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Immunoreactive Human Chorionic Gonadotropin and Its Free β Subunit in Serum and Ascites of Patients with Malignant Tumors[1]

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ABSTRACT

Human chorionic gonadotropin (hCG) is a clinically relevant marker of trophoblastic and nontrophoblastic malignancies. In the present studies, in addition to determining serum hCG, we investigated the presence and properties of hCG immunoreactivity in ascites of patients with nontrophoblastic malignant tumors and, for comparison, in ascites caused by cirrhotic liver disease. Total hCG immunoreactivity [hCG (+hCG-β)] was found to be elevated above the reference value (>5 IU/liter) in the serum of 2 of 20 patients with cirrhosis and 11 of 20 patients with malignant tumors. For comparison, in ascites, hCG (+hCG-β) concentrations were frequently higher than in the corresponding serum samples and exceeded 10 IU/liter in 0 of 20 cirrhotic samples and in 16 of 20 malignant samples.

In order to elucidate the nature of the hCG immunoreactive material, all samples were then assessed by immunoradiometric assays specific for the intact hCG molecule (holo-hCG) and the free hCG-β subunit, respectively. In the holo-hCG assay, elevated values were detected in 0 of 20 (0 of 20) cirrhotic ascites (serum) samples and 0 of 20 (1 of 20) malignant ascites (serum) samples. In the free hCG-β assay, on the other hand, no positive results were obtained in the ascites or serum of 20 patients with liver cirrhosis; however, 8 of 20 serum samples and 16 of 20 ascites samples derived from tumor patients were positive. In accord with the immunological data, gel chromatographical studies of malignant ascites revealed the abundance of free hCG-β subunit rather than that of holo-hCG. In contrast to malignancy-related ascites, in ascites of patients receiving hCG injections for treatment of infertility, holo-hCG was more abundant than free hCG-β immunoreactivity. Incubation experiments of purified holo-hCG in ascites for 24 h at −20, 20, or 37°C showed no substantial dissociation of the hCG molecule and release of free hCG-β immunoreactivity, thus arguing against production of free hCG-β by degradation of holo-hCG and in favor of its tumor-related secretion.

In conclusion, hCG-β immunoreactivity is frequently elevated in malignancy-related ascites and appears to be related to the presence of free β subunit of hCG rather than that of the intact hCG molecule. Interestingly, hCG-β determination in ascites proved to be clearly superior to serum measurement in discriminating between tumor and cirrhosis. Thus, hCG-β might be a useful marker of malignancy-related ascites and should be prospectively assessed for possible clinical use in comparison with other well-established parameters, such as cytology and protein determination. For this purpose, according to our results, only assays that exhibit a high sensitivity for free hCG-β subunit appear to be suitable.

INTRODUCTION

hCG[3] is a glycoprotein hormone with a molecular weight of 38,000 which is made up of two dissimilar noncovalently linked subunits, termed α- and β-subunit (1). Furthermore, the hCG molecule contains a relatively high amount of carbohydrate that makes up approximately 30% of its weight (2). Apart from its physiological role in maintaining corpus luteum function during human pregnancy, hCG is secreted by tumors of trophoblastic origin and, therefore, has long been used as an important tumor marker for the diagnosis and management of patients with testicular cancer (3–8). In addition, serum hCG immunoreactivity has been reported to be elevated in a significant portion of patients with various nontrophoblastic malignancies, such as cancers of the ovary, cervix, gastrointestinal tract, lung, and breast (9). In this respect, most studies have focused on serum determinations, whereas hCG immunoreactivity in other body fluids, such as ascites, has received scant attention. The detection of malignancy-related ascites, however, is of considerable clinical importance, and the currently used markers, such as cytological examination and protein determination, are not very efficient in separating benign from malignant ascites (10, 11).

We have therefore undertaken the present studies to evaluate the presence and properties of immunoreactive hCG in malignancy-related ascites. To this end, we determined hCG immunoreactivity in both serum and ascitic fluid of patients with various malignant tumors and, for comparison, in patients with cirrhotic liver disease. In addition, in order to further elucidate the nature of the hCG immunoreactivity, the samples were assessed by immunoradiometric assays that specifically recognize holo-hCG and the free β subunit of hCG, respectively. Finally, the hCG immunoreactive material present in malignant ascites was characterized by gel chromatography.

