

Choriogonadotropin-Like Antigen in an Anaerobic Bacterium, *Eubacterium lentum*, Isolated from a Rectal Tumor

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Using the indirect fluorescein-labeled and indirect peroxidase-antiperoxidase-labeled immunohistochemical techniques, and utilizing both antiserum specific for the β -subunit of choriogonadotropin and antiserum for the total hormone, we have demonstrated the presence of a choriogonadotropin-like immunoreactive material in a strain of *Eubacterium lentum* that was originally isolated from a rectal tumor. In contrast, both immunohistochemical reactions were negative when applied to a strain of *Corynebacterium parvum* and to pathogenic and nonpathogenic strains of *Agrobacterium tumefaciens*. Our results demonstrate for the first time the expression of the choriogonadotropin-like antigen in an obligate anaerobe and support our previous findings that the choriogonadotropin-like material appears to be expressed only in "cancer-associated bacteria" but that not all bacteria associated with the malignant neoplasms have the capacity to express the antigen, at least in amounts detectable by immunohistochemistry.

Our previous investigations (1) demonstrated the de novo synthesis and expression of a wall-associated choriogonadotropin (CG)-like immunoreactive material in subcultures of a group of aerobic bacterial strains that were originally isolated from tissues of patients bearing a variety of malignant neoplasms. These microorganisms were identified on the basis of standard bacteriological procedures as *Staphylococcus epidermidis* (12 strains), *Escherichia coli* (2 strains), and *Pseudomonas maltophilia* (1 strain, ATCC 13637).

The absence or lack of expression of the CG-like "antigen" in other "cancer-associated bacteria," *Streptococcus faecalis* ATCC 12818 from gingival cancer and a strain of *Pseudomonas aeruginosa* isolated from a carcinoma of the colon, suggested that not all cancer-associated bacteria have the capability to synthesize and express the material, at least in detectable amounts. Negative results were also obtained with 48 strains of "noncancer control" bacteria, which included 19 strains from the American Type Culture Collection and 29 laboratory isolates, indicating that the expression of the CG-like immunoreactive material is not ubiquitous.

We and others have also found de novo biosynthesis and expression of a membrane-associated CG-like antigen by every cell classified as a cancer cell that has been tested from tissue cultures or from experimental animals (2, 13)

and from the great majority of human malignant neoplasms that have been studied (9, 11). We have initiated a systematic investigation of the occurrence of the CG-like antigen in bacteria from proven sources (known origin) to determine whether or not the antigen is limited to bacteria isolated from human or animal malignant neoplasms that either developed spontaneously or were experimentally induced.

Eubacterium lentum ATCC 25559, *Propionibacterium acnes* (*Corynebacterium parvum*) ATCC 11829, and pathogenic and nonpathogenic forms of *Agrobacterium tumefaciens* were examined for the presence of the CG-like antigen. The first two microorganisms were selected because of their anaerobic characteristics and because some strains of *P. acnes* (*C. parvum*) are utilized for the preparation of vaccines for the treatment of cancer (3, 5). *A. tumefaciens* was selected because pathogenic strains induce malignant growths in plants. The results of these investigations are presented.

MATERIALS AND METHODS

Bacteria. *E. lentum* ATCC 25559 (Prévot strain 1899B, VPI 0255) is an obligately anaerobic, gram-positive, pleomorphic, nonmotile microorganism which was isolated in 1938 from a rectal tumor by A.-R. Prévot in Paris. An updated description of the microorganism and designation of ATCC 25559 as the neotype strain were reported in 1971 by Moore et al. (10).

P. acnes (*C. parvum*) ATCC 11829 (NCTC 10387, VPI 0210) is another gram-positive, anaerobic microorganism, also isolated by Prévot, apparently from acne pustules. Extensive work done by Johnson and Cummins (6) has demonstrated its homology to *P. acnes*, type II, based on cell wall and deoxyribonucleic acid similarities. These bacteria are usually nonpathogenic and are part of the normal skin and intestinal flora (6).

The other two microorganisms examined were the pathogenic and nonpathogenic strains of the gram-negative bacterium *A. tumefaciens*, strains H-38-9 and H-38-7, respectively. These strains were obtained from the Department of Microbiology, Pennsylvania State University, University Park, Pa., and the Department of Biology, Edinboro State College, Edinboro, Pa. The pathogenic strains of *A. tumefaciens* induce crown gall tumors in many (mostly dicotyledonous) plants because of the Ti plasmids present in these microorganisms (12, 14).

