Macromolecular Growth Requirements of Human Cells in Continuous Culture.* (25196)

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It is now well established that human cells in continuous culture provide valuable tools in studies of metabolism and genetics. There remain, however, 2 important limitations which frequently give rise to conflicting results(1,2); the necessity of incorporating serum proteins in assay media and the unpredictable evolution of cell populations. It is well known that dialyzed serum contains numerous components, active biologically in trace quantity such as enzymes, hormones, vitamins and trace metals. It is not surprising that experimental results may be affected by the source of sera(2,3). To eliminate the variable introduced through serum proteins, investigations have been directed chiefly towards development of chemically defined media. Indeed, preliminary reports indicate that variants of human epithelial-like cells derived from skin have been successfully adapted to propagate in chemically defined media(4). While this development undoubtedly represents an important advance in tissue culture methodology, the importance of the reported existence of intracellular proteins immunologically related to various serum proteins should not be minimized(5). With this view in mind, the staffs of the Dept. of Microbiology, Harvard University and the Protein Foundation have jointly undertaken a long term study to elucidate the roles of various serum macromolecules in the physiology of human cells in continuous culture. This report describes the isolation of serum fractions capable of promoting and inhibiting cell growth. While there are reports on the isolation of alpha globulin-like substances capable of enhancing cell attachment to glass surfaces(6,7), studies on promotion of prolonged cell growth are lacking. To the best of our knowledge, there also has been no report of serum fractions which when diluted in a complete growth medium, cause cellular degeneration.

Materials and methods. Preparation of serum and plasma fractions. Serums were collected and stored as described previously(8). Plasmas were prepared both from blood collected by standard procedures (in ACD anticoagulant solution) and from blood collected through resin columns and separated in the Cohn-ADL blood fractionator(9). Plasma was always held refrigerated until fractionated, and after the lability of the growth promoting factors had been noted, it was processed immediately after collection or was frozen and held in the frozen state until fractionated. Human serums and plasmas were fractionated in pools of 3 to 10 donors while equine serums were fractionated individually. Cold ethanol method 6(10) was used throughout this study. Zinc method 12(11) was equally promising in initial studies but was abandoned because of the more extensive knowledge of the subfractionation of the cold ethanol fractions. Both methods were designed for human plasma. To obtain more reproducible results, we have found it desirable to complete the fractionation procedure within 7 days and to remove the ethanol from the fractions by dialysis at 0°C against 2 changes of 2 liters each of 0.85% NaCl for 24 hours instead of by lyophilization. The fractions were prepared in concentrated form and were kept frozen at below –60°C. *Human cells.* The HeLa(12) and the conjunctival cells(13) derived respectively from malignant and normal human tissues were used. Stock cultures were nourished in the minimal growth medium containing 10% dialyzed horse serum described by Eagle(14) and modified in this laboratory by omission of biotin, addition of meso-inositol to 10 μM and substitution of Earle's balanced salt solution by Hank's. These cultures have been examined at regular intervals and found free of con-

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TABLE I. Propagation of Conjunctival Cells in Fractions from a Pool of Human Serums.

Fraction	Concentration S.E.,* %	Concentration Protein†	No. fold increase per weekly subculture 1st	2nd	3rd
II + III	50	.93	<2	deg.§	
"	10	.19	<2	"	
IV-1	50	.24	deg.		
"	10	.05	"		
IV-4	50	.22	5	12	16
"	10	.04	4	2	deg.
V	50	1.52	2	deg.	
V	10	.30	deg.		
Combined‡	50	3.29	5	8	3
"	10	.66	6	4	deg.
Serum‖		.66	20	18	14

* S.E. = Serum equivalent.
† Protein concentration in g/100 ml media as calculated from values reported by Cohn, *et al.*(10).
‡ Combination of fractions II + III, IV-1, IV-4, and V.
§ Complete degeneration.
‖ Serum from same pool without fractionation but dialyzed; used at dilution of 1/10.

tamination by pleuropneumonia-like organisms. Methods used in assaying cell multiplication have also been described(8). *RNA and RNAse determination.* Ribonucleic acid was determined by incubating 2 ml of serum fraction with 10 μg of crystalline ribonuclease at pH 5 for 24 hours at 37°C. The mixture was dialyzed against an equal volume of water at 0°C for 48 hours and the dialyzing fluid was assayed for ribosides by the orcinol reaction as well as by UV light absorption at 260 mμ. Ribonuclease activity was tested by incubating approximately 1 mg of yeast RNA with 2 ml serum fraction at pH 7.6 for 48 hours at 37°C; the amount of dialyzable ribosides was then assayed as described for determination of RNA. These conditions were selected to mimic those of cell cultivation. *Vitamin assays.* Total niacinamide and B_{12} content of various fractions were measured by the standard microbiological technic(15).