MATERIALS AND METHODS

Highly purified preparations of intact hCG (CR121) and the β-subunit of hCG (CR123-β) were supplied by the Hormone Distribution Program of the National Institute of Diabetes and Digestive and Kidney Diseases. The third International Reference Preparation 75/537 for hCG and the first International Reference Preparation 75/551 for the β subunit of hCG used for standardization of IRMAs were kindly provided by the National Institute for Biological Standards (London, United Kingdom).

mab 11/6 (against holo-hCG and holo-LH), mab 3/6 [against hCG (+hCG-β)], mab 12/17 [against hCG (+hCG-β), different epitope from that recognized by mab 3/6], and mab 2/6 (specific for free hCG-β subunit) were kindly donated by Dr. K. Siddle (University of Cambridge, Cambridge, United Kingdom). They had been raised in BALB/c mice and characterized as described previously (12).

Patients

Patients with Liver Cirrhosis. Group I consisted of 20 patients with ascites due to cirrhotic liver disease (13 men, 7 women; age, 28–79 years; mean ± SD, 56 ± 13 years). Diagnosis had been established in these patients by liver biopsy and histological examination. With respect to the etiology of liver disease, 12 patients had alcoholic cirrhosis and 6 patients had posthepatitic liver cirrhosis. Other causes included primary biliary cirrhosis ($n = 1$) and Budd-Chiari syndrome ($n = 1$).

Patients with Malignant Tumors. Group II consisted of 20 patients with advanced malignant diseases and ascites (7 men, 13 women). Their mean age was 57 ± 13 years (range, 33–80 years). In all patients, histological proof of the primary tumor was obtained and diagnoses were as follows: carcinomas of the breast ($n = 4$), stomach ($n = 3$), colon ($n = 3$), pancreas ($n = 1$), ovary ($n = 3$), cervix ($n = 2$), and

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[3] The abbreviations used are: hCG, human chorionic gonadotropin; holo-hCG, intact hCG molecule; mab, monoclonal antibody; IRMA, immunoradiometric assay; NIH, National Institutes of Health.

bladder (n = 1); hepatocellular carcinoma (n = 1); carcinoid (n = 1); and adenocarcinoma of unknown origin (n = 1).

Patients Treated with hCG. Ascites samples were obtained from two 29-year-old women who had received hCG injections for treatment of infertility and developed ascites due to ovarian overstimulation.

hCG Determinations

Three specific IRMAs, which were previously been described and characterized in detail (13–15), were used to measure total hCG-related immunoreactivity [hCG (+hCG-β)] in serum and ascites and to discriminate between the presence of holo-hCG and free hCG-β.

The hCG (+hCG-β) IRMA uses mab 3/6 and mab 12/17 and recognizes the β subunit of hCG in its combined form within the holo-hCG molecule as well as in its free form (cross-reactivity of 720% on a molar basis).

The holo-hCG IRMA utilizes mab 3/6 and mab 11/6 and measures the intact hCG molecule with negligible cross-reactivity with free hCG-β subunit (<0.01%) and other pituitary hormones (15).

The free hCG-β IRMA with mab 2/6 and mab 12/17 is highly specific for the free β subunit of hCG and exhibited cross-reactivities of 0.36–2.7% with purified holo-hCG from various sources (15, 16).

The assays use two monoclonal antibodies each and were carried out by coupling the first antibody (dilution 1:5000 of native ascitic fluid in 50 mmol/liter ammonium bicarbonate, pH 8.0) to polystyrene tubes (Star-Tubes; Nunc, Wiesbaden, Germany) and using the second antibody as ^{125}I-labeled tracer (Chloramine-T method)(14). The antibody-coupled tubes were incubated with 100 μl of serum or ascites and 100 μl of IRMA buffer (phosphate-buffered saline containing 1% bovine serum albumin) under constant shaking at room temperature for 2 h. After the tubes were washed twice with 10 mmol/liter Tris-Cl buffer, pH 8.0 (1 ml), containing 0.05 g Tween-20/liter, incubation with the ^{125}I-labeled second antibody (approximately 100,000 cpm; 2–3 ng in 100 μl) was performed under the same conditions. After the tubes were again washed, the radioactivity bound was determined by the use of a gamma counter (Multigamma; LKB, Freiburg, Germany).

