97.5, 2.5	2	<1	4	4	pH remained >7.5
	4	<1	2	3	
90, BEE–10	2	<1	4	4	Cells granular, pH lower than in cultures without BEE, *i.e.*, 7.2-6.9
	4	<1	3	4	
70, HS–20, BEE–10	2	1	4	>4	pH lower than in cults. with serum only, *i.e.*, 7.1-6.9 at time fluid changed
	4	2	4	>4	
85, 5, 10	2	1	4	>4	"
	4	2	4	>4	
90, 5, 5	2	2	4	>4	"
	4	1	4	>4	
87.5, 2.5, 10	2	1	4	>4	"
	4	1	4	>4	
92.5, 2.5, 5	2	1	4	>4	"
	4	<1	4	>4	
Regular medium (no BAF)	2	1	4	4	Retraction more marked than in cults. with BAF, pH dropped to 6.8 or lower
	4	2	4	4	

* For explanation of abbreviations see Table I.
† Fluids of 4 cultures changed Mondays, Wednesdays and Fridays.
‡ Fluids of cultures maintained with same medium but changed Mondays and Fridays.
§ Numerals indicate mean outgrowth of cells in 4 comparable cultures estimated by simple inspection and recorded as 1+, 2+, etc.

ultrafiltrate mixture. This property has permitted us to replace BAF medium in such cultures at intervals of 4 instead of 2 days as is necessary to maintain the pH above neutrality when the regular medium is employed. Thus, as the data included in Table II show, cultures nourished with BAF, BEE and horse serum and in which the medium is changed only at the longer interval exhibited continuing growth of cells which was essentially as luxuriant as that observed in cultures changed every 2 or 3 days. When large numbers of cultures are required, the saving in labor and materials achieved by adopting the 4-day interval for change of medium is considerable. In the presence of BAF variation in the quantities of horse serum and BEE within the range that has been studied has effected little or no discernible difference in cell growth as shown in Table II. We have, therefore, now adopted more or less arbitrarily but with consideration of economy of materials in mind a medium for routine purposes consisting of 5% BEE, 5% normal horse serum and 90% BAF. This medium has afforded consistently good growth not only of cells derived from human uterine tissue but also those from human kidney and prepucial tissues removed at operation and from a variety of tissues of the human embryo which include skin and muscle, liver, amniotic sac and umbilical cord (from embryos of 2-3 months gestation).

Multiplication of poliomyelitis virus in cultures maintained with BAF in the medium. Considerable experience has now been gained in the propagation of poliomyelitis viruses in cultures of various human tissues maintained with media containing BAF. The concentrations of virus developing in such cultures have equaled or exceeded those found in comparable cultures nourished with "regular" medium. Furthermore, the capacity of cultures maintained with BAF medium to support multipli-

TABLE III. Assay of Brunhilde Virus Grown in Roller Tube Cultures of Human Uterus Maintained with BAF and Regular Media and Titrated in Cultures Maintained with These Media.

Source of virus	Medium in cultures for titration	Dilution of virus inoculated				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
BAF culture*	BAF‡	1/1‖	2/2	2/2	2/2	0/2
Reg. med. culture†	BAF	1/1	1/2	1/2	0/2	0/2
BAF culture	Reg. m.§	1/2	2/2	2/2	0/2	0/2
Reg. med. culture	Reg. m.	1/1	0/2	1/2¶	0/2	0/2

* Pooled virus derived from 2 cultures of human uterine tissue maintained with BAF-85, HS-5, BEE-10 and each inoculated with 10 ID_{50} (tissue culture infecting doses).

† Pooled virus derived from 2 similar cultures maintained with Hanks-Simms' sol. 85, HS-5, BEE-10 and each inoculated with 10 ID_{50}.

‡ Cultures of human uterine tissue used for titration and maintained with medium mentioned in footnote 1.

§ Cultures of human uterine tissue used for titration and maintained with medium mentioned in footnote 2.

