Human chorionic gonadotropin (hCG), the hormone of life and death: a review

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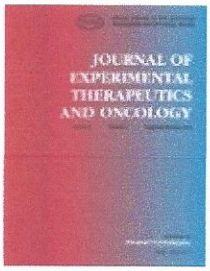

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INTRODUCTION

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Human chorionic gonadotropin (hCG) is a member of the glycoprotein hormone family. These hormones exhibit similar evolutionary, immunological and biochemical characteristics. hCG was the first hormone to be described as the "pregnancy hormone" by Ascheim and Zondek in 1928 (1–3), and was the last hormone to be purified and crystallized (4,5). hCG is also considered as a hormone of development. The whole (dimeric) hormone (hCG holo) is composed of two non-covalent linked glysolated subunits, the α and the β.

Paradoxically, it appears that human reproduction and malignant transformation share common genetic (evolutionary) and biochemical pathways related to hCG. Thus, synthesis of biologically active hCG by the human fetus and embryos was reported as early as 1983 (6,7) followed by the demonstration by Rothman and his associates that extraplacental human fetal tissues express translatable levels of hCGβ mRNA (8). Then, Cosgrove and his associates (9), in 1989, gave the first evidence of the synthesis of hCG, steady-state levels of hCGβ mRNA and gene structure by human tumor cell lines.

Three years later, using quantitative analytical flow cytometry and a battery of monoclonal antibodies directed to different epitopes of the hCG molecule, Acevedo and his associates demonstrated the expression of membrane-associated hCG, its subunits and fragments by *live* cancer cells of every type and origin (10–12). Using the same methodology, and the nude mouse model, Acevedo and Hartsock pointed out that there was a direct *in vivo* correlation between human cancer cells that metastasize spontaneously in nude mice, and the expression of membrane-associated complete hCGβ. This correlation indicated a) that the hCGβ is a characteristic metastatic phenotype marker, b) that the marked ratio variations indicated that the metastatic phenotype is an unstable event, and c) that growth and local invasion *in vivo* correlates with the expression of hCG-holo, that is the whole hCG molecule (13). More recently, it has been shown that the AP-2 family members can regulate the basal and cAMP-induced expression of hCG (14), that hCGβ expression in both tumor tissue and serum can predict the outcome in colorectal cancer (15), and that hCGα protein can be used as a clinical marker for the management of ER-alpha-positive breast cancer (16).

At the molecular level, the basic work of Cosgrove (9) was confirmed by Acevedo *et al* in 1995 (17) and in 1997 by Bellet and his co-workers (18). It is important to note that work by Acevedo's team in 1995 (19) demonstrated immunologically as well as at the genetic level that the expression of membrane-associated hLHβ in cultured human cancer and fetal cells correlated with hLHβ gene expression, indicating that the whole hCGβ-hLHβ gene cluster becomes activated during the process of malignant transformation. Thus, it is tempting to define the dual activity of these hormones as a process having two personalities, like the classical creation of R.L. Stevenson, as a biochemical Dr. Jekyll and Mr. Hyde.

Evolutionarily, the hCG genes appear to have existed before the appearance of "Homo sapiens". When Dr. Donald

Johnson discovered the skeleton of Lucy in Ethiopia in November of 1974 (20), it was calculated to have existed about 3.5 million years ago. Lucy, her paleontological name "Australopitecus aferensis", is the oldest hominid that has been found, was a five-foot erectus female. She was part of a family, since marks of bipedal "beings" have been found in the zone. Logically, her existence required the activity of hCG (Hominid CG).

Later, the discovery of cancer in *cold blooded animals*, including fish and reptiles (21), pushed the existence of hCG or its subunits *back* to the Triassic period during the Mesozoic era which was about 230 million years ago when the first dinosaurs evolved. The first true mammals appeared during the Jurassic period, about 181 million years ago, when the dinosaurs were at their peak. Cultured cells from the spleen metastasis of a Russell's viper tumor (VSW cells), available from the American Type Culture Collection (ATCC CCL 129), presented evidence of hCG by immunoperoxidase and immunofluorescence techniques (22) indicating that hCG existed *before* mammalian evolvement. These data show that hCG is a very old glycoprotein, well preserved during evolution.