The hCG (+hCG-β) IRMA and the holo-hCG IRMA were standardized at the third International Reference Preparation for hCG, while the free hCG-β IRMA was standardized with the first International Reference Preparation for hCG-β. For that reason, results expressed in IU/liter have a different meaning for the different assays and are not to be compared directly among the assays. For comparison, 1 ng holo-hCG (NIH CR121) showed an immunoreactivity of 10 mIU in the hCG (+hCG-β) IRMA and of 8 mIU in the holo-hCG IRMA. On the other hand, 1 ng hCG-β (NIH CR123-β) displayed an immunoactivity of 60 mIU in the hCG (+hCG-β) assay and of 1.6 mIU in the free hCG-β assay.

Reference values for ascites were not known for any of the assays prior to the present study and were defined according to the results obtained in cirrhotic ascites samples. Reference values for serum are given in Table 1.

The analytical performance of the assays was reported in detail previously (14). Briefly, the coefficients of intraassay variation were between approximately 5 and 10% at different concentrations, and the interassay coefficients of variation ranged between 9 and 13%.

Chromatography of Ascites on a Column of AcA 44

To separate holo-hCG and its subunits, ascites samples obtained from two patients with carcinoma of the bladder and hepatocellular carcinoma (0.5 ml) containing large amounts of hCG (+hCG-β) immunoreactivity (14,492 and 50,422 IU/liter) were directly applied to a column of Ultrogel AcA 44 (Serva, Heidelberg, Germany) (1 x 100 cm) preequilibrated with 0.01 M Tris-Cl, 0.15 M NaCl, pH 8.0. Fractions obtained upon eluting the column with the same buffer were measured by specific holo-hCG and free hCG-β IRMA as described above. The column was calibrated by dextran blue and several molecular weight markers. In some experiments, elution of ^{125}I-hCG was studied concomitantly. The elution position of hCG-β subunit (CR123-β) was determined in a separate experiment after chromatography of ascites.

Table 1 *Limits of detection and reference values for serum for hCG assays*

	Solid phase	Tracer	Limit of detection[a]	Reference value[b] (IU/liter)
hCG (+hCG-β) IRMA	mab 3/6	mab 12/17	2 IU/liter	<5
holo-hCG IRMA	mab 3/6	mab 11/6	2 IU/liter	<5
free hCG-β IRMA	mab 2/6	mab 12/17	0.05 IU/liter	<0.2

[a] Limits of detection, the concentration interpolated from the curve at 3 SD above the zero point.

[b] Reference values, obtained by using the 95th percentile of 200 normal subjects of both sexes including 46 postmenopausal women.

Incubation Experiments of hCG in Ascites

To determine its stability, a defined amount of holo-hCG (CR121) was added to an hCG (+hCG-β) negative ascites sample, a control serum, and the IRMA buffer (phosphate-buffered saline containing 1% bovine serum albumin). The samples were kept at −20, 20, or 37°C for 24 h and then measured by hCG (+hCG-β) IRMA, holo-hCG IRMA, and free hCG-β IRMA. In other experiments, dilution and recovery of hCG immunoreactivities in ascites were studied.

Statistical Methods

For statistical analysis of data, Wilcoxon tests for paired or unpaired observations were used.

RESULTS

hCG Determinations in Serum. Initially, total hCG-related immunoreactivity was assessed in the sera from patients by hCG (+hCG-β) IRMA that recognizes the β subunit of hCG in its free form as well as in the holo-hCG molecule. Levels above normal (>5 IU/liter) were found in 2 of 20 (10%) patients with liver cirrhosis (group I) and in 11 of 20 (55%) patients with malignancies (group II). Serum concentrations of both intact holo-hCG and free hCG-β subunit immunoreactivity were then determined in the two groups of patients by the use of the two specific IRMAs described in "Materials and Methods." The data are shown in Fig. 1. In group I, values above the normal range for holo-hCG were found in 0 of 20 patients and, for free β subunit of hCG, in 1 of 20 (5%) patients. In group II, on the other hand, holo-hCG was elevated in 1 of 20 (5%) patients, and hCG-β was elevated in 8 of 20 (40%) patients.

hCG Determinations in Ascites. hCG (+hCG-β) immunoreactivities were mostly higher in ascites as compared to serum values, particularly in tumor patients. Fig. 2 shows the corresponding results in serum and ascites for each patient.