‖ Numerator: cultures exhibiting cell degeneration within 10 days following inoculation. Denominator: cultures inoculated.

¶ Cytopathogenic effect not evident until 9th day after inoculation; in all other cases this was apparent within 5 days.

cation of the virus following the introduction of minimal inocula has usually exceeded that of cultures with the regular medium. Illustrative data are presented in Table III which summarizes the results of an experiment in which the Brunhilde strain was first propagated in each of 2 cultures maintained respectively with the 2 kinds of medium. The virus suspensions thus produced were then titrated *in vitro*(3,9). It is apparent that in cultures with BAF medium the yield of virus as well as the end point of viral activity as denoted by cell degeneration were higher by a factor of about 10. It should be pointed out in respect to this experiment that somewhat higher yields of virus have frequently been achieved with the regular medium(9). However, in subsequent experiments with BAF medium evidence has been obtained that more virus developed than had previously been found in cultures maintained with the regular medium. The *greater sensitivity* of the cultures maintained with BAF in respect to the detection of small quantities of virus has also been apparent when the inocula have consisted of suspensions of feces from patients with poliomyelitis. In experiments with such materials carried out by Dr. Sidney Kibrick in this laboratory roller tube cultures supported with BAF medium were found to reveal the presence of approximately 1/10th the quantity of virus detectable by cultures containing "regular" medium. Moreover, in a reexamination of 24 10% stool suspensions from 24 patients from which no virus had previously been isolated in cultures maintained with regular medium, 5 agents were revealed through the use of cultures in which BAF medium was employed.

Multiplication of other viruses. Other viruses that include herpes simplex, the Boston strain of Coxsackie virus which displays cytopathogenic properties(10,11) and four agents isolated from fecal material which do not appear to belong to any of the known agents of this class(4) have been successfully propagated in cultures with BAF medium. These viruses as well as those of the poliomyelitis group have induced their cytopathogenic effects more clearly and promptly in cells growing in a mixture consisting of 90% BAF, 5% BEE and 5% horse serum.

Effect of BAF medium in cultures used for virus neutralization tests. Because of the possibility that BAF might in some way interfere with the neutralization of virus by specific antibody *in vitro* virus neutralization tests were done in parallel using cultures maintained respectively with the two types of medium. Three specimens of serum from a patient with poliomyelitis taken at various periods after the onset were tested. The results presented in Table IV suggest no interference by BAF with the action of antibody which was shown

TABLE IV. Neutralization of Brunhilde Virus by Serum of a Poliomyelitis Patient in Roller Tube Cultures of Human Uterine Tissue Maintained with BAF and Regular Media.

Serum of polio patient drawn*	Medium used in test cultures	Final serum dilution					
		1/8§	1/16	1/32	1/64	1/128	1/256
9/23/52	BAF†	0/2‖	2/2	ND	ND	ND	ND
	Reg.‡	ND	1/2	ND	ND	ND	ND
10/11/49	BAF	ND	ND	0/2	2/2	1/2	2/2
	Reg.	ND	ND	0/2	1/2	1/2	2/2
1/21/52	BAF	ND	ND	0/2	0/2	0/2	1/2
	Reg.	ND	ND	ND	0/2	0/2	1/2

* Specimens of serum obtained on dates noted from patient with paralytic poliomyelitis. Type I virus (Brunhilde) was isolated from feces of this patient during acute phase of disease.

† BAF = medium consisting of 5% horse serum, 10% beef embryo extract, 85% bovine amniotic fluid.

‡ Reg. = medium of 5% horse serum, 10% beef embryo extract, 85% Hanks-Simms' sol.

§ .1 ml of a mixture of diluted serum and virus added to each culture. 100 ID_{50} of Brunhilde tissue culture virus employed in each instance.

‖ Numerator: cultures exhibiting cell degeneration within 10 days after inoculation. Denominator: cultures inoculated.

ND = not done.

to increase in concentration with passage of time.