Results. Assays of growth promoting activity. The results obtained with fractions from a pool of human serum are presented in Table I; growth promoting activity is associated chiefly with fraction IV-4. It should be emphasized that while some growth promoting activity has been consistently detected in most fractions IV-4, the results obtained with different pools of human serum or plasma show important differences. There has been a tendency for less active fraction IV-4 to be obtained from pools in which the cytotoxic activity described below is most pronounced. Two out of 18 lots of fraction IV-4 has been found completely inactive at 50% serum equivalent concentration, and several lots of IV-4 were incapable of sustaining growth for more than one subculture. Recombination of fractions, even when an active fraction IV-4 has been obtained, frequently failed to sustain growth. Addition of serum ultrafiltrate to a final concentration of 10% generally improved and prolonged cell growth but did not alter qualitatively the results of such experiments. Subfractions of fraction IV-4(16) have failed to show the growth promoting activity of the parent fraction. Three pools of human serums were also fractionated by zinc method 12,(11); growth promoting activity was detected in zinc insoluble fractions (PGP). Contrary to human sera, growth promoting activity of equine serum was found to be associated chiefly with fraction II + III as illustrated in Table II. Two individual equine sera have been studied thus far with similar results. No significant qualitative difference has been observed thus far between the conjunctival and HeLa cells. In consideration of these results it must be remembered that a fractionating system designed for human plasma has been applied to equine serum

TABLE II. Multiplication of Conjunctival Cells in Fractions from An Equine Serum.

Fraction	Conc., %*	Fold increase/weekly subculture 1st	2nd	3rd
II + III	50	17	20	16
	10	4	deg.†	
IV-1	50	10	"	
	10	2	"	
IV-4	50	10	"	
	10	deg.		
V	50	2	"	
	10	deg.		
Combined‡	50	12	10	8
	10	12	5	9
Serum§		13	18	11

* % serum equivalent concentration.
† Complete cellular degeneration.
‡ Combination of all fractions.
§ Unfractionated but dialyzed; tested at 1/10 dilution.

and that little is known about the distribution of proteins in the fractions so obtained, and, indeed it is unlikely that the distribution would be the same as that obtained from application of the system to human plasma or serum.

Assays for growth inhibitory activity. Our observation that combination of all human serum fractions frequently were incapable of sustaining cell growth suggested that certain fractions may be growth-inhibiting or cytotoxic. Screening for cytotoxic activity was performed by assaying cell growth in the modified Eagle's basal medium containing 10% horse or human serum with the further addition of test fractions. Results of several screenings consistently indicated that cytotoxic activity was associated with human fraction II + III. No toxic fraction has yet been found with equine sera. Quantitative study of cytotoxic activity of fraction II + III for the HeLa cell is presented in Table III. Of interest is the fact that human serum pool C consisted of sera from 4 selected donors whose sera had been pretested, found satisfactory and used regularly during the past 4 years for cell cultivation. Fraction II + III from this pool was interestingly of low cytotoxicity. Similar results were obtained for the conjunctival cell. In one single experiment, fraction II + III from pools A, B, and D were markedly cytotoxic for primary explant of human amnion cells. Two lots of human fraction II + III were further subfractionated by the method of Oncley, *et al.*(17). In both instances, cytotoxicity was present in subfraction II + IIIw. When II + IIIw was further subdivided into fractions II-1,2,3 and III, neither of these was cytotoxic. When II-1, 2,3 and III were recombined, however, cytotoxicity was again demonstrable. Of interest is the absence of demonstrable cytotoxicity in subfractions III-0, III and II-1,2,3 in which lipoprotein, proteolytic enzymes and gamma globulins are concentrated respectively. To exclude the possibility that cytotoxicity of fraction II + III may have been artificially introduced through fractionation, cell propagation in 4 human serums were tested undiluted and at 10% concentration; in all instances, advanced degeneration were noted in undiluted serums in contrast to the 10-25 fold increases in 10% serum.

TABLE III. Propagation of HeLa Cells in Minimal Growth Medium with the Addition of Fraction II + III.

Serum pool	Conc. II + III tested, %*	No. cell × 10^3/culture 0 day	7th
Human A	100	16	0, 0†
	25	"	88, 70
B	100	"	0, 0
	25	"	6, 14
C	100	"	238, 272
	25	"	311, 249
D	100	"	0, 0
	25	"	90, 62
Control	0	"	290, 274
Equine 1	100	24	456, 474
2	100	"	552, 638
Control	0	"	276, 374

* Conc. fraction II + III diluted in modified Eagle's basal medium containing 10% dialyzed human or horse serum; conc. expressed as percent of equivalent conc. in unfractionated serum.

† Results of replicate culture.

Vitamins, RNA and RNAse contents of growth promoting fractions. Since some tissue culture investigators tend to consider serum fractions as homogeneous macromolecules, the presence or absence of molecular species (Vitamin B_{12} and Niacin), non-protein macromolecules (RNA) and enzymes of low molecular weight (RNAse) was determined. Table IV tabulates such results which are self-explanatory.

Discussion. Bazely and his associates, assaying serum fractions with primary explants of monkey kidney cell by a crude short term method, reported growth promoting activity in fractions IV and V of human, equine and bovine serums(18). Sanford and her associates found growth promoting activity for the L strain of mouse fibroblast in all fractions (19). Since the cells assayed by these workers were capable of surviving or slowly multiplying in the chemically defined medium employed as diluent for the duration of their experiments, the activity measured may have been related to stimulation of growth (as determined by net increase in cell number).