Furthermore, in ascites samples derived from patients with malignant diseases, significantly higher hCG (+hCG-β) activities were found than in samples from patients with liver cirrhosis (median, 19.1 *versus* 2.2 IU/liter, $P < 0.01$). When 10 IU/liter was defined as the upper limit of the normal range, 20 of 20 (100%) of the cirrhotic ascites samples were judged to be normal and 16 of 20 (80%) of the malignant ascites samples were considered pathological (Fig. 3).

Further assessment of the same samples by the more specific IRMAs against holo-hCG and free hCG-β revealed that elevation of hCG (+hCG-β) activity was caused by the presence of free hCG-β activity rather than that of holo-hCG (Fig. 3).

In malignant ascites, immunoactivities of free hCG-β ranged between <0.05 and 102 IU/liter (median, 0.85 IU/liter) and were elevated >0.2 IU/liter in 16 of 20 (80%) samples. Holo-hCG immunoactivities, on the other hand, were undetectable in 17 of 20 (85%) malignant ascites samples and exceeded the normal range (>5 IU/liter) only in one case. For comparison, the 20 cirrhotic ascites samples showed all normal free hCG-β

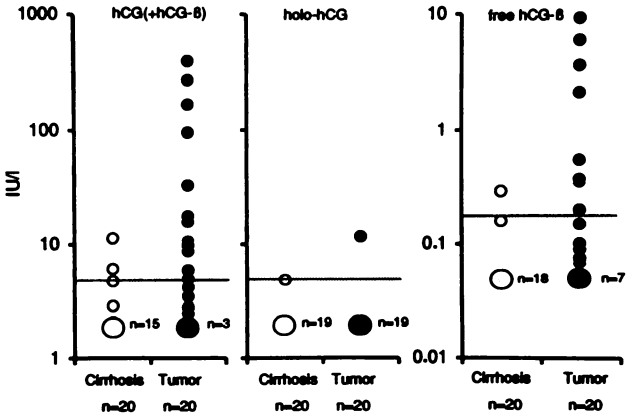

Fig. 1. Determination of hCG (+hCG-β), holo-hCG, and free hCG-β immunoreactivities in serum of patients with cirrhotic liver disease and malignant tumors. Three specific IRMAs were used to determine total hCG-β-related immunoreactivity [hCG (+hCG-β)] and to distinguish between the presence of the holo-hCG or the free hCG-β. Immunoreactivities for hCG (+hCG-β), holo-hCG, and free hCG-β were elevated in 2 of 20, 0 of 20, and 1 of 20 patients with cirrhosis and 11 of 20, 1 of 20, and 8 of 20 patients with malignant tumors, respectively. *Big circles*, the specified number of values that were below the detection limit of the assay; *dashed lines*, the upper limit of the normal range of each assay. Note that a different scale was used for free hCG-β. Also, because of different standardization of the assays with hCG or hCG-β, results expressed in IU/liter have a different meaning for each assay and are not directly comparable among the IRMAs (see "Materials and Methods").

