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Choriogonadotropin-Like Antigen in a Strain of *Streptococcus faecalis* and a Strain of *Staphylococcus simulans*: Detection, Identification, and Characterization

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The presence of choriogonadotropin- and α-subunit-like materials in two species of bacteria identified as *Staphylococcus simulans* and *Streptococcus faecalis* have been demonstrated by the indirect fluorescein-labeled and the indirect peroxidase-labeled immunocytochemical techniques, utilizing antiserum for human choriogonadotropin, for its α and β-subunits and the β-subunit COOH-terminal peptide. The bacteria were originally isolated from the urine of two patients with advanced forms of cancer. Chromatography done on the water-soluble extract of acetone powder preparations of the bacterial cultures revealed the presence of a material similar to the complete trophoblastic hormone and to its β-subunit in the culture media of *S. simulans*, and to the β-subunit in the media of *S. faecalis*. No free α-subunit was detectable. Furthermore, the choriogonadotropin-like factor demonstrated biological activity in in vivo assay systems. From the present results, it can be concluded that some species of "cancer-associated" bacteria can synthesize a human trophoblastic hormone-like glycoprotein with physicochemical properties similar to those of the human trophoblastic hormone that is biologically active and that is either released complete or as one of its subunits in the culture media.

The presence of choriogonadotropin (CG)-like immunoreactive material in a group of aerobic and anaerobic bacterial strains, originally isolated from tissues of patients having a variety of malignant neoplasms, has been demonstrated by the use of the indirect fluorescein-labeled and the indirect peroxidase-labeled techniques, utilizing antiserum to human CG as well as to its α and β subunits (CGα and CGβ) and fragments (CGβ COOH-terminal peptides) as first antibodies (1, 2). Immunoelectron microscopy revealed that the material was associated mainly with the membranes of the cell wall (15). The CG-like antigen was detected neither in other bacteria from similar sources nor in a wide variety of "noncancer" control bacteria that included strains from the American Type Culture Collection (ATCC) as well as laboratory isolates. These findings suggested that not all bacteria isolated from cancer patients (hereafter called cancer-associated bacteria) have the capability to synthesize the CG-like immunoreactive compound, at least in detectable amounts, and that the expression of the antigen by bacteria is not ubiquitous (1, 2; H. F. Acevedo, E. A. Campbell-Acevedo, and M. Slifkin, *in* S. J. Segal, ed., *Chorionic Gonadotropin*, in press).

Further work done by Maruo and his associates (12; T. Maruo and S. S. Koide, Abstr. 62nd Annu. Meet. Endocrine Soc. 1980, abstr. no. 10, p. 77) demonstrated that the material obtained from one bacterial culture possessed physicochemical and biological characteristics of the human trophoblastic hormone including in vivo biological activity in the rat uterine weight assay. The investigators concluded that except for a difference in biological potency, all of the properties of the bacterial material and native human CG were similar.

The findings of a de novo biosynthesis and expression of a membrane-associated CG-like antigen by malignant cells tested from tissue cultures or experimental animals, as well as from the great majority of human malignant neoplasms that have been studied (1, 2), have led us to undertake a systematic investigation for the presence and localization of the CG-like antigen in bacteria to determine (i) if the presence of the CG-like antigen is limited to bacteria isolated from humans and animals with cancer, (ii) to which extent bacteria not associated with clini-

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cal cancer, to the best of our knowledge, express the CG-like antigen, and (iii) the clinical frequency of the phenomenon. As part of this investigation, we wish to report here the detection, identification, and characterization of the trophoblastic hormone-like protein from two laboratory isolates, a strain of *Streptococcus faecalis* and a strain of *Staphylococcus simulans*.

MATERIALS AND METHODS

Bacteria. The strain of *S. faecalis* was isolated from the urine of a patient (AK no. 082788) confined at Queens Hospital, Honolulu, Hawaii. The patient had an invasive adenoid cystic carcinoma of the nasopharynx and ethmoid sinus with bone metastases. The second isolate, *S. simulans*, was also isolated from the urine of a patient with generalized metastatic carcinoma of undetermined origin. ATCC strain 19433 of *S. faecalis*, Lancefield group D, and ATCC strain 27848 of *S. simulans* were used as reference strains for identification. *S. faecalis* ATCC 12818, isolated from a patient with advanced epidermal carcinoma of the gingival ridge, *Pseudomonas maltophilia* ATCC 13637, obtained from the oropharyngeal region of a patient with mouth cancer, *Escherichia coli* strain M3, isolated from the neoplastic tissue of a patient with carcinoma of the colon, and *E. coli* strain TZ, isolated from the urine of a woman in week 32 of pregnancy, were used as positive and negative cell controls (1, 2, 12; Acevedo et al., in press).

