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Glycoprotein Synthesized by Cultured Cells: Effects of Serum Concentrations and Buffers on Sugar Content[1] (40490)

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There have been a number of reports in recent years which indicate that the concentrations of serum used to supplement cell culture media can have profound effects on the rates of synthesis and degradation of proteins by cells grown in culture (1–3). These effects, moreover, are not necessarily dependent upon the stage of growth of the cultures (4), nor are they limited to intracellular proteins. Recently, Chen *et al.* (5) demonstrated a decrease in the synthesis of a major fibroblast surface glycoprotein when supplemental serum levels in the medium were lowered from 10% to 0.7%. We report in this paper the changes in the sugar composition of a glycoprotein secreted by murine parietal yolk sac carcinoma (PYSC) cells (6) grown in medium supplemented with different concentrations of fetal bovine serum (FBS). This soluble glycoprotein is a component of epithelial basement membranes (EBM) and has been isolated from cultures grown in medium supplemented with 10% FBS, purified and characterized as to amino acid composition, sugar content and molecular weight (7, 8).

Serum contains a number of biochemical components which are necessary for normal cell growth and behavior. Several investigators have identified many of these components such as hormones, including growth factors, which Hayashi *et al.* consider the most important components (9), and essential fatty acids (10). Incorporating these substances into growth medium makes the use of serum unnecessary. In order to further study the phenomenon observed as the result of serum concentrations, we chose to grow the cells in chemically-defined medium, thus allowing for more precisely controlled manipulation of medium components. This study also reports the result of manipulation of one component, the buffer system, and the effects that HEPES[2] and bicarbonate buffers have on the degree of glycosylation of EBM glycoprotein.

Materials and methods. Murine carcinoma cell cultures, stored in liquid nitrogen, were started in Dulbecco and Vogt's medium supplemented with 10% FBS.[3] To study the effects of lowered serum concentrations, one-fourth of the cultures (group A) were maintained in this medium throughout the studies, while one-fourth (group B) were adapted to growth in Dulbecco and Vogt's medium supplemented with 1% FBS. This adaption was accomplished by decreasing the FBS to 5%, 2% and finally 1% on sequential medium changes. No apparent differences were detected in growth rate or morphology as a result of lowering FBS. To study the effects of different buffers, one-fourth of the cultures (Group C) was washed with sterile medium containing no FBS and subsequently fed with the chemically-defined medium described by Higuchi and Robinson (11), buffered with 13.6 m*M* $NaHCO_3$ and 28 m*M* HEPES. Group D cultures were grown in the same chemically-defined medium except that buffering was achieved with 44 m*M* $NaHCO_3$. Cultures were given fresh medium every three to 5 days. Cultures grown in each type of medium were assessed for the presence of EBM glycoprotein by indirect immunofluorescence according to the method of Coons (12).

The media from each culture group was collected and pooled for the isolation of EBM glycoprotein. Medium from group B cultures was collected in two separate pools. Pool B1 was medium collected during the first fifteen days in which the cells were grown in 1% FBS, and pool B2 was medium collected

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[2] *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

[3] Flow Laboratories, Rockville, Maryland.

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during the following 24 days (days 16 through 39). The media collected from culture groups C and D were initially discarded to minimize the effects of residual fetal bovine serum. Medium was replaced every 3–5 days. If medium was not immediately carried through the procedure for isolation of EBM glycoprotein, it was stored at 4° in the presence of 0.01% sodium azide.

Glycoproteins were isolated from each of the five pools as described previously (8) except that the glycoproteins were eluted from a column of CPG-10-350[4] with 5 m*M* Tris buffer, pH 8.9, containing 38 m*M* glycine.

The fraction which eluted with the void volume was identified as EBM glycoprotein by a passive hemagglutination assay (PHA) using sheep red blood cells (SRBC) to which was bound the glycoprotein isolated from each type of growth medium. The PHA assay was that previously described by Johnson *et al.* (13), utilizing antiserum directed against each of the glycoproteins. These antisera were raised in albino rabbits using the immunization schedule previously described (7). Additional immunological characterization was obtained by immunodiffusion of EBM glycoprotein against each of the antisera according to the method of Megaw (14).

The EBM glycoprotein from each type of growth medium was also characterized with respect to amino acid composition, sugar composition and molecular weight. Total amino acids were determined on lyophilized glycoprotein, hydrolyzed in 6 *N* HCl for 22 hr at 110° *in vacuo*. The amino acids were separated in a Joelco model JLC-6AH analyzer according to the method of Moore and Stein (15). Terminal amino acids were also determined. The amino terminals were reduced and alkylated according to Raftery and Cole (16) and after cleavage from the glycoprotein were analyzed by the method of Stark and Smyth (17). The carboxy-terminal amino acids were determined by the method of Fraenkel-Conrat and Tsung (18).