Moreover, a peptide similar to the vertebrate gonadotropin system in the central nervous system of the cockroach (*Periplaneta Americana*) (23) was described in 1986. LH-like material was detected in such extracts by imunocytochemistry. Furthermore, treatment with extracts of the nervous system of the cockroach produced a significant stimulation of testosterone synthesis. This bioactivity was established using mouse interstitial (Leydig) cells. As early as 1979 Charlet and his associates (24) reported evidence for a neuroendocrine control of ecdysone biosynthesis in grasshoppers (*Locusta migratoria*) and Hagedorn and his associates demonstrated that in adult mosquitoes the ovarian ecdysone secretion was controlled by a brain hormone (25). All the aforementioned results prove that these hormones are very old and have been maintained throughout evolution, since insects appeared at the beginning of life on our planet Earth.

It is important to note that plants get a form of cancer known as crown gall disease. This condition is characterized by the formation of neoplasms (galls) that usually appear near the plant crown (26). It was not until 1992 that a bacterium, *Agrobacterium tumefaciens*, was identified as the cause of the disease by a DNA transfer (27).

Unicellular organisms like bacteria and yeasts have been identified to acquire genes of certain hormones. Of fundamental importance is the fact that some bacteria isolated from cancer patients expressed hCG, but *not* every isolated bacteria did (28–33). Moreover, the morphologic changes observed by electron microscopy (34) indicated that some of the bacteria expressing the hCG-like material are revertants of cell wall deficient (CWD) variants. In 1993, Caticha and his associates isolated and characterized an hCG-like protein from *Candida albicans*, a yeast, that was not isolated from a cancer patient (35).

Subsequently, Grover *et al*(36), using molecular biology techniques, demonstrated the large degree of homology of the protein isolated from the cell wall of *Xanthomonas maltophilia* with that of the hCGβ subunit as well as its carboxy-terminal peptide. These investigators concluded that this great similarity suggested that the hCGβ gene(s) has not evolved from the hLHβ gene, having been present in prokaryotes well before. Furthermore, the hCGβ as it is now, may have evolved from this primordial gene, perhaps by natural selection. Investigators from the same group also reported that bacterial hCG, as well as hCG, *but not hLH*, stimulated both growth and change in morphology in the bacteria (37).

Since common carriers of bacteria are insects, and it has already been proved that insects produce material similar

to hCG or LH (23–25,34), *it is possible that tumors in plants may also be associated with hCG material.*

Chemistry and Biochemistry

Because of its biochemical and biophysical characteristics, human chorionic gonadotropin, hCG, can be expressed in different forms. The whole hormone (hCG-holo), known as the hormone of pregnancy (1–3), and is the basis for the pregnancy test, is synthesized by the syncytiotrophoblast, after placenta evolves. Chemically, hCG is a 38 KD heterodimeric glycoprotein containing approximately 30% of oligosaccharides. The hormone is composed of two subunits, the α and the β-subunits, noncovalently attached, having a total of 244 amino acids.

The 99 amino acid α-subunit, common to the four dimeric hormones of the adenohypophysis, that is, human luteinizing hormone (hLH) (also known in earlier literature as interstitial cell stimulating hormone (ICSH), follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and the pituitary chorionic gonadotropin, is coded by a single gene located in chromosome six in normal tissue and in tumor derived cell lines (38,39). The α-subunit gene is activated independently of the hCGβ-hLHβ genes, constituted by a cluster of six genes and pseudogenes located in chromosome 19, described as β1, 2, 3, 5, 7 and 8. Alleles for β3 and β7, called β9 and β6 respectively, have been identified (40).

Structurally, while the 145 amino acid hCGβ is considerably different from that of TSHβ and FSHβ, there is a substantial homology with the first 115 amino acids of the β-subunit of hLH. Thus hCGβ contains an additional 30 carboxy-terminal amino acid residues, rich in serine, known as the carboxy-terminal peptide (CTP).

The purification and crystallization of hCG reported in 1994 (4,5) revealed the presence of a central loop in the hCGβ molecule; a cystine knot that is common to three growth factors, the transforming growth factor β (TGFβ), the nerve growth factor (NGF), and the platelet derived growth factor β (PDGFβ), characterized by distinctive folds stabilized by disulfide bonds. This fundamental discovery explained the growth activity of hCG on cultured cells reported in the 1980's by Melmed and Braunstein (41), Mukherjee and Das (42), and Raikow, Acevedo, Kellen and Agarwal (43).