All bacteria were identified by standard morphological, nutritional, and biochemical tests. Gram stain and the Kinyoun acid-fast test procedures were performed on all the microorganisms. The bacteria were grown in Trypticase soy broth at 35°C and 5% sheep blood agar with Columbia base (BBL Microbiology Systems) for 18 h at 35°C.

Hormones and special reagents. Highly purified human CG (CR-119), CGα (CR-119-1), and CGβ (CR-119-2), used as standard preparations for radioimmunoassay (RIA) and radioreceptor assay (RRA) systems, were gifts from the Center for Population Research of the National Institute of Child Health and Human Development, Bethesda, Md., and the Department of Medicine, Columbia University, College of Physicians and Surgeons, New York, N.Y.

The origins, characteristics, and sources of the specific antibodies against the complete hormone and its subunits have been previously reported (1, 2, 12; Acevedo et al., in press). To test by immunocytochemistry for the presence of sialic acid in the CG-like material, we used antiserum R-141, an anti-CGβ COOH-terminal peptide, a sialic acid-sensitive antiserum which, at a given concentration, binds only asialo CG (and does not bind the native hormone), and antiserum R-525, another anti-CGβ COOH-terminal peptide that binds both asialo CG and complete CG (S. Birken, R. Canfield, G. Agosto, and J. Lewis, Abstr. 62nd Annu. Meet. Endocrine Soc. 1980, abstr. no. 249, p. 137; S. Birken and R. Canfield, *in* S. J. Segal, ed., *Chorionic Gonadotropin*, in press). Both antisera were obtained from the Department of Medicine, Columbia University, College of Physicians and Surgeons, New York, N.Y. All of these antisera were used as first antibodies.

Fluorescein-labeled goat anti-rabbit IgG and peroxidase-labeled goat anti-rabbit IgG, utilized as second antibodies, nonimmunized rabbit serum, total as well as IgG fraction (light and heavy chain specific), and rabbit anti-horse antiserum, utilized as controls for specificity, were obtained commercially from Cappel Laboratories, Downingtown, Pa., and from GIBCO Laboratories, Grand Island, N.Y.

Neuraminidase (mucopolysaccharide *N*-acetylneuraminylhydrolase; EC 3.2.1.18) was obtained from Behring Diagnostics, Summerville, N.J. This brand of *Vibrio cholerae* neuraminidase (VCN) does not contain proteases, aldolase, or lecithinase C. The solution contains no preservatives, and has an activity of 500 U/ml.

Immunocytochemical methods. Detection of the CG-like immunoreactive material was done at the light microscope level by utilizing the indirect fluorescein-labeled and the indirect peroxidase-labeled reactions by procedures previously published in detail (1). For examination, a drop of sterile water was placed in each one of the circles of a Fluoro slide (Curtin Matheson), and then the surface of a colony of the respective microorganisms was gently touched with a bacteriological loop and mixed into the drop of water in each circle. After air drying, the preparations were immediately processed or were stored at −70°C.

Neuraminidase incubations. Testing for the presence of sialic acid in the CG-like material in situ required VCN treatment of the bacteria. This was done by suspending the microorganisms (moderate turbidity) in 0.2 ml of sterile water and adding 50 μl of VCN. The bacteria were incubated for 30 min at 37°C and then centrifuged in a clinical centrifuge for 10 min at 2,800 rpm. After the supernatant was discarded, the samples were washed three times with sterile water. After the last wash, the microbes were suspended in 0.2 ml of sterile water and blended briefly in a Vortex mixer. Portions (25 μl) from these suspensions were used for immunocytochemical analysis.

