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Antibodies to Bacterial Vaccines Demonstrating Specificity for Human Choriogonadotropin (hCG) and Immunochemical Detection of hCG-Like Factor in Subcellular Bacterial Fractions

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Investigations were done to determine whether vaccines prepared with chemically killed *Staphylococcus haemolyticus* RU1 and *Streptococcus bovis* AV46 (bacteria that have been demonstrated to express human choriogonadotropin [hCG]-like material on their surface) elicited antibodies in rabbits with specificity for hCG determinants. In addition, the anatomical locus of the hCG-like factor was determined by separation of bacterial subcellular fractions. The results demonstrated that these bacterial vaccines elicited antibodies immunologically similar to those antibodies produced in response to the whole human trophoblastic hormone, a similarity extending even to cross-reactivity with human luteinizing hormone. The bacterial hCG-like material appeared to be localized in the membranes of the cell wall, and most was present in the soluble membranous and cytoplasmic constituents. Its expression in bacteria was a strain characteristic and not a species characteristic.

Bacteria exist that express a human choriogonadotropin (hCG)-like material with in vitro and in vivo biological activity similar to that of the human trophoblastic hormone (1, 3–5, 7, 10, 18). Because the hCG-like material has been demonstrated on the surface of whole bacterial cells (1, 3–5, 22), an investigation was done to determine whether immunization of rabbits with vaccines prepared with hCG-producing bacteria elicit antibodies with specificity for the hCG epitopes. We also attempted to determine the anatomical locus of the hCG-like material by separating subcellular fractions while preserving the cell structures that have shown hCG-like material by immunoelectron microscopy (22).

MATERIALS AND METHODS

Staphylococcus haemolyticus RU1 (1) (previously described as *Staphylococcus simulans* [4]) (Fig. 1), which was originally isolated from a patient with metastatic cancer of unknown origin, and *Streptococcus bovis* AV46, a cell wall-deficient variant (by cultural and electron microscopic morphologic characteristics) (11, 13) isolated from the blood of a patient with a fever of unknown origin and with a possible brain abscess, were killed with 0.5% phenol or with 1% formalin and used as immunogens in New Zealand White rabbits. Immunizations were done by intramuscular injection of 10^6 cells emulsified in the squalene-Arlacel A system described by Stevens et al. (23). Preparations of non-hCG-producing *Staphylococcus haemolyticus* KL14 (1) (Fig. 2) and *Streptococcus bovis* Zak were used as control immunogens. The rabbits were immunized three times at 28-day intervals. Blood was taken 35 days after primary immunization and weekly thereafter.

Antibody levels were measured as previously described (20). Briefly, the method is a radioimmunoassay with chloramine-T ^{125}I-labeled hCG (15). With a ratio of 10 μg of hCG (CR119; Center for Population Research, National Institute of Child Health and Human Development, Bethesda, Md.) per mCi of ^{125}I and a reaction time with chloramine-T of 45 s, this iodination procedure gave specific activities of 35 to 45 μCi/μg of hCG. Separation of the labeled hCG from free iodide was done by gel filtration with BioGel P-60 and phosphate-buffered saline. The eluted protein was further diluted in phosphate-buffered saline containing 1% bovine serum albumin for use in assay tubes. Sera to be tested were serially diluted in a buffer (pH 7.4) containing phosphate-buffered saline, 0.05 M EDTA, and 20% normal calf serum. Each tube contained 0.2 ml of diluted serum and 0.1 ml of labeled antigen. The serial dilutions were incubated for 120 h at 4°C with 1.3 pmol of [^{125}I]hCG mixed with three amounts of unlabeled antigen (1 to 50 ng) to ensure antibody saturation. Bound hCG and unbound hCG were separated by the addition of 1.0 ml of 25% polyethylene glycol 6000, followed by centrifugation. The precipitate containing the bound hCG was counted in a gamma spectrometer. Antibody binding was calculated as moles of hCG (based on a molecular weight of 38,000) bound per liter of serum (20). Values for multiple dilutions in which binding was less than 30% bound/total (B/T) were averaged.

The anatomical locus of the hCG-like material in the bacterial cell was determined from analysis of subcellular fractions prepared as shown by the flow diagram in Fig. 3 (12). The sediment from a 500-ml culture of *Staphylococcus haemolyticus* RU1 grown in brain heart infusion broth was suspended in distilled water, disrupted in a French press, and fractionated by differential centrifugation. The fractions were not washed because hCG is highly soluble in water, and solubilized hCG-like material would, therefore, be lost.