The human pituitary hCG was isolated and characterized by Birken and his associates in 1996 (44), from acetone preserved human pituitary glands. In contrast to the trophoblastic hormone that is highly sialylated, the pituitary hCG contained both sulfate and sialic acid. Using highly sensitive hCG assays, *pulsatile hCG secretion into the blood of nonpregnant individuals has been detected.* As early as 1980, Matsuura *et al*(45) described the physicochemical and immunological characterization of the hCG-like material from human pituitary glands. The most important aspect of the pituitary hCG is that in contrast to the membrane-associated hCG from trophoblastic, embryonic, and cancer cells, the pituitary hCG, produced in very small amounts, is only a secretory (soluble) product, and its secretion, as the secretion of all the other protein hormones of the adenohypophysis, is pulsatile (46).

The stimulating biological activity of hCG depends on the presence of receptors. hCG *per se* has no receptor; it uses the hLH receptors. Since the attachment to the receptor requires the soluble whole hormone, that is the α-β dimer, the hCGβ has to be an assembly-competent subunit to be able to have the right structural configuration. Such incompetent forms of the β and α subunits do exist as free subunits (47,48).

In a study of various hCG isoforms at the level of the hLH receptor, using a radio-receptor assay based on cell membranes expressing recombinant human LH receptors, H-H Ho and his associates at the University of California (Davis) and at Columbia University (NY), demonstrated once and for all, that only the intact heterodimeric hCG has tropic (stimulatory) hormonal biological activity (49). Besides the whole hCG, the study included the major polypeptide variants of hCG which are found in biological fluids, which are free α-subunit, free β-subunit, β core fragment and nicked dimeric hCG, which results from a peptide bond breakage of the hCGβ between residues 47 and 48 or 44 and 45. Isoforms of intact hCG may bary in their carbohydrate moieties, but these variations are not yet well characterized. hCG isoforms that lack glycosylation because of chemical treatment or genetic mutations, have been shown to possess binding activity for the LH receptor which is equal to or greater than that of the glycosylated intact hCG, but having a much lower, *or total absence of the hormonal biological activity* when compared to glycosylated intact hCG (50,51).

The nature of the oligosaccharides of these glycoproteins is of fundamental importance. The α-subunit contains two chains of N-linked oligosaccharides attached to asparagine in a horse-shoe like form, with two molecules of n-acetyl-neuraminic acid, also known as sialic acid, while the β-subunit contains four chains of O-linked oligosaccharides attached to the four serines of the hCGβ carboxy-terminal peptide, with a total of six molecules of sialic acid as shown in Figures 1 and 2(52–54). Because of these chemical characteristics, hCG is a sialoglycoprotein. The high content of sialic acid gives the hCG molecule a *very high negative charge*, superior to any other normal or abnormal mucopolysaccharide.

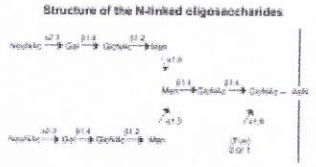

Figure 1. Structure of the N-linked oligosaccharides on the α and β subunits of hCG with the corresponding frequencies indicated (52,53).

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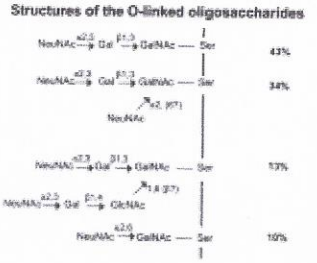

Figure 2. Structure of the O-linked oligosaccharides on the α and β subunits of hCG with the corresponding frequencies indicated (54).

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This characteristic was reported for the first time by VanBeek *et al* in 1973 (55), and was followed by a series of publications which proved the immunological and biophysical effects of the presence of sialic acid (n-acetyl-neuraminic acid) on the cell membrane of cancer cells and embryonic and fetal cells. To mention a few, Brummett

and Dumont (56) reported the localization of *negative charges* on the surface of developing oocytes; Yogeeswaran and Salk (57) reported that the metastatic potential was positively correlated with the cell surface sialylation of cultured murine tumor cell lines; Stanford and his associates (58) discussed the role of tumor cell surface carbohydrate in experimental metastasis; Van Rinsum *et al*(59) reported that the inhibition of human natural killer cell mediated cytotoxicity *was due to the sialic acid and sialo-oligosaccharides of the cell membrane*.