Preparation of extracts from bacterial cultures. The preparation of extracts was done as previously described (12). Briefly, the bacteria were grown in Trypticase soy broth for 24 h at 37°C. The pH of the culture was then adjusted to 4.5 with glacial acetic acid (5 ml/liter). Five volumes of cold acetone were added under constant stirring, and the mixture was stored at 4°C overnight. The supernatant was aspirated off. The sediment was suspended in 100 ml of water and homogenized. After 1 h, the homogenate was centrifuged for 10 min at 10,000 × *g*. The supernatant was lyophilized.

RIA and RRA. All RIAs were performed by the double-antibody techniques as described by Maruo et al. (12). RRA was done by the method of Saxena et al. (13). The plasma membranes of bovine corpus luteum for RRA were obtained from Wampole Laboratories, Cranbury, N.J. For these assays, the acetone powder of the cultures was suspended in distilled water, dialyzed, and centrifuged. Assays were done on the supernatant.

Chromatography. Subsequent purification of the water-soluble portion of the acetone powder extract

was done by Sephadex column chromatography, using Sephadex G-100, concanavalin A-Sepharose column chromatography, and ion-exchange chromatography on diethylaminoethyl Sephadex A-50 performed as previously described (12).

Polyacrylamide gel electrophoresis. Electrophoresis of the purified material obtained from diethylaminoethyl Sephadex A-50 chromatography was performed in a 7.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate by the method of Weber and Osborn (17). The gels were stained with 0.2% Coomassie brilliant blue.

Bioassay. In vivo biological activity of the purified CG-like material was determined by uterine and ovarian weight assays by the classical bioassay method of Diczfalusy and Loraine (7), using the second international standard human CG as a reference.

RESULTS

Identification of microorganisms producing immunoreactive CG. The bacteria isolated from the patient with disseminated carcinoma of the nasopharynx were gram-positive cocci. On the blood agar plate, there were small, off-white entire colonies without hemolysis. Scanty growth was obtained on eosin-methylene blue agar. The growth was slightly cloudy with sediment in Trypticase soy broth. Optimal temperature for growth on the Columbia blood agar plate was 30 to 35°C. Acid fastness was not demonstrated. The results of the tests done as compared with those given by the reference strain *S. faecalis* ATCC 19433 identified the microorganisms as a strain of *S. faecalis* Lancefield group D enterococcus, designated as strain AK.

The microorganisms isolated from the patient having an unknown primary with generalized metastasis were also gram-positive cocci. The microbes formed dry, white colonies with a complete margin interspersed with gray-white colonies with slight hemolysis when grown on blood agar. Acid fastness was not demonstrated. The results of the tests done (9, 14) as compared with those given by the reference strain *S. simulans* ATCC 27848 permitted us to identify the bacteria as *S. simulans*, designated as strain RU-1.

Immunocytochemistry. Both cancer-associated bacterial strains *S. simulans* RU-1 and *S. faecalis* AK presented a positive reaction when screened with the indirect immunofluorescein reaction utilizing antiserum to total CG. Positive results were also obtained when they were subsequently tested with the indirect immunoperoxidase reaction and subjected to all the reagent and cell controls that we have previously described (1, 2). Both bacteria also gave positive results with both reactions when tested using antiserum to CGα (R-112) and antiserum to CGβ (H-9 and R-126) as first antibodies. Examples of these results are illustrated in Fig. 1 to 5. Identical results were also obtained with both anti-CGβ COOH-terminal peptides R-141 and R-525. The reactivity to antiserum R-141 of both bacteria was clearly influenced by VCN treatment, as illustrated by one of the titrations depicted in Table 1, whereas the treatment did not produce any effect with antiserum R-525, the anti-CGβ COOH-terminal peptide insensitive to the presence of sialic acid, as shown in Table 2.

Culture extracts. The results of the studies performed with the acetone powder preparations of the culture of *S. simulans* RU-1 were basically those described in a previous publication (12). Briefly, the extracts contained CG-like material as determined by RIA with antiserum to CGβ and to CGβ COOH-terminal peptide and RRA with bovine corpus luteum membranes. In contrast to the immunocytochemical results, no free α subunit was detected. The CG-like material was eluted at the same position as standard human CG and CGβ in Sephadex G-100. It was also adsorbed by concanavalin A-Sepharose and