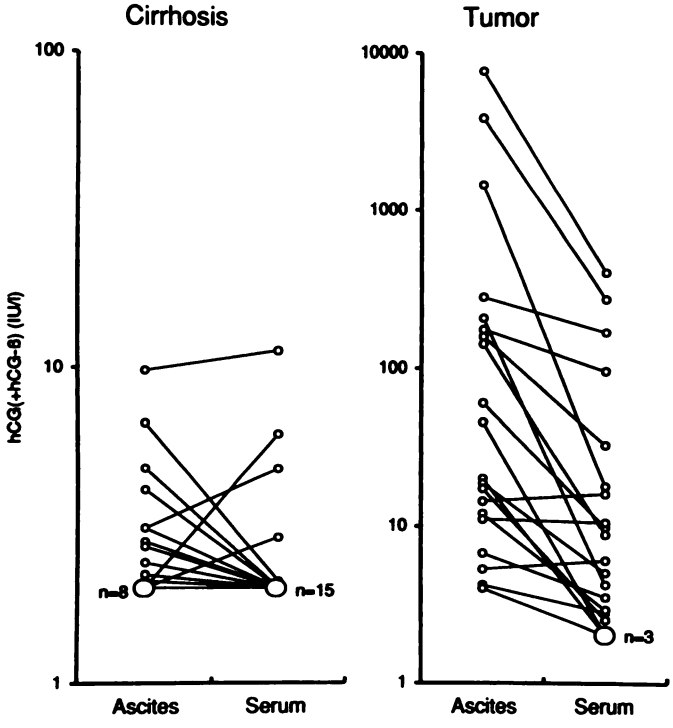

Fig. 2. Comparison of hCG (+hCG-β) immunoreactivities in ascites and corresponding serum samples of patients with cirrhotic liver disease and malignant tumors. The data show that hCG (+hCG-β) concentrations are frequently higher in ascites than serum. Statistical evaluation by Wilcoxon signed rank test: for cirrhosis, $n = 20$, $P > 0.05$; for tumor group, $n = 20$, $P < 0.001$).

activities (<0.2 IU/liter) ranging from <0.05 to 0.18 IU/liter (median, <0.05 IU/liter), as well as normal, mostly undetectable holo-hCG activities (Fig. 3).

In contrast to malignancy-related ascites samples, ascites samples obtained from two women who had received hCG injections and developed ascitic fluid due to ovarian overstimulation displayed elevated activities of both holo-hCG (65.5 and 21.2 IU/liter) and free hCG-β subunit (0.59 and 0.48 IU/liter). Corresponding serum values were 139 and 11 IU/liter, respectively, for holo-hCG and 0.38 and 0.22 IU/liter, respectively, for free hCG-β.

Identification of Free hCG-β in Malignant Ascites. Two ascites samples from patients with carcinoma of the bladder and hepatocellular carcinoma were selected on the basis of highly elevated hCG (+hCG-β) immunoreactivities and subjected to gel chromatography on a column of AcA 44, as described in "Materials and Methods" (Fig. 4). Fractions eluted from the column were measured by specific holo-hCG and specific free hCG-β IRMA, respectively. They contained a significant amount of free hCG-β activity, whereas any appreciable activity of intact holo-hCG was lacking. Furthermore, the position of elution of the hCG-β immunoreactive material in ascites was virtually identical with that of purified hCG-β subunit (NIH CR123-β) of pregnancy origin and clearly distinguishable from the ^{125}I-holo-hCG elution pattern.

In order to determine its stability in various media, purified holo-hCG was added to ascites, serum, and IRMA buffer. After the samples were incubated at different temperatures (−20, 20, 37°C) for 24 h, hCG (+hCG-β), holo-hCG, and free hCG-β immunoreactivities were measured. The results are shown in Fig. 5. As can be seen, there was no substantial dissociation of holo-hCG into its subunits under these conditions, although at 37°C a small increase in free hCG-β activity was detectable in all three media.

Dilution of highly positive malignant ascites samples, as well as of ascites samples to which holo-hCG- or hCG-β standard preparations had been added, resulted in a linear decrease of immunoreactivities measured (data not shown). Furthermore, dose-response curves of holo-hCG and hCG-β standards proved to be parallel in serum and ascites in holo-hCG and free hCG-β IRMA, respectively. Also, recoveries of holo-hCG and hCG-β in ascites were similar to those in serum (data not shown).

DISCUSSION

In the present study we evaluated the presence and nature of hCG immunoreactivity in serum and ascites of patients with various malignant tumors and, for comparison, patients with cirrhotic liver disease.