Since all the normal cells from our immune system, macrophages, NK cells and B cells, express in their membranes a "normal" negative charge, the high negative charge of hCG and its subunits demonstrated to be present in the cell membranes of embryonic and fetal cells, in sperm cells in every stage of development (Figures 3,4) (60–62), and in all cancer cells irrespective of type or origin as membrane-associated hCG (10–13), make all these cells *immunologically inert*. The cells from the immune system cannot approach, cannot touch, since negative charges repel. That is the reason why the embryo and fetus, which under normal conditions are 50% foreign to the mother, are able to survive the immune system of the mother, and why sperm cells and cancer cells also survive.

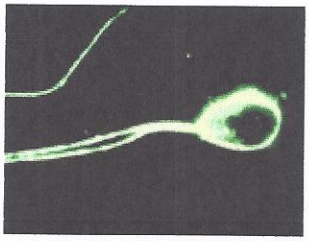

Figure 3. Human spermatozoa demonstrating the presence of hCG by immunofluorescence reaction with anti-hCG antibody (61,62).

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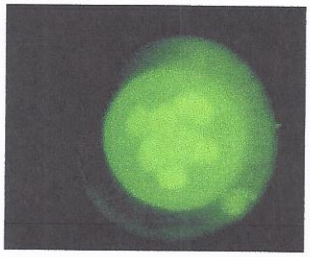

Figure 4. Eight cell rabbit morula demonstrating the presence of hCG by immunofluorescence reaction with anti-hCG antibody (60).

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Up to this point, we have discussed hCG mainly from the point of view of its powerful biological activity, as a water soluble tropic hormone. The thorough mapping of the hormone and its isoforms, the availability of a large group of monoclonal antibodies (MoAb) directed to specific, well characterized, antigenic determinants of the hCG molecule (Figure 5) and the development of a quantitative method using analytical flow cytometry, permitted us to demonstrate with *live* cancer and fetal cells, that hCG in all of its forms is an integral part of the cell membrane in all of these cells, irrespective of their type and origin. The membrane-associated material can only be isolated by rupturing the cells and the use of detergents such as Triton X (10–13).

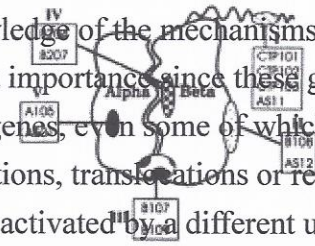

Figure 5. Scheme of the hCG molecule showing the monoclonal antibodies which react with specific antigenic determinants. Group I reacts only with determinants on the carboxy-terminal peptide of the molecule. Group II reacts with determinants on hCGβ, the whole hormone or hCGβ-free. Group III reacts only with the whole hormone (conformational epitopes). Group IV reacts on with hCGβ-free and Group V reacts with the hCGα free, or as part of the whole hormone.

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The knowledge of the mechanisms governing the activation of the hCGα gene and of the hCGβ-hLHβ gene cluster is of great importance since these genes are maintained in every cell throughout life and evolution. In contrast to all other genes, even some of which are expressed in different types of cancer, which are activated by amplifications, translocations or rearrangement, although certain polymorphisms have been observed, the hCG genes are activated by a different unique mechanism, that is, patterns of methylation. The first communication that dealt with this process was by R.K. Iles and his associates who demonstrated that amplification or rearrangement of the hCGβ-hLHβ gene cluster was not responsible for the ectopic production of hCGβ by bladder tumor cells (63).

Methylation of cytosines is a common occurrence in vertebral DNA and the loss of such methylations was postulated to be one of the regulatory mechanisms of gene activation in cancer (64–66). Furthermore, Benvenisty *et al*(67) showed that sequential changes in DNA methylation patterns occur in the rat during development and Razin and his associates (68) reported the replacement of 5-methyl-cytosine by cytosine as a mechanism for transient DNA methylation during differentiation. Also, Shmookler Reis and his associates demonstrated that clonal variations in gene methylation occur independently in human embryonic and fetal cells (69). The human fibroblast lineages used in the investigation were embryonic and fetal cells. *Normal cells like fibroblasts cannot be maintained in culture*. Shmookler Reis *et al*, at that time, honestly believed that they were working with *human fibroblasts*.