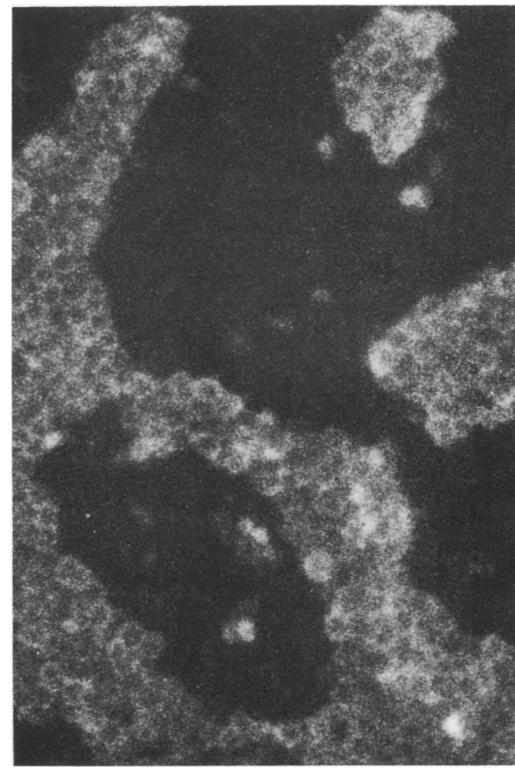

FIG. 1. *S. simulans RU-1 positive indirect immunofluorescein reaction with antiserum to total CG (1:20) as first antibody and fluorescein-labeled goat anti-rabbit IgG (1:20) as second antibody. Screening test.* ×*1,520.*

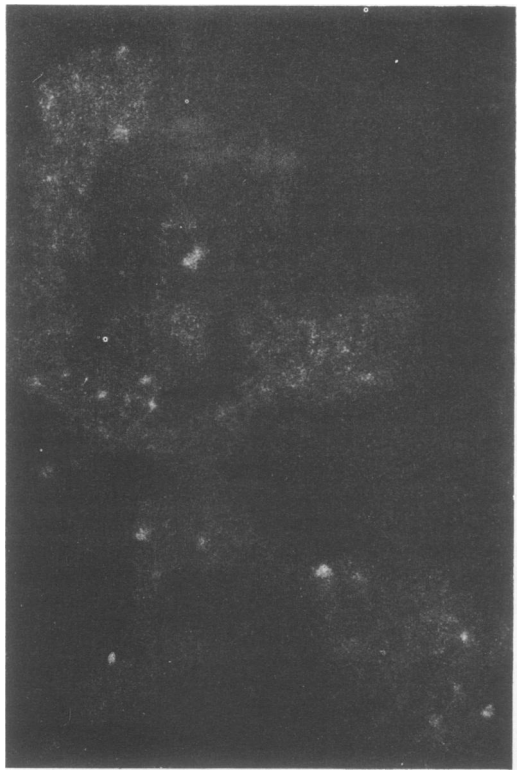

FIG. 2. *Reagent control of reaction shown in Fig. 1. The first antibody was replaced by nonimmunized rabbit serum. Conditions were as in Fig. 1. Nonspecific attachment of second antibody did not occur. ×1,520.*

eluted with 0.2 M methyl α-D-glucopyranoside, indicating the presence of carbohydrate moieties. When the concanavalin A-adsorbed material was applied to a column of diethylaminoethyl Sephadex A-50 and eluted with a linear gradient, the CG-like material was eluted in a single peak, with the elution position corresponding to that found for trophoblastic human CG. This purified material dissociated into bands corresponding to the standards of human CGα and CGβ, respectively, when subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The bacterial CG-like material also stimulated the uterine and ovarian weights in immature female rats, demonstrating gonadotropin biological activity in vivo.

The results of the studies performed with the preparations of the culture of *S. faecalis* AK demonstrated that the extract contained about 40 pg of CG and CGβ-like immunoreactive material per mg by weight, as assayed by homologous RIA for CGβ. As in the case of the *S. simulans* RU-1 extracts, no free CGα was detected, but in contrast to the results obtained with the extracts of *S. simulans* RU-1, the extracts of the *S. faecalis* AK cultures demonstrated immunoreactivity only with anti-CGβ when subjected to gel filtration on Sephadex G-100, as shown in Fig. 6.