RESULTS

The results showed that sera from animals immunized with hCG-producing bacteria bound [^{125}I]hCG and ^{125}I-

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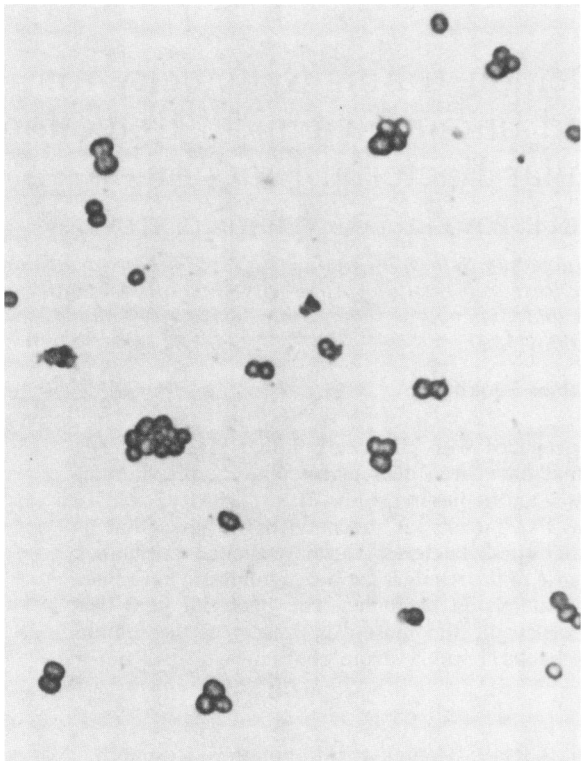

FIG. 1. Immunocytochemical detection of hCGβ-like material in *Staphylococcus haemolyticus* RU1. Indirect immunoperoxidase reaction with a mouse monoclonal antibody (337W; Chemicon International, Inc., El Segundo, Calif.) to hCGβ as first antibody. This antibody is a purified IgG1 from mouse ascites fluid and it was used at a concentration of 20 μg/100 μl. The antibody is directed against an epitope of hCGβ, and also cross-reacts with the complete hCG. It does not react with the COOH-terminal peptide of hCGβ. Peroxidase-labeled sheep antimouse IgG was used as second antibody, and the immunocytochemical reaction was done on unfixed air-dried cells. Replacement of the first antibody by ascites fluid supplemented with mouse IgG (Cooper Biomedical, Inc., West Chester, Pa.) at the same concentration was used as a reagent control. Peroxidase staining was done with 3-amino-9-ethlycarbazole (Sigma Chemical Co., St. Louis, Mo.). This bacterial strain has also shown reactivity with another monoclonal antibody to hCGβ that reacts with an epitope different to the one reacting with antibody 337W, and with polyclonal antisera to whole hCG, to hCGβ, to the hCGβ COOH-terminal peptide residues 115 through 145, and to hCGα (1). *Streptococcus bovis* AV46 has also shown a positive reaction with the same reagents. Magnification, ×1,600.

labeled human luteinizing hormone (hLH) (Table 1). Sera from control animals immunized with bacteria from the same species that did not produce hCG failed to show detectable binding to hCG and hLH ($<0.5 \times 10^{-10}$ M).

The results of the studies done to determine the anatomical locus of the hCG-like material in the bacterial cell showed that most of the hCG-like material (20 mIU/mg) appeared in the 144s120 fraction (2 and <0.7 mIU/mg appeared in the 20p30 and 144p120 fractions, respectively). Immunocytochemistry (1, 3–5) (Fig. 1) and immunoelectron microscopy (22) have revealed the hCG-like material on the cell surface and in the membranes of the bacterial cells, respectively, a finding confirmed by the present observations indicating that the material needed to be disrupted from the bacterial membranes before it could dissolve in the aqueous phase.

DISCUSSION

The results of this study demonstrated for the first time that vaccines prepared with chemically killed bacteria containing hCG-like material elicited in rabbits antibodies that reacted with the human trophoblastic hormone. These antibodies were immunologically similar to the antibodies produced by hCG, since we found that the antibodies to the bacteria containing the hCG-like material also cross-reacted with hLH (Table 1). The data also demonstrated that the bacterial material could be extracted by disruption of the cell membranes and that the expression of the hCG-like protein by bacteria is a strain characteristic and not a species characteristic, confirming the results from our immunocytochemical studies (1). Bacteria within the same species shown by immunocytochemistry to be devoid of the hormone-like protein (Fig. 2) did not give rise to antibodies cross-reacting with hCG or hLH.

A number of the strains of bacteria expressing hCG-like material have been isolated, primarily from cancer patients (1, 3–5, 10, 18, 22). Tumor cells of many different types from different species have also been shown to contain hCG-like material in their membranes (2, 9, 19, 27). This poses the following question: what is the relationship of the hCG-like material in bacteria to that in cancer cells and to hCG elaborated by trophoblasts before implantation (6) and during pregnancy? The production of the trophoblastic hormone

FIG. 2. Results of the immunocytochemical reaction of *Staphylococcus haemolyticus* KL14, which was used as negative cell control, showing the lack of reactivity to the hCGβ monoclonal antibody. The analysis was done in parallel with the same reagents and conditions as the reaction described in the legend to Fig. 1. This bacterial strain has also been tested with all the polyclonal antisera listed in the legend to Fig. 1 and has demonstrated no reactivity (1). Similarly, *Streptococcus bovis* Zak has shown no reactivity when tested with the same reagents. Magnification, ×1,600.