All bacteria were identified by standard morphological, physiological, nutritional, and biochemical tests. Gram stains and the Kinyoun acid-fast procedure were performed on all strains. The anaerobic bacteria were grown on 5% sheep blood agar with Columbia base (BBL) for 48 h at 35°C in anaerobic jars (GasPak, BBL). They were also grown in prerduced peptone yeast glucose broth under similar conditions. *A. tumefaciens* was grown on 5% sheep blood agar with Columbia base for 18 h at 35°C.

Methods. Detection of the CG-like immunoreactive material was done at the light-microscope level by utilizing two highly sensitive immunohistochemical techniques, the indirect immunofluorescein- and the indirect immunoperoxidase-antiperoxidase-labeled reactions. For examination, the surfaces of several colonies of the respective microorganisms were touched with a bacteriological loop and gently smeared on a microscope slide. After air drying, the preparations were immediately processed or were stored at -70°C. Details of the procedures, origin of first and second antisera, and details of the optical system have been published previously (1). Briefly, rabbit antiserum specific for the β -subunit of CG and rabbit antiserum for the complete hormone were utilized as first antibodies. Fluorescein-labeled or peroxidase-antiperoxidase-labeled goat anti-rabbit antisera were used as second antibodies. BeWo cells, a tissue culture line of non-clonal human malignant trophoblasts that produces an average of 1,000 IU of human CG/10⁸ cells per 24 h, were used as "positive cell controls." MA-160, a cultured line of human adenomatous prostatic hyperplasia, obtained from Microbiological Associates, Bethesda, Md., was utilized as the "negative cell control" (1). Standard controls for immunohistochemistry were used with all cells that gave a positive reaction for the presence of CG-like antigen. These controls were (i) utilization of CG-absorbed first antibody (obtained by incubation of the first antibody with an excess of human CG [APL, Ayerst Laboratories, New York, N.Y.] for 30 to 60 min at 37°C) and (ii) elimination and replacement of the first antibody by phosphate-buffered saline, nonimmunized rabbit serum, or rabbit anti-horse antiserum (Cappel Laboratories, Downingtown, Pa.).

RESULTS

The results of the indirect immunofluorescein reaction as applied to the positive and negative cell controls are illustrated in Fig. 1 and 2, respectively. The indirect immunoperoxidase-antiperoxidase reaction gave similar positive and negative reactions, respectively. These results demonstrate the specificity of the technique. The high concentration of the membrane-associated antigen CG in the human malignant trophoblast is notable. In contrast, as seen in Fig. 2, the nonmalignant human prostatic cells showed no reaction. Since the indirect immunofluorescein reaction is sensitive to 1,000 or 2,000 molecules of antigen in the plasma membrane of a cell (7), the negative cell control reaction demonstrates that nonmalignant prostatic cells in tissue culture have only minute amounts of the CG-like antigen or do not express the information for its biosynthesis at all.

Figure 3 illustrates the results of one of the standard controls for specificity of the second antibody, in this particular experiment, the fluorescein-tagged second antibody. The cell used for this control was the human malignant trophoblastic cell. The negative results obtained when the specific first antibody was replaced by phosphate-buffered saline indicate that in spite of the high concentration of CG and all of the polypeptide hormones synthesized by these cells, nonspecific attachment of the second antibody molecules did not occur. Similar negative results were also obtained when the first antibody was replaced by rabbit serum, rabbit anti-horse antiserum, or CG-absorbed first antibody.

The results of the indirect immunofluorescein and indirect immunoperoxidase-antiperoxidase reactions with *E. lentum* ATCC 25559 are illustrated in Fig. 4 and 5, respectively. The strong reaction indicating the presence of the CG-like material on the cell wall is notable. The corresponding "reagent controls" for both reactions were negative and are not shown.

In contrast to the results obtained with *E. lentum*, results with *P. acnes* (*C. parvum*) ATCC 11829 and the pathogenic and nonpathogenic forms of *A. tumefaciens* were consistently negative.