The constituent sugars were analyzed primarily by gas–liquid chromatography. Monosaccharides were cleaved from the oligosaccharide chains and methylated. The methyl monosaccharides were converted to trimethylsilyl derivatives, separated and quantitated as previously described (7). The content of sialic acids was determined colorimetrically by the thiobarbituric acid assay of Codington *et al.* (19).

The molecular weights of the glycoproteins were estimated by electrophoresis of reduced, denatured glycoproteins on SDS-acrylamide gels[5] according to Laemmli (20). A group of standard proteins ranging in molecular weight from 12,400 (cytochrome C) to 90,000 (transferrin) was simultaneously electrophoresed for comparison. The gels were stained with Coomassie blue and destained by diffusion into 7.5% acetic acid.

Results. The carcinoma cells appeared to grow as well in chemically-defined medium as they did in Dulbecco and Vogt's medium supplemented with FBS. Specific staining of fibrillar extracellular material, which represented secreted EBM glycoprotein, was noted in all culture groups. This staining pattern was similar to that previously described when cultured cells have been examined (21). The secreted EBM glycoprotein, isolated from different types of growth media was found to be anitgenically identical to the completely glycosylated EBM glycoprotein by both immunodiffusion and passive hemagglutination assay (PHA). The immunodiffusion patterns indicated lines of identity between each glycoprotein as shown in Fig. 1. The results of PHA are shown in Table I. Antibody titers against EBM glycoprotein obtained from 10% FBS supplemented medium tended to be higher than antibody titers against EBM glycoprotein isolated from chemically-defined media. As the data in Table I show, each antiserum agglutinated SRBC's sensitized with the glycoprotein from each type of medium. Agglutinating ability could be removed if each antiserum was absorbed with any antigen prior to incubation with sensitized cells. Agglutination did not occur if unsensitized cells were incubated with specific antiserum or if sensitized cells were incubated with normal rabbit serum. These data indicate that the glycoprotein secreted into the medium is immunologically the same regard-

[4] Controlled-pore glass from Electro-Nucleonics, Inc., Fairfield, New Jersey 07006.

[5] Either 5.0 or 7.5% acrylamide.

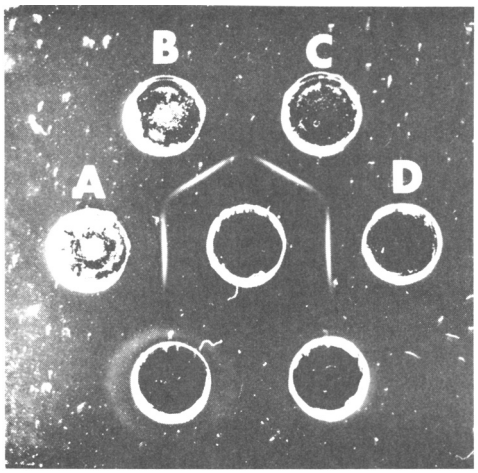

FIG. 1. Immunodiffusion patterns produced by reacting glycoproteins isolated from medium pools A, B, C and D (peripheral wells) with antiserum specific for maximally glycosylated EBM glycoprotein (central well). The reactions of the glycoprotein from each type of growth medium with the antiserum were identical.

TABLE I. PHA OF EBM GLYCOPROTEIN-SENSITIZED SRBC.[a]

Antisera	Antigens				
	A	B	C	D	FBS
Anti-A	>1024	>1024	>1024	>1024	0
Anti-B	1024	1024	512	512	0
Anti C	512	512	256	256	0
Anti D	256	256	256	256	0
Anti A (abs)	0	0	0	0	0
Anti B (abs)	0	0	0	0	0
Anti C (abs)	0	0	0	0	0
Anti D (abs)	0	0	0	0	0

[a] SRBC were sensitized with the glycoprotein (antigens) isolated from the medium of culture groups A, B, C or D or with FBS. The antigen from group B cultures was that from later cultures (B_2). Sera were also absorbed (abs.) with each of the glycoproteins prior to incubation with sensitized SRBC. The numbers represent the reciprocal of the final antiserum dilution which resulted in hemagglutination.

less of the type of medium in which the cells are grown.

The amino acid composition of the EBM glycoprotein from each type of medium is shown in Table II. No appreciable differences were noted. The amino terminal residue of the EBM glycoprotein was glycine, and the carboxyl terminus was found to be proline.