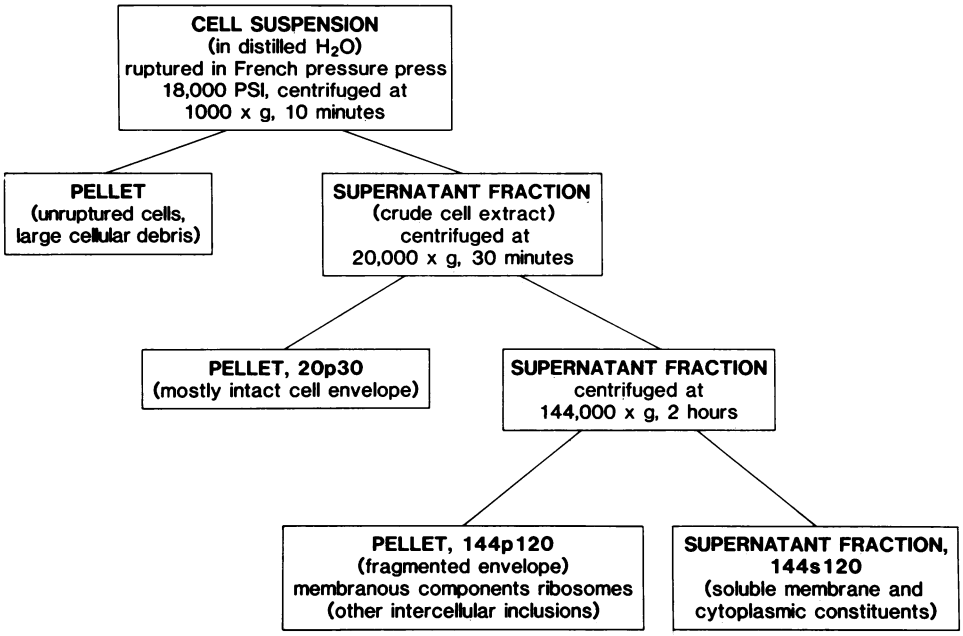

FIG. 3. Fractionation procedure.

before implantation makes hCG the first de novo gene product identifiable in development. Observations demonstrating hCG-like material in nontrophoblastic and nonembryonal neoplasms of different species provide a biochemical verification of the concept that malignant cells may represent a functional reversion to an early embryonal phase of development. The expression of hCG-like material by strains of different species of bacteria may indicate that hCG is a primeval molecule, its genes originating very early in evolution, or that hCG genes arose in bacteria and vertebrates by convergent evolution (5, 18, 21). Since most of the hCG-producing bacteria have been isolated from cancer patients, an alternative hypothesis is that the expression of hCG genes in bacteria is a consequence of a genetic exchange between transformed or malignant cells and bacteria (5, 18). This appears unlikely, inasmuch as the two subunits of hCG are made by separate genes (8, 14), and because specific enzymes are needed to add the sugar moieties. Thus, an unlikely series of recombination events would have to occur for the bacteria to express biologically active hCG (4, 18).

The presence of hCG-like material in bacteria may prove to be useful as a practical source of biologically active hCG-like material, heretofore available only through the isolation and purification of the trophoblastic hormone from human urine obtained during pregnancy. Furthermore, animal models suggest that de novo induction of antibodies to hCG may be of value as a potential method of birth control in women (24–26) and also for the prevention of some types of cancer (16–17). In this regard, bacterial vaccines containing material similar to hCG or its subunits may have the potential for development as immunogens of practical utility.

TABLE 1. Summary of antibody levels in sera from rabbits immunized with hCG-producing bacteria or non-hCG-producing bacteria

Immunogen used, killing agent	Antibody level (10^{-10} M)[a]		hLH/hCG ratio
	hCG	hLH	
Staphylococcus haemolyticus RU1[b]			
Phenol	4.8 ± 3.7	1.7 ± 0.5	0.35
Formalin	5.2 ± 4.3	2.6 ± 1.0	0.50
Staphylococcus haemolyticus KL14 (Formalin)[c]	ND[d]	ND	
Streptococcus bovis AV46[b]			
Phenol	10.9 ± 4.2	3.8 ± 1.6	0.35
Formalin	8.9 ± 5.3	2.1 ± 1.1	0.24
Streptococcus bovis Zak (Formalin)[c]	ND	ND	

[a] Mean of the peak levels of four animals plus or minus the standard error. Peak levels of hCG were obtained after 70 days from rabbits injected with Staphylococcus haemolyticus RU1 and after 77 days from rabbits injected with *Streptococcus bovis* AV46.
[b] hCG-producing bacteria.
[c] Non-hCG-producing bacteria.
[d] ND, Not detectable ($\leq 0.5 \times 10^{-10}$ M).

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