DISCUSSION

Antibody to the complete hormone was used as a screening procedure because it is highly sensitive, relatively inexpensive, and commercially available. However, it cross-reacts with luteinizing hormone. If cells do not react when antiserum to CG is used as the first antibody, the results are definitive and there is no need to use the less sensitive but more specific (and

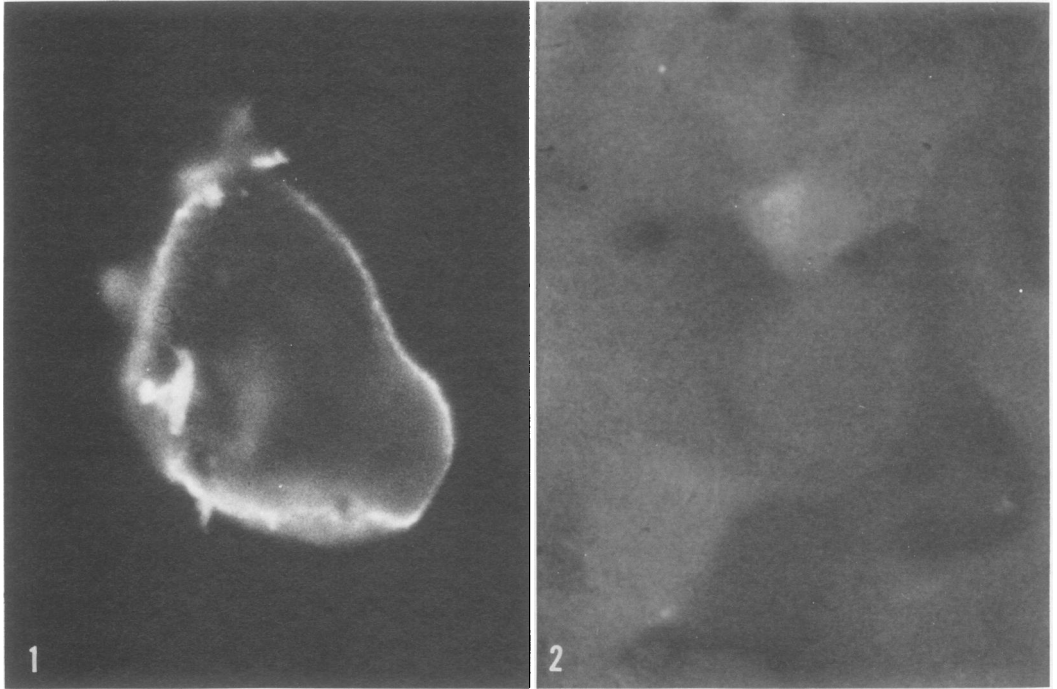


FIG. 1. BeWo cell, nonclonal human malignant trophoblast, used as a positive cell control. Indirect immunofluorescein reaction with antiserum to the β -subunit of CG as first antibody. Cross-reactivity of this antiserum is 0.16% for luteinizing hormone, 0.05% for follicle-stimulating hormone, 0.01% for TSH, 0.004% for prolactin, and 0.005% for α -human CG. The cross-reactivity is calculated from the amount of each polypeptide required to reduce the binding of ^{125}I -labeled human CG (obtained by the chloramine-T method) by 50%, with the amount of unlabeled human CG standard required to do the same, as analyzed by radioimmunoassay. All cells in this work are unfixed and air dried. $\times 1,391$.

FIG. 2. MA-160, cultured human nonmalignant prostatic cells, utilized as a negative cell control. Indirect immunofluorescein reaction with antiserum to total CG as first antibody. This antibody cross-reacts significantly with luteinizing hormone (LH) from all animal species that have been tested. Cross-reactivity with other polypeptide hormones is negligible. Because of the high sensitivity and the high cross-reactivity with LH, a negative reaction with this antiserum is conclusive. $\times 1,391$.

more expensive) antiserum to the β -subunit of CG.

Antiserum to the β -subunit of CG is utilized after a positive reaction has been demonstrated with antiserum to CG. Positive results with the second test confirm the presence of a CG-like material rather than luteinizing hormone-like material. The demonstration of the presence of a CG-like material suggests a biological relationship between these cancer-associated bacteria and cancer cells, spermatozoa, and trophoblasts (1).

Our findings have demonstrated for the first time the biosynthesis of CG-like immunoreactive material by an anaerobic bacterium, *E. lentum* ATCC 25559. This result and the negative results obtained with *P. acnes* (*C. parvum*) ATCC 11829 and strains H-38-9 and H-38-7 of *A. tumefaciens* support our previous findings (1) that the CG-like antigen appears to be expressed

only in cancer-associated bacteria but that not every bacterial strain associated with malignant neoplasms has the capacity to express the material, at least in detectable amounts.