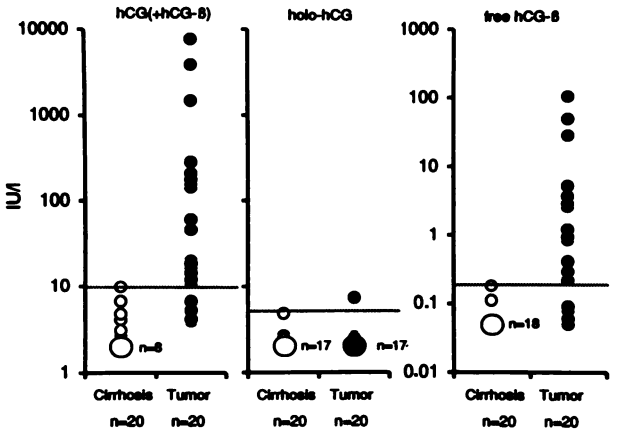

Fig. 3. Determination of hCG (+hCG-β), holo-hCG, and free hCG-β immunoreactivities in ascites of patients with cirrhotic liver disease and malignant tumors (see legend to Fig. 1). Immunoreactivities for hCG (+hCG-β), holo-hCG, and free hCG-β were elevated in 0 of 20, 0 of 20, and 0 of 20 patients with cirrhosis and 16 of 20, 1 of 20, and 16 of 20 patients with malignant tumors, respectively.

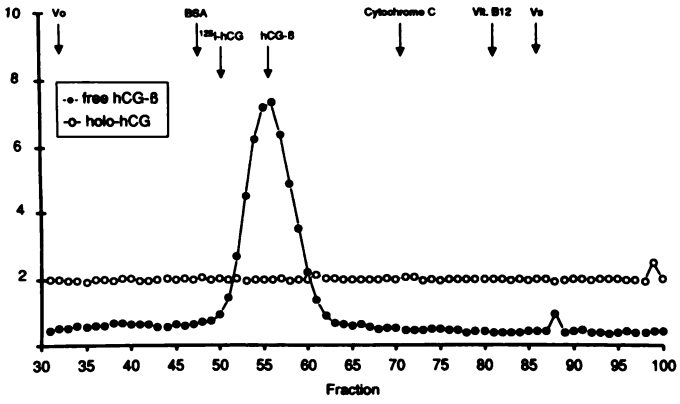

Fig. 4. Chromatography on an AcA 44 column of ascites obtained from a patients with carcinoma of the bladder. The data indicate the abundance of free hCG-β subunit in the absence of holo-hCG in this ascites sample. Native ascites (0.5 ml) containing 14,492 IU/liter of hCG (+hCG-β) immunoreactivity and 203 IU/liter of free hCG-β activity was applied to the column. Upon eluting with 0.01 M Tris-Cl, 0.15 M NaCl (pH 8.0), fractions were measured by IRMAs specific for holo-hCG and free hCG-β subunit. *Arrows*, elution positions of ^{125}I-holo-hCG, free hCG-β, and molecular weight markers [dextran for detection of void volume (Vo); bovine serum albumin (BSA), M_r 67,000; cytochrome, M_r 12,000; and vitamin (vit.) B_{12} M_r 1,200]. Please note that limits of detection of the two assays are different and that their results are not directly comparable on the basis of the IU/liter values, because those relate to different standards (see "Materials and Methods").

In accord with previous reports (9, 17–22), serum concentrations of total hCG-related immunoreactivity [holo-hCG or free hCG-β subunit, hCG (+hCG-β)] were elevated above the normal range in a substantial fraction of our tumor patients (55%). In comparison, elevated serum hCG (+hCG-β) activity was rare in patients with liver cirrhosis (2 of 20, 10%). More interesting, however, we found that hCG (+hCG-β) immunoreactivities in patients with malignancies were usually higher and more often elevated in ascites as compared to serum. The differences could not be accounted for by the use of different media, since dose-response curves showed parallel behavior in serum and ascites, and recovery of hCG standard preparations was comparable in the two media (data not shown). As a result, measurements in ascites were superior to serum determinations in discriminating between tumor patients and patients with cirrhosis. At a cutoff value of 10 IU/liter, hCG (+hCG-β) determination correctly identified 80% of tumor samples and 100% of cirrhotic ascites samples. There have apparently been no reports in the literature on the value of hCG determination in ascites of tumor patients as compared to serum assessment. With respect to hCG measurement in malignant effusions, a few studies are available (23–25). Couch (23) reported a sensitivity of 36% for hCG (+hCG-β) (>10 IU/liter) in malignant ascites, which is much lower than our data indicate.

This discrepancy may be due to the following reasons. There may be differences between the study groups regarding type and stage of tumors. Furthermore, the IRMA technique generally offers an enhanced sensitivity compared to formerly used radioimmunoassay methods. Finally, the particular hCG (+hCG-β) IRMA we have used in the present study has a remarkably high cross-reactivity with free β-