More closely related to the present study are the reports of Lieberman and Ove(6), Fisher, Puck and Sato(7) and Chang and

TABLE IV. B_{12}, Niacin, RNA and RNAse Contents of Human Fraction IV-4 and Equine Fraction II + III.*

Serum	Fraction	B_{12}, mμg	Niacin, μg	RNA (μg) Orcinal	RNA (μg) UV	RNAse
Human #1	Unfractionated†	.2	.02	3.4	2.6	+
	IV-4	.01	.01	0	0	+
#2	Unfractionated	.5			3	+
	IV-4	.01	.02	0	0	+
#3	IV-4	<.01	.04	0	0	+
Equine #1	Unfractionated	2.9	.07		2	+
	II + III	.13	.03	0	0	+
#2	Unfractionated	1.2	.05			+
	II + III	.17	.04	0	0	+

* Expressed as amt/ml; fractions were diluted to equivalent concentrations in sera.
† All unfractionated sera were extensively dialyzed.

Geyer(8). Lieberman and Fisher reported that an alpha-globulin fraction actively promoted cell attachment to glass and that cell multiplication would occur for the short duration of their experiments if autoclaved peptone or albumin were further added. Chang and Geyer found that fractions soluble in 17.5% Na_2SO_4 were capable of sustaining cell growth for at least 2 months. More recently, Lieberman reported complete loss of activity upon purification of fetuin, an alpha-globulin which has been suggested to be active in promotion of the growth of tissue cells(20).

Our results showed that the essential growth factors of serum proteins were concentrated chiefly in fraction IV-4 (46% alpha-globulin, 38% beta-globulin and 16% albumin) of human serum. When zinc method 12 was employed, however, the growth promoting activity accompanied the zinc precipitable proteins (PGP) which are, in general, dissimilar to the proteins of fraction IV-4. The alpha-globulins are generally not zinc precipitable. The presence of growth sustaining activity in horse serum fraction II + III rather than IV-4 indicated possible differences in the physicochemical properties of these factors between those of human and equine origin. Frequent variations in the growth promoting activity of fraction IV-4 from different lots of human serum served to reiterate the importance of testing several lots of serum in the study of cell physiology to minimize conflicting results.

The presence of cytotoxic fraction in most human serums tested deserved comment. This finding may explain the fact that cell cultures often do better with serum at 10-25% rather than 50-100% concentrations; it also suggests that toxicity of some serums is inherent in the serum rather than introduced through faulty technic. Of interest is the absence of cytotoxicity in fraction III-0 (rich in lipids), fraction II-1,2,3 (rich in gamma globulins) and in fraction III (rich in proteolytic enzymes); these factors have been considered by some investigators as deleterious to *in vitro* cell growth. The possibility that the cytotoxic factors may act as a barrier to hematogenous migration of somatic cells and as growth inhibitor *in vivo* deserves further exploration.

The co-existence of growth promoting and inhibiting factors in varying concentrations substantiates an earlier observation of the desirability in selecting serums for establishment of cell lines from primary explants(12). The ability of established cell lines to multiply subsequently in most unselected serums(21) is most probably due to the selection of cell population capable of multiplying in higher concentration of toxic factor as demonstrated by Fredoroff(22).

The current status of this study is not inconsistent with the suggestion that both growth promoting and cytotoxic effects noted are the result of the relationship between 2 or more serum protein factors. Neither of these activities has been successfully concentrated in a subfraction of the active parent fraction, yet cytotoxicity, at least, can be regained by recombination of the subfractions.

It is also noteworthy that the growth promoting fractions of both human and equine se-

rums contain the major portion (50% or more) of the niacin of the parent serum which had been extensively dialyzed.

Summary. The essential growth factors associated with serum proteins are present in cold ethanol fraction IV-4 for most human serum pools and in fraction II + III for 2 individual equine serums. Cytotoxic activity has been found in fraction II + III from most human serum pools. Further subfractionation of IV-4 and II + III by the established cold ethanol methods fails to yield active subfractions. The significance of this finding is discussed.

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Is Multiple Sclerosis Caused by *Spirochaeta myelophthora* (Steiner)?* (25197)

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Ichelson(1) reported regular cultivation of a spirochete resembling *Spirochaeta myelophthora,* previously described by Steiner(2). She claims to have isolated from spinal fluid of 78% of 76 persons with clinical diagnosis of multiple sclerosis, a large spiral organism morphologically resembling members of the genus, Borrelia. In contrast she was unable to obtain a single positive culture from spinal fluid of 28 persons with diagnoses of other illnesses. We believed that several aspects of this report demanded clarification and/or confirmation. First, the report that one can cultivate "Borrelia-like" organisms from 78% of the spinal fluid of persons with multiple sclerosis regardless of stage of disease should be confirmed. Secondly, no exact data were included in the original report as to time following inoculation when each culture first be-

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