STUDIES ON A SERUM SUBSTITUTE FOR MAMMALIAN CELLS IN CULTURE

2. Analyses of Fractionated Peptone Dialysate and the Use of Glycine Peptides

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SUMMARY

Amino acid analyses of two biologically efficacious fractions from a peptone dialysate (PD) revealed high concentrations of glycine, presumably present in PD as glycine-rich peptides. Before undertaking further analyses of PD, the growth-promoting abilities of several commercially available glycine peptides were tested with strain L (NCTC 2071) cells continuously subcultured in chemically defined medium NCTC 135, and with RH-PD cells which require peptone or peptone fractions for continued survival and proliferation. NCTC 2071 populations 5 to 10% greater than control cultures were obtained with 0.18 to 0.72 mM peptide-supplemented medium NCTC 135; slightly better results were obtained with peptides containing an even number of glycine residues. No amplification of this response was observed after 28 to 29 weekly culture transfers in 0.72 mM supplemented NCTC 135, and evidence suggested that the peptides were poorly hydrolyzed to monomeric glycine. Addition of the glycine peptides may permit use of a reduced minimum inoculum for NCTC 2071. Marked growth enhancement of peptone-dependent RH-PD cells was obtained only when glycine peptides were tested in the presence of 0.5 mg of PD per ml.

Key words: tissue culture, serum-free medium, glycine peptides, peptone.

INTRODUCTION

Serum has been used successfully for many years as a supplement to chemically defined tissue culture media (CDM). As in the use of embryo extracts or ultrafiltrates, a biological material such as serum can contribute to in vitro microbial contamination (1-3), and introduces a heterogenous mixture of natural products such as enzymes (4) and hormones (5) which themselves vary in type and concentration between lots. These variations may explain in part the variation in growth-promoting ability commonly observed between lots of sera. Attempts to characterize the biologically efficacious serum "growth factor(s)" represent a complex and incomplete task. Moreover, recent studies (6-8) suggest that a diversity of serum components is responsible for different in vitro cellular responses, and work in several laboratories suggests that the role of serum is more complex than was earlier thought (9-16). Although a variety of medium formulations is available (17), few will support survival and growth for a broad spectrum of cells in the absence of serum. Unfortunately, little work has been done on the development of adequate serum substitutes.

Two recent developments underscore the need for an effective, heat-stable, and preferably characterized, serum substitute for cancer research. First, Merrill et al. have demonstrated that viable bacteriophages contaminate some lots of com-
mercially available serum (18) and have also presented evidence for the transcription and expression of the viral genome in human cells (19). Chu et al. (20) have confirmed the adventitious presence of *Escherichia coli* bacteriophage in fetal calf serum, and reported a negative correlation between the titer of phage detected and the growth-promoting properties of the contaminated sera. Secondly, Evans and Andresen demonstrated the influence of serum on malignant transformation in vitro (21), and subsequently, Price et al. (22) implicated the low molecular weight serum fraction in this transformation process. The importance of these findings to clinical or research applications is obvious.

Several investigators (23-27) have used Difco Bacto-peptone as an autoclavable serum substitute for mammalian cells in culture. Pumper et al. demonstrated the activity to be dialyzable (28), and subsequently Taylor et al. (29) separated this dialysate (PD) into two fractions of varying activity. Amborski and Moskowitz (30) and Hsueh and Moskowitz (31) have reported similar results. The objectives of these studies were to analyze further the fractionated PD and to ascertain whether the biological efficacy of PD is associated with low molecular weight glycine peptides.

**Materials and Methods**

*Sephadex G-10 fractions.* Fractions of whole peptone dialysate (PD) (28) were prepared by gel filtration chromatography and assayed for biological activity as previously described (29). The biologically efficacious fractions, designated G-10(I) and G-10(II), were recycled under the same chromatographic conditions as were used in the original fractionation. Total amino acid analyses of G-10(I) and G-10(II) were performed following hydrolysis under vacuum in re-distilled 5.8 N HCl, 104 °C, 22 hr. Free amino acid determinations were made by reconstituting untreated fractions in pH 2.2 citrate buffer and analyzing the fluids as physiological solutions. All determinations were made with a Beckman model 120C amino acid analyzer by the method of Spackman et al. (32). A correction factor of 5% was used to compensate for threonine, cystine, and tyrosine decomposition, and a 10% correction was made for serine decomposition (33).

The ultraviolet spectra of whole PD, and of fractions G-10(I) and G-10(II), were obtained with a Perkin-Elmer 202 ultraviolet-visible recording spectrophotometer using an aqueous 1-mg per ml sample. Whole PD, G-10(I), and G-10(II) were analyzed for reducing sugars by means of the Molisch test at a sensitivity of 0.01%, w/w.

**Preliminary glycine peptide growth studies.** Since the biologically efficacious fractions G-10(I) and (II) were observed to have high glycine concentrations, the growth-promoting ability of several glycine peptides was assayed with strain L cells (34) continuously subcultured in protein-free, chemically defined medium NCTC 135 (35) (CDM); this cell line is designated NCTC 2071. The peptides included glycyglycine (gly)2, glycylglycylglycine (gly)3, tetraglycine (gly)4, pentaglycine (gly)5, and hexaglycine (gly)6 (Schwarz-Mann, Orangeburg, N.Y.). Peptides were tested at five concentrations, 0.045 to 0.72 mM; these represent multiples of the glycine concentration used in NCTC 135, i.e. 0.18 mM or 13.51 mg per liter. Appropriate weights of each peptide were reconstituted in triple glass-distilled water, autoclave-sterilized, and each mixed with volumes of 2× Earle’s saline, 1× Earle’s saline, and 2.5× NCTC 135 sufficient to prepare an isotonic 1× culture medium.

In preliminary studies, cells from several 5- to 6-day confluent cultures were suspended in 150 to 250 ml of unsupplemented NCTC 135 and replicate cultures in T-15 flasks prepared with 3 ml of cell suspension as previously described (36-38). Cultures were incubated 18 to 24 hr at 37.5 ± 0.15°C, randomized into control and experimental sets, and the planting medium replaced with the appropriate test fluid. At this time, three to five replicate cultures were counted to ascertain the “time-zero” cell population. Seventy-two hours later, a complete fluid renewal was performed on all cultures; a second complete fluid change was done at 120 hr if cultures were carried beyond this point. Following incubation, cell populations were harvested and counted as described (36), and the data were expressed as the average cell number observed in three to five T-15 cultures per variable.

To test the influence of these peptides on long-term growth, derivative cell lines, designated NCTC 6797-6802, were subcultured continuously in NCTC 135 supplemented with one of the test peptides at 0.72 mM. After 28 to 29