The sugar compositions of the glycoproteins are given in Table III. The total sugar contents of the glycoprotein from group A

TABLE II. AMINO ACID COMPOSITION OF EBM GLYCOPROTEINS (RESIDUES SPECIFIC AMINO ACID/1000 TOTAL RESIDUES).[b]

Amino acid	A	B_1	B_2	C	D
Lysine	62	61	62	60	59
Histidine	20	19	19	19	18
Arginine	40	42	43	43	43
Aspartic acid	98	98	99	99	97
Threonine	61	62	64	62	61
Serine	73	73	73	72	74
Glutamic acid	100	101	102	102	100
Proline	48	47	47	46	47
Glycine	101	102	99	104	105
Alanine	100	103	104	104	105
Half-cystine	21	20	21	20	21
Valine	65	66	67	64	66
Methionine	9	9	10	10	9
Isoleucine	42	44	45	46	42
Leucine	90	90	90	91	89
Tyrosine	26	26	26	27	26
Phenylalanine	40	39	38	39	38
Amino terminus	Gly	Gly	Gly	Gly	Gly
Carboxy terminus	Pro	Pro	Pro	Pro	Pro

[b] Lyophilized glycoprotein isolated from each of the types of growth medium was hydrolyzed at a concentration of 1 mg/ml in constant boiling HCl for 22 hr at 110° *in vacuo*. Amino acid analyses were performed on a Joelco Model JLC6AH analyzer, according to the method of Moore and Stein (15). The amino terminal residues were determined on reduced, alkylated glycoprotein (16) by the cyanate method (17). The carboxy terminal residue was liberated from the peptide following heating in the presence of hydrazine (18).

cultures, and from medium pools B1 and B2 were 13.5, 5.0 and 1.7% by weight, respectively. Cells grown in media C and D secreted EBM glycoprotein containing 7.5% and 3.6% sugar respectively.

The SDS-acrylamide gel electrophoresis of reduced, denatured glycoproteins from medium pools A, B, C and D resulted in a single, usually rather broad, band in each case (Fig. 2). The molecular weights approximately were 30,000, 27,000, 28,000 and 26,500 daltons respectively, and the differences can be attributed to the differences in sugar content of the glycoproteins. Occasionally, more than one band could be seen when a glycoprotein was electrophoresed. In all cases, additional bands were polymeric forms and could be eliminated by more extensive reduction and denaturation. This phenomenon was noted when the EBM glycoprotein was initially characterized (8).

Discussion. As indicated in this paper, the sugar composition of the glycoprotein we have studied is dictated to a great degree by

TABLE III. SUGAR COMPOSITION OF EBM GLYCOPROTEINS EXPRESSED IN μg SUGAR/mg OF LYOPHYLIZED GLYCOPROTEIN.[a]

	Glycoproteins				
Sugar	A	B_1	B_2	C	D
Fucose	1.55	1.30	1.21	1.42	1.14
Mannose	18.00	12.20	1.05	3.11	0.70
Galactose	20.60	11.90	3.65	7.10	0
Glucose	10.00	3.95	2.70	10.10	0
N-acetylgalactosamine	41.50	3.70	3.40	27.70	0
N-acetylglucosamine	40.30	16.40	3.85	22.68	1.80
Sialic acids	3.40	0.90	0.70	2.20	0
	135.35	50.35	16.56	74.40	3.64

[a] The heterosaccharides of glycoproteins from all medium pools were cleaved with 500 m*M* HCl in anhydrous methanol. The resultant methylmonosaccharides were separated as the trimethylsilyl derivatives by gas–liquid chromatography. Sialic acids were determined by the thiobarbituric acid assay following hydrolysis of each glycoprotein with 0.1 *N* H_2SO_4 at 80° for 1 hr.

the composition of the medium in which the murine carcinoma cells are grown; however, there does not appear to be any appreciable difference in the amount of EBM glycoprotein synthesized under the different growth conditions.

With regard to the role of serum, the extent of decrease in sugar content appears to depend upon the length of time that the cultures have been growing in medium supplemented with only 1% FBS, as is indicated by the difference in sugar content of the glycoprotein isolated from the medium pools B1 and B2. When this phenomenon was initially observed, the cells had been in 1% FBS-supplemented medium for several months. The only detected sugars were hexosamines, predominantly *N*-acetylglucosamine, thus indicating further failure of glycosylation (22). The progressive decline in sugar content of the glycoprotein with time may reflect progressive depletion of sugar pools by the cells, coupled with diminished availability of sugars supplied by the serum. Alternatively, it may reflect depression in the activity of glycosyltransferases. A third possibility is that some factor in FBS may repress endogenous glycosidase activity. However, with regard to exogenous glycosidases, Block *et al.* (23) recently reported that glycosidic activity detectable in various sera did not alter cell