Immunoreactive CG-like material or its subunits were not detected by RIA in the acetone powder preparations of the cultures of *S. faecalis* ATCC strain 12818 and *P. maltophilia* ATCC strain 13637 utilized as cell controls.

Finally, examination of the acetone precipitates of the bacterial cultures revealed the presence of intact bacteria, indicating that the CG and CGβ-like material found in the purified acetone powder preparations corresponded to material found in the culture media, not in the bacteria themselves.

DISCUSSION

The two microorganisms under study were isolated from cancer patients and identified as *S. faecalis* and *S. simulans*. The initial identification of *S. faecalis* AK, done at Queens Hos-

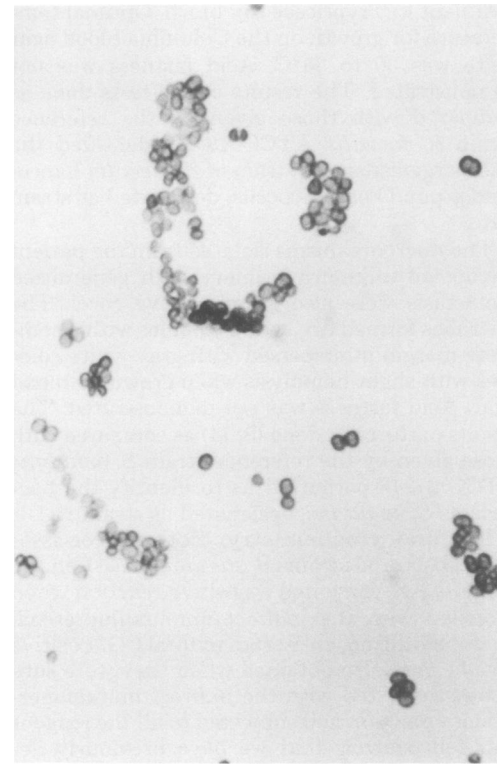

FIG. 3. *S. simulans RU-1 positive indirect immunoperoxidase reaction with antiserum to CGα (R-112) (1:10) as first antibody. ×1,600.*

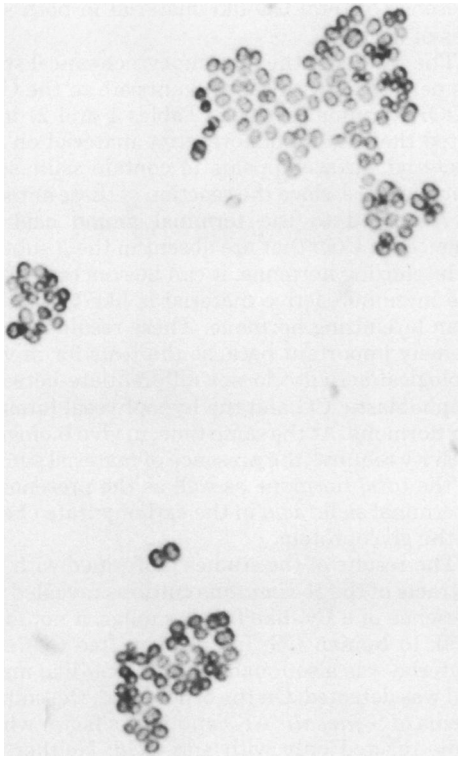

FIG. 4. *S. simulans RU-1 positive indirect immunoperoxidase reaction using antiserum to CGβ (R-126) (1:10) as first antibody. ×1,600.*

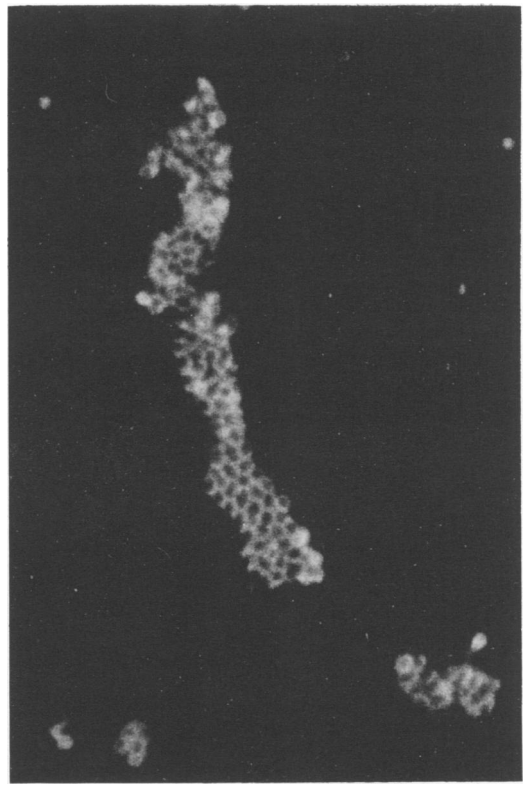

FIG. 5. *S. faecalis AK positive indirect immunofluorescein reaction using antiserum to CGα (R-112) as first antibody. ×1,520.*