The presence of the CG-like immunoreactive material in certain strains of cancer-associated bacteria can only be explained by its de novo biosynthesis. It is unlikely that its presence is due to absorption or binding of the material to these microorganisms because the strains have been subcultured many times since their isolation. *E. lentum* (ATCC 25559) was isolated in 1938, *P. maltophilia* ATCC 13637 was isolated in 1961, and the *E. coli* strains were isolated in 1975. The culture media contain no material resembling the CG-like antigen. All the microorganisms examined in this investigation were cultured on sheep blood agar, but only one, *E. lentum*, demonstrated the presence of the CG-like immunoreactive material. Furthermore, ra-

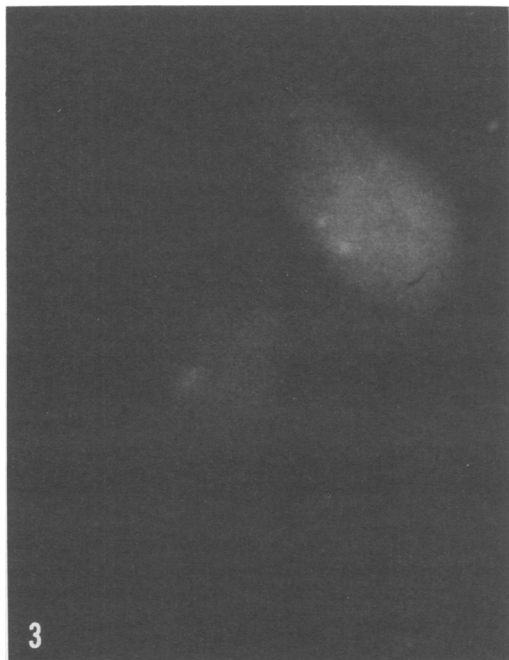


FIG. 3. Reagent control for specificity of the second antibody, fluorescein-tagged goat anti-rabbit antise-

rum, utilizing *BeWo* cells. The first antibody was replaced by phosphate-buffered saline. The negative results demonstrate that even in the presence of high CG antigen in these cells, nonspecific attachment of the second antibody does not occur. $\times 1,345$.

dioimmunoassay of the medium or of ultrafiltrates of the medium have given negative results. Bacterial synthesis, and this time by an obligate anaerobe, of a material with antigenic sites common to those of the human trophoblastic hormone and spermatozoa has important biological implications: first, because the immunoreactive material isolated from some of such strains demonstrated biochemical, physical, and biological characteristics similar to those of the human trophoblastic hormone (4), and, second, because it is increasingly apparent that the phenomenon occurs only in bacterial strains that have been grown with, or exposed to, malignant cells (1, 4, 8).

Demonstration of the synthesis of a polypeptide hormone-like material by a procaryote only when associated with malignant eucaryotic cells may suggest the presence of a transferable plasmid that could have an important relationship to the incidence of malignant transformation.

rum, utilizing *BeWo* cells. The first antibody was replaced by phosphate-buffered saline. The negative results demonstrate that even in the presence of high CG antigen in these cells, nonspecific attachment of the second antibody does not occur. $\times 1,345$.



FIG. 4. Positive indirect immunofluorescein reaction in *E. lentum* ATCC 25559. Note the intensity of the reaction at the cell wall. $\times 1,391$.

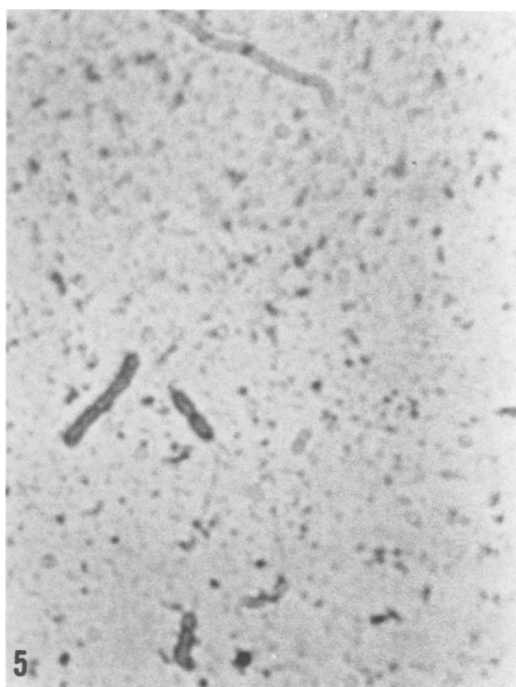


FIG. 5. Positive indirect peroxidase-antiperoxidase reaction in *E. lentum*. $\times 1,464$.

The phenomenon may also be explained, as in the malignant eucaryotic cells, by a derepression or activation of genetic information normally present in all bacteria as a result of conservation during evolution or as an example of convergent evolution.

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