Discussion. The results presented here as well as much additional experience recently gained make it clear that BAF forms an excellent basis of a medium, when enriched by the addition of other materials, for the propagation *in vitro* of certain human cells. This finding is of value from the utilitarian standpoint since it has enabled us to obtain regularly excellent cell growth which has been accompanied by increase in the yield of poliomyelitis viruses as well as in the capacity of the tissue culture to reveal the presence of small quantities of these agents.

The observation that BAF alone without the addition of other factors promoting cell growth leads to a more restrained migration and proliferation of tissue cells is perhaps of general biologic interest. So far as we are aware the physiologic role of amniotic fluid has not been satisfactorily elucidated except that it may protect the embryo against injury induced by mechanical means. Although something is known concerning the chemical nature of the simpler constituents of the fluid(12,13), little or nothing has been recorded in respect to its possible content of certain biologically active entities such as vitamins, hormones or growth-promoting factors. The evidence for the presence of the latter which our investigation has afforded would seem to encourage attempts to define more exactly the biochemical properties of this interesting material. It is possible that the results of such studies might lead to the hypothesis that the amniotic fluid may represent at least one source whence the cells of the early embryo before the vascular system is established derive the materials for their maintenance and growth.

Summary. Bovine amniotic fluid induced active cell growth when substituted for balanced salt solution and bovine serum ultrafiltrate in a medium used for the cultivation of embryonic and mature human tissues in roller tube cultures. The growth was more luxuriant than that observed in comparable cultures maintained with the balanced salt mixture. Moreover, superior yields of poliomyelitis viruses have been obtained in cultures nourished with bovine amniotic fluid as a constituent of the medium and in such cultures the minimal infecting dose of these agents has also proved to be smaller. Bovine amniotic fluid does not interfere with the neutralization of poliomyelitis virus by specific antibody. Through the use of bovine amniotic fluid the labor and expense involved in the preparation and maintenance of tissue cultures applied to the propagation of poliomyelitis virus and certain other agents of this class have been reduced.

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Effects of Cortisone Acetate on Fluid and Electrolyte Balance in Normal Rabbits.* (20036)

JERRY K. AIKAWA.† (With the technical assistance of Eloise L. Rhoades.)

From the Department of Internal Medicine, Bowman Gray School of Medicine of Wake Forest College, Winston-Salem, N. C.

Although the effects of cortisone on the metabolism of sodium, potassium and water have been extensively investigated in diseased man and in the normal rat and dog(1-4), comparable data in the rabbit are lacking. Most studies in immunology, however, have been performed in the latter species of animal. Because of the variability in the response of different species to drugs, a study of the effects of cortisone on fluid and electrolyte metabolism in the rabbit appeared to be desirable.

Recent studies have suggested that immune reactions in the rabbit might be associated with alterations in the distribution of body fluid and electrolytes, and that the adrenal gland may be involved in these changes(5,6). Before the role played by the adrenal gland in the phenomena of immuno-physiology can be interpreted, it is first necessary to determine the effects of adrenal hormones in normal animals. The availability of radioactive potassium (K^{42}) has made possible the direct measurement of the exchangeable potassium content in intact animals. The purpose of the present study was to determine the effects of two different dosages of cortisone acetate on sodium, potassium, and water metabolism in normal rabbits.

Materials and methods. Fourteen normal adult male rabbits of mixed breeds were used. They were kept in individual metabolism cages and were fed a stock diet of compressed pellets. Tap water was given without restriction. In order to acclimate the animals to their new environment, they were placed in the cages one week prior to the initiation of the baseline studies. *Procedure.* Two groups of 7 rabbits were used. External balance studies of sodium and potassium were performed daily for 4 weeks in Group 1 and for 5 weeks in Group 2. Direct measurement of the exchangeable potassium content (Ke) was made with K^{42} at weekly intervals. During the first week, baseline studies were done on each group. Starting on the 8th day

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