pital in Hawaii, was confirmed by the Department of Infectious Diseases of the Memorial-Sloan-Kettering Cancer Center, New York, and the Section of Microbiology and Immunology, Department of Laboratory Medicine, Allegheny General Hospital, Pittsburgh, Pa. The final identification of *S. simulans* RU-1 was also done in Pittsburgh, and was confirmed by the *Staphylococcus* and *Streptococcus* Section of the Center for Disease Control, Atlanta, Ga.

It is important to note that the identification of these two strains, which we refer to as cancer-associated bacteria, continues the trend observed in our previous reports; that is, the capability to synthesize CG-like material appears to reside only in species belonging to the so-called normal flora and not to overt pathogens (1, 2; Acevedo et al., in press).

The presence of the membrane-associated CG-CGβ and CGα-like materials in both bacterial strains was clearly demonstrated by immunocytochemistry. Because of the cross-reactivity of the anti-CGβ or anti-CGβ COOH-terminal peptide with total CG, the simultaneous pres-

TABLE 1. *Effects of VCN treatment of S. simulans RU-1 in the indirect immunoperoxidase reaction with sialic acid-sensitive antiserum (R-141) to CGβ COOH-terminal as first antibody*[a]

Slide no.	Antiserum concn	VCN treated		Slide no.	Untreated	
		Test reaction	Reagent control reaction		Test reaction	Reagent control reaction
1	1:2	+	−	1a	+	−
2	1:5	+	−	2a	+	−
3	1:10	+	−	3a	−	−
4	1:20	+	−	4a	−	−
5	1:40	−	−	5a	−	−

[a] +, Positive reaction for the indirect immunoperoxidase reaction, identical to those illustrated in Fig. 3 and 4. The test reaction is considered negative (−) when it becomes indistinguishable with that of the reagent control reaction that is always performed simultaneously on the same slide. Since all the reactions are done at the same time with the same reagents, the conditions of the experiment are maintained identically.

ence of total CG-like and CGβ-like materials cannot be ascertained by this test. On the other hand, a positive immunocytochemical reaction obtained with anti-CGα indicates the possible presence of CGα-like material because anti-CGα does not cross-react with the complete hormone. It is also important to note that cross-reactivity of anti-CGα and serine proteases is unlikely since the CAGY sequence, common to proteases and CG, is found only in the β subunit of CG (10). Therefore, the positive reactions observed with anti-CGα indicated the expression of a free CGα-like material in the cell membrane of both species of bacteria and gave virtual assurance of the presence of total CG-like material in both species of bacteria.

TABLE 2. *Effects of VCN treatment of S. simulans RU-1 in the indirect immunoperoxidase reaction with antiserum to CGβ COOH-terminal R-525, insensitive to the presence of sialic acid as first antibody*[a]

Slide no.	Antiserum concn	VCN treated: Test reaction	VCN treated: Reagent control reaction	Slide no.	Untreated: Test reaction	Untreated: Reagent control reaction
1	1:2	+	−	1a	+	−
2	1:5	+	−	2a	+	−
3	1:10	+	−	3a	+	−
4	1:20	+	−	4a	+	−
5	1:40	+	−	5a	+	−
6	1:80	±	−	6a	±	−
7	1:160	−	−	7a	−	−

[a] See Table 1, footnote *a*.