Finally, one of the most important questions concerning this extraordinary molecule is still unanswered. An important question is what are the biochemical and biophysical mechanisms that intervene in the synthesis and coupling of the carbohydrates, sialic acid and others, to the main body of the molecule? While we know most, if not all, the molecular biology of the systems, in spite of all the research around the world, this question has never been answered. Perhaps it will never be, since this knowledge deals with the secret of life itself.

Biological and Biochemical Studies

Birth control vaccines In humans, hCG is localized on the cell membrane of the syncytiotrophoblast facing the maternal blood stream. The syncytiotrophoblast is formed from the trophoblast cells of the mammalian blastocyst. As early as 1972 Vandeputte and Sobis (70) and in 1974 Martin and his associates (71) suggested that the hCG on human syncytial plasma membranes may, in part, be responsible for the lack of maternal immunological rejection of the fetus, *indicating for the first time*, that hCG may have an immunological function during pregnancy. In the same year Lynn D. Wiley described the presence of an hCG-like substance on the surface of preimplanted mouse embryos, and the detectable amount is maximal in the morula stage (72). Ten years later, Fishiel, Edwards and Evans at Cambridge, UK (73) described the "secretion" of hCGβ by preimplantation human embryos. Human oocytes were collected by laparoscopy, fertilized and cultured *in vitro*. hCG was detected in the medium surrounding the two embryos cultured for more than 7 days after fertilization. It is logical to conclude that if secretion of hCG by the human oocytes occurred, their cell membranes must also have membrane-associated hCGβ. It is important to note that in 1979 Asch and his associates (60) obtained rabbit morulae. Immunofluorescence cytochemistry revealed the presence of hCGβ in the cell membrane of the eight-cell rabbit morula (Figure 4).

The expression of membrane-associated hCGβ at such an early stage after fertilization gave a sound basis for the development of an antifertility (birth control) vaccine. Two programs were developed, one funded by the World Health Organization (WHO) in Geneva, Switzerland, and the other funded by the Population Council, The Rockefeller University, New York, NY.

Dr. Vernon C. Stevens and his associates at the Department of Obstetrics and Gynecology, Ohio State University directed the WHO program. The product of this investigation was a birth control vaccine incorporating a synthetic peptide representing the amino acid sequence 109-145 of the carboxy-terminal peptide of the β-subunit of hCG conjugated to diphtheria toxoid (74–76). The Phase I clinical trial of this vaccine was done at the Flinders Medical Centre, Adelaide, Australia, using thirty surgically sterilized female volunteers divided into 5 equal groups for different vaccine doses. Over a six-month follow-up there were no adverse reactions, and potentially contraceptive levels of antibodies to hCG developed in all subjects. In the group receiving the highest dose of vaccine, the results gave promise of a contraceptive effect of six months duration (77). For socio-political reasons, subsequent phases of these studies are being done in Europe.

The Population Council program was based on an immunogen composed of hCGβ linked to tetanus toxoid, using different vehicles and adjuvants (78–83). This program was terminated and Dr. G.P. Talwar and his associates continued their work at the National Institute of Immunology, the All India Institute of Medical Sciences and the Indian Council of Medical Research, New Delhi, India. In 1994, they reported the results of the clinical trials of their vaccine consisting of a heterospecies dimer of the hCGβ associated noncovalently with the α-subunit of ovine LH and conjugated to tetanus and diphtheria toxoid as carriers which induces anti-hCG antibodies of high avidity. Fertile women exposed to conception over 1224 cycles recorded only one pregnancy at antibody titers of less than 50 ng/ml (hCG bioneutralization capacity). The antibody response declines with time. Fertility was regained when titers fell to less than 35 ng/ml (84,85).