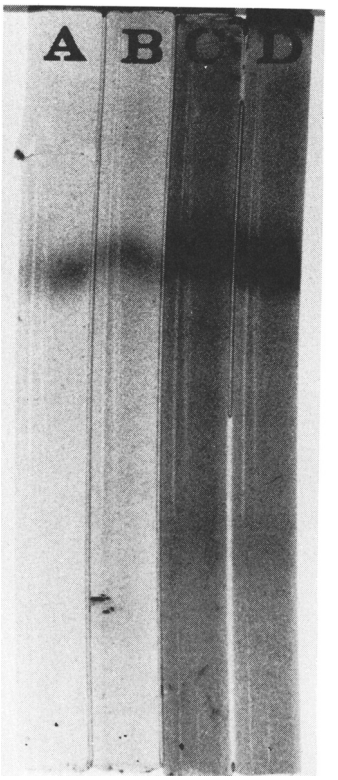

FIG. 2. SDS-acrylamide gel electrophoresis of glycoproteins isolated from medium pools A, B, C and D. Lyophilized glycoproteins were dissolved in 0.1 *M* Tris–HCl buffer, pH 8.2 containing 1% sodium dodecyl sulfate and 5% 2-mercaptoethanol. After incubation at 100° for 5 min followed by 2 hr at 37°, the reduced, denatured glycoproteins were electrophoresed on 7.5% acrylamide gels at 4 ma per tube at pH 8.2. Gels were stained with Coomassie blue. After destaining, only one characteristically diffuse band with an apparent molecular weight of approximately 28,000–30,000 daltons was identified in each gel. The width of the band is probably the result of overloading gels to insure that only one protein was present. Less heavily loaded gels yielded a much more compact band with the same apparent molecular weight.

growth or differentiation, indicating little if any modification of cell surface glycoproteins or glycolipids. Assuming that no degradation occurs, the secretion of minimally glycosylated protein is of interest from the standpoint of determining the unifying functional role of sugars covalently linked to proteins. According to most hypotheses, the presence of sugar determines the destination of the protein within the organism (24, 25). For example, glycoproteins are predominantly, if not

solely, extracellular. It has been proposed that the sugar component of a glycoprotein acts as a signal to the cell which indicates that the glycoprotein should be transported to an "extracellular" site. If this is indeed the case, our data suggests that surprisingly little sugar is required to fulfill this role. As yet, we cannot comment with regard to the effect decreased glycosylation might have on the biological behavior of this glycoprotein. This glycoprotein interacts with type IV collagen to produce the basement membrane associated with epithelial cells. Low sugar content might well be expected to alter or abolish this interaction resulting in a basement membrane with abnormal properties.

As the studies reported in this paper indicate, the choice of buffer is also critical in terms of the sugar composition of the isolated EBM glycoprotein. The impetus for examining the effects of HEPES buffer on glycosylation of this protein came from the studies of Daniel and Wolf (26) in which they found impaired incorporation of [^{3}H]fucose into a cell surface glycoprotein synthesized by tracheal epithelium. However, they observed no inhibition of incorporation into secreted glycoproteins, indicating some differences in the effect of HEPES on the glycosylation of these two types of glycoproteins, with respect to fucose incorporation. They did note a rather marked inhibition of glucosamine incorporation into secreted glycoproteins. Daniel and Wolf were not able to explain the inhibitory effects of HEPES on glucosamine incorporation, and we cannot explain the apparent stimulatory effects of HEPES in our studies. Although HEPES is presumably metabolically inert and resistant to passage across cell membranes (27), there are several reports of enhanced enzyme activity attributable to this zwitterionic buffer (28–30). Although none of these reports deal with glycosyltransferases, the possibility of their enhancement cannot be ruled out.

Whatever the biological significance of the data produced in this study, the results do demonstrate the need for careful delineation of growth conditions before drawing conclusions about glycoproteins synthesized and secreted by cells in culture.

Summary. A cell line derived from a murine teratocarcinoma was cultured in growth medium supplemented with either 10% or 1% fetal bovine serum or was grown in chemically-defined medium buffered with either a combination of HEPES and sodium bicarbonate or with bicarbonate alone. The sugar content of a particular glycoprotein secreted by these cells was markedly different in each case. No differences were noted in antigenicity or amino acid composition, including amino and carboxyl terminal amino acids. Differences in molecular weight were attributable to the difference in carbohydrate content. These data indicate that the level of glycosylation of proteins in vitro is dependent on the medium composition, including apparently unsuspected components such as buffers.

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