The results of the immunocytochemical studies performed with the two antisera to the CGβ COOH-terminal peptide (Tables 1 and 2) indicated that the immunoreactive material on the bacterial surface appears to contain sialic acid. Furthermore, since the reaction of these antisera is restricted to the terminal amino acid sequences of CGβ that are absent in the β subunit of luteinizing hormone, it can be concluded that the immunoreactive material is like CG rather than luteinizing hormone. These results are extremely important because the tests for in vivo biological activity do not differentiate between trophoblastic CG and the hypophyseal luteinizing hormone. At the same time, in vivo biological activity requires the presence of material similar to the total hormone as well as the presence of a terminal sialic acid in the carbohydrate chains of the glycoprotein.

The results of the studies performed with the extracts of the *S. simulans* cultures revealed the presence of a CG-like factor similar, if not identical, to human CG. In addition, free CGβ-like material was also found, but no CGα-like material was detected. On the other hand, the culture media of *S. faecalis* AK contained a factor which cross-reacted only with anti-CGβ. Neither the complete hormone nor its α subunit was detected in the chromatographically purified material.

Based on all these results, it appears that the microbes synthesize material similar to both

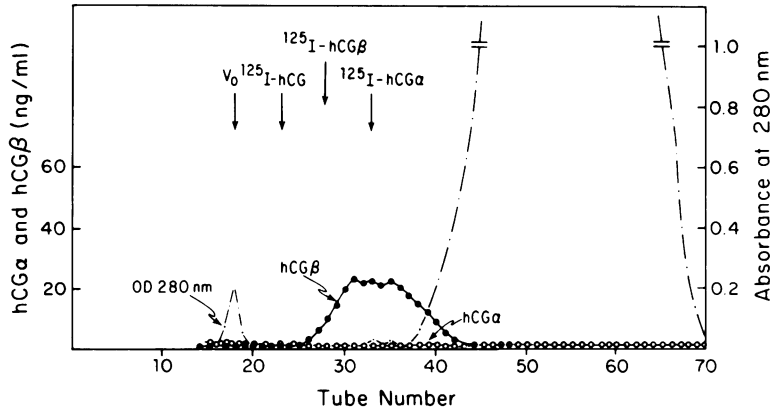

FIG. 6. *Gel filtration of an acetone powder-water extract of a subculture of S. faecalis AK on Sephadex G-100. The lyophilized acetone powder was purified by dissolving 100 mg in 2.0 ml of 0.01 tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.4)–0.15 M NaCl. The mixture was then applied to a column (1.4 by 85 cm) of Sephadex G-100, equilibrated at 4°C with 0.01 M Tris-hydrochloride–0.15 M NaCl. Each 2.0-ml fraction was assayed by RRA and RIA systems for human (h)CG, hCGα, hCGβ, and CGβ COOH-terminal peptide. Protein content of the eluted fractions was estimated by measuring absorbance at 280 nm. V_o represents void volume determined by elution of blue dextran. The vertical arrows indicate the elution positions of ^{125}I-labeled human CG, CGβ, and CGα.*

subunits of CG and that both microbes release free immunoreactive CGβ-like material, whereas only the complete hormone-like material of *S. simulans* RU-1 appears to find its way into the culture media.

The discrepancy between some of the immunocytochemical findings and the results of the analysis of the extracts may be explained by the demonstration that the bacterial walls were not broken by the cold acetone treatment and may account for the inability to extract the membrane-associated glycoproteins (15). This lack of physical interaction may also explain the negative results obtained in the analyses of the extracts of *P. maltophilia* ATCC 13637, a cancer-associated bacterial strain that was previously shown to express CG-like material by immunocytochemistry (1).

The present findings support the thesis that in the bacteria, as in mammalian trophoblasts, the biosynthesis of each subunit appears to be regulated by separate genes (6), although the production of large species of CG-like substances cannot be excluded (12; T. Maruo, S. J. Segal, and S. S. Koide, Acta Endocrinol. [Copenhagen], in press).

The apparent excessive release of CGβ-like protein is unusual. Significant levels of free CGα are found in the sera of pregnant women and in placental tissue, whereas the level of free CGβ present is low or absent (3–5, 8, 11, 16). The observed differences may be due to a variation in production or in release mechanisms or to both. This variability in the production of the α and β subunits suggest that the biosynthesis of CG by human trophoblasts and of CG-like material by bacteria may be under different control mechanisms.

Finally, the findings of the present studies added to those of our previous investigations (1, 2, 12, 15) continue to support the possibility of the existence of a biological association between these intriguing microorganisms and clinically manifested cancer.

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