Anti-cancer vaccines It was logical that the *demonstration of membrane-associated hCG in all cancer cells*, irrespective of type or origin (10–13), indicated the possibility of using the anticonceptional vaccine as a vaccine against cancer. The first experimental studies using the Population Council vaccine were done in 1982 by Kellen, Kolin and Acevedo (86,87) at the Departments of Clinical Biochemistry and Pathology at Sunnybrook Medical Centre, Toronto, Canada, and the Department of Pathology, Allegheny General Hospital, Pittsburgh, PA, USA. The experimental neoplasm used was R3230 rat mammary adenocarcinoma in Fisher 344 female rats. A test group of 70 animals and two matched groups of 15 animals each were used. One of the control groups was untreated and the other received tetanus toxoid only. All the control animals had multiple lung foci of neoplastic cells ten days after seeding, and no hCG antibodies were detected. In contrast, a significant titer of anti-hCG antibody was found in all preimmunized animals. *The protective effects of the preimmunization with hCGβ-tetanus toxoid vaccine* were further demonstrated by the animals living 20 days post-seeding, the absence of lung pathology in the ones killed thereafter, and by six animals which were left alive and did not show any deterioration for more than six months after the administration of the cancer cell suspensions. This work was repeated using the mammary adenocarcinoma in Fisher 344 rats and the implanted 5123 1-1 hepatoma in Buffalo rats. Autopsies performed 20 days after vaccination demonstrated that the vaccine retarded significantly ($P < 0.01$) the growth of the two transplantable tumors. As before, no antibodies to hCG were found in the control animals. To our knowledge, this is the first time in the history of clinical science that a vaccine against the hormone of life (hCG) was effective in

controlling and eliminating two types of deadly cancers.

It was not until 1987 that we were able to study the effects of preimmunization against hCG on the growth of the transplantable Lewis lung carcinoma in C57BL/6J mice, and on the spontaneous mammary carcinoma in C3H/OuJ mice using the WHO vaccine. The results were that preimmunization significantly ($P < 0.05$) retarded the growth of Lewis lung tumors as measured by tumor weight 18 days following transplantation. Furthermore, the weights of the tumors in the preimmunized animals were inversely correlated ($r = 0.61$) with the levels of circulating antibodies against hCGβ, whereas no statistical correlation was found between tumor weights and the levels of antibodies reactive to diphtheria toxoid. The number of conjugated treated C3H/OuJ mice that developed mammary tumors (due to Bittner mammary tumor virus) was significantly reduced ($P < 0.05$) when compared to the vehicle-treated cohorts. Pretreatment with the synthetic muramyl dipeptide analog, a potent stimulant of the immune system, used as an adjuvant in the WHO vaccine, did not show any direct effect on the tumor growth in either tumor system (88).

Since a prerequisite for a successful immunotherapeutic approach to cancer is the preferential expression by the malignant neoplasms of a membrane-associated antigen, and taking into consideration that the embryonic and fetal cells, like cancer cells of every type and origin expressed membrane-associated hCG in its different forms (10–13), it was obvious to test the WHO antifertility vaccine in different types of cancers. A phase I clinical trial of the anti-hCG vaccine was done at the Ohio State University Comprehensive Cancer Center, the Arthur G. James Cancer Hospital and Research Institute, Columbus, Ohio, the city where the WHO vaccine for fertility control was developed (89). Twenty-three patients with non-trophoblastic cancers participated in this study. Diphtheria toxoid hypersensitivity developed in one patient. The immunization elicited anti-hCGβ IgG antibodies that persisted for more than 10 months. Disappearance of circulating hCG in the few patients who had it, as well as tumor regression were observed. The cellular response was quite interesting. All patients were skin treated with autologous tumor prior to immunization and 30 days after the third (and final) immunization. No patient showed positive delayed type hypersensitivity (DTH) to autologous tumor. The largest area of induration observed was 4 mm in one patient. Skin test reactivity to purified hCG was done up to 12 months after the third immunization and also after booster immunization. No positive tests were observed. Peripheral blood mononuclear cell cytotoxicity was also assessed pre-immunization and 30 days after the third immunization using standard CR ^{51}-release assays. No patient developed significant enhancement of cytotoxicity versus NK-sensitive K-562 cells. Furthermore, cytotoxicity versus NK-resistant Daudi cells and/or hCG positive HeLa cells was not observed in any patient pre- or post-immunization. The authors concluded that active specific immunotherapy with the WHO (CTP37) vaccine was well tolerated and has biological activity in patients with cancer.

The data obtained in the phase I clinical trial in 1994 (89) did not explain the mechanism of action of the vaccine. This mechanism was elucidated in 1998 by Kalantarov and Acevedo (90), who demonstrated that one of the three antibodies against the hCGβ-carboxy-terminal peptide that the vaccine elicits, has a direct dose-related cytotoxicity at 37° C. At the time this review is being written, extensive phase II and phase III multicenter clinical trials, involving different types of cancer, are being performed in different locations in the United States. Since licensing trials are currently underway, it is possible that the vaccine, Avicine (AVI Biopharma), may be available to the public in approximately three years.

Antiviral activity of hCGβ One of the least mentioned or recognized biological characteristics of this extraordinary hormone is its antiviral activity. One of the most important studies in the immunodeficiency virus-1 (HIV-1) was the European Collaborative Study on the natural history of vertically acquired HIV-1 (91). 600 children born to HIV-infected mothers by June 15, 1990, in ten European centers, were followed to study the natural history of HIV infection and the vertical transmission rate. They were seen at birth, every three months up to 18 months of age, and every six months thereafter. At the last follow-up only 64 children were judged to be HIV-infected and 343 had lost antibody and were presumed uninfected. The estimated transmission rate was only 13% in this study.

In December 1991, to examine the epidemiology and the natural history of mother-to-infant transmission of HIV-1, Goedert, his associates, and the International Registry of HIV-Exposed Twins studied data on twins and triplets born to women infected with the virus (92). The findings of this investigation revealed that among the 66 evaluable sets, HIV-1 infection was more common in the first born than in second born twins ($P = 0.004$). In 22 sets, only one twin was infected, 18 first-born, 4 second-born, 50% of first-born twins delivered vaginally and 38% of first-born twins delivered by cesarian were infected, compared with 19% of second-born twins delivered by either route. This finding suggested that a substantial proportion of infections, if not all, occurred as the first twin encountered the cervix and birth canal. *This is important because it indicates that no infection occurred prior to any type of delivery*.

Based on the European Collaborative Study, Bourinbaiar and Nagorny, working at the Population Council Center for Biomedical Research in New York, investigated the *in vitro* effect of hCG on the reverse transcriptase activity in HIV-1 infected lymphocytes and monocytes. They used commercial hCG from Sigma, and *pure hCG* acquired from the Population Council. Their results clearly demonstrated the inhibition of viral replication *in vitro* with the best results being obtained with the pure hCG (93). There was little doubt that hCG is anti-reverse transcriptase, showing that hCG is an antiviral agent against all RNA viruses.

Furthermore, in a subsequent publication, the same investigators tested the effects of hCG on HIV-1 transmission from lymphocytes to trophoblasts (94). They used an *in vitro* model consisting of choriocarcinoma-derived ENAMI trophoblasts (obtained from Dr. N. Matsuzaki, Osaka University Medical School, Japan) exposed to HIV-infected MOLT-4 lymphocytes. The results of the hCG (Sigma) treatment showed a U-shaped anti-viral dose effect and suggested that hCG may contribute to protection against intrauterine transmission of HIV-1. It was not until 1995 that Bourinbaiar and Lee-Huang described that the anti-HIV effect was located in the β-subunit of human chorionic gonadotropin (95). They tested purified α and β subunits of hCG for the inhibition of p24 gag protein synthesis in virus-producing ACH-2 lymphocytes and U1 monocytes.

In 1995, Dr. Robert Gallo, working at the time at the Laboratory of Tumor Cell Biology, NCI, in a collaborative study encompassing scientists from the NCI, the Gynecology and Obstetrics Medical Center in Paris, France, the University Libre de Bruxelles, Belgium and the University of Southern California School of Medicine, Los Angeles, California, studied the effects of hCG in the tumorigenesis and metastasis of neoplastic Kaposi's sarcoma cell lines in immunodeficient mice. The investigators found that both native hCG and hCGβ inhibited Kaposi's sarcoma, while the hCGα did not have any effect (96). At that time, Dr. Gallo ought to have known the *in vitro* effects of hCG on the HIV virus, and the anti-reverse transcriptase activity previously described by Bourinbaiar

and his associates (87–89), since he supplied the HIV virus to those investigators in 1992. It is very possible that due to his heavy work load Dr. Gallo did not remember those basic studies.

In the same year, 1995, working independently, Dr. Pamela Harris reported the effects of the treatment of AIDS-induced Kaposi's sarcoma with hCG injected im in very high escalating doses (97), based on the report of Gallo and his associates at the 1994 International AIDS conference in Yokohama, Japan (98). In that meeting, Gallo described the treatment of nude mice with human Kaposi's sarcoma induced by injecting cells from an HIV induced Kaposi's sarcoma cell line. They used im injections of hCG at doses of 50,000 to 100,000 IU per 10 gr of mouse weight three times per week, producing complete remission of the disease. Harris used escalating doses for her patients ranging from 150,000 to 700,000 IU of hCG im three times per week, with marked tumor progression. The only side effects of the treatment were a mild sensation of skin retraction around the lesions and tolerable pain at the sites of injection.

The problem with the work of Gallo and Harris is the very high cost of treatment. At a pharmacy price of $40 for 10,000 IU of hCG (Serono's Profasi), 700,000 IU would cost $2800. Injections required three times a week would put the cost at $8400 per week. It would be difficult for the U.S. health care insurance system to cover such an expense considering the great number of HIV infected individuals in the United States.

Another possible method of administration is the slow release form, which involves the use of a vehicle combined with any water-soluble material. hCG is soluble *ad infinitum*. Acevedo and his co-workers have already used this slow release form of administration to eliminate virus induced leukemias and lymphomas (99), so the possibility exists for its use in the treatment of AIDS.

In 1997, in one of the most important reports concerning AIDS, Bourinbaiar, Powell and Stevens, the last two of whom are the creators of the WHO anti-hCG vaccine, described and demonstrated that the antiviral effect of hCG is located in the carboxy-terminal peptide of the hCGβ, the same part of the molecule that is the target for the anti-hCG vaccine. In order to identify the active site responsible for the antiviral activity, twelve overlapping peptides spanning across the hCGβ were examined for their effect against HIV-caused cell death. The core region was biologically inert. The most potent activity was observed with the fragment representing the carboxy-terminal peptide of hCGβ. The dose response curve to serial dilution of the peptide, containing amino acid residues 109–145 had a bell-shaped appearance, characteristic of hCGβ and of hCG. The peak activity corresponded to 100 ng/ml, the dose at which two thirds of virus-exposed MT-4 T-lymphocytes survived. None of the tested peptides were toxic to MT-4 T-lymphocytes. While the mechanism of action still remains unclear, the results indicate that the unique carboxy-terminal peptide of hCG and hCGβ confers the anti-HIV activity (100).

Finally, in 1998, the origin of the HIV epidemic and the speed of its expansion around the world was discussed by Wain-Hobson (101) and by Zhu and his associates (102). Wain-Hobson and Zhu's group are in agreement that both AIDS viruses, HIV-1 and HIV-2, originated in Africa in what at that time was known as the Congo, with mangabeys (HIV-2) and chimpanzees (HIV-1). Both authors give the year as around 1959. Since then HIV-1 has been wreaking global havoc. Although HIV-2 is capable of generating an AIDS epidemic, HIV-1 has a more rapid disease course and more efficient transmission. The big problem with these viruses is that, like the influenza viruses, they are completely unstable, constantly changing their faces, thus precluding the use of *any* effective vaccine. All the treatments that are now in use are based on drugs that have anti-reverse transcriptase properties.

Unfortunately, most of these are expensive and toxic.

An article written by Angus Shaw of the Associated Press appeared in the September 9, 1999 issue of the Pittsburgh Tribune Review and was entitled, "Africa's AIDS silence a tough hurdle to jump". It was a report of a four-day AIDS conference being held in Lusaka, Zambia, sponsored by the United Nations. Shaw stated that the African continent accounts for *more than four-fifths of the world AIDS-related deaths* and that the burden of AIDS has brought health service to the brink of collapse and reduced food production in several countries.

It is puzzling how a virus known to have existed only in simians (simian adenovirus), transferred by sexual contact and possibly by mother's milk, and having apparently no lethal effect, jumped from simians to the human. One can speculate that a disease that is sexually transmitted and attacks the immune system may have a great attraction as a biological warfare product. Such a product will require a *stable virus* that can be controlled by a vaccine. HIV viruses *are not stable*. This characteristic eliminates the possibility of any effective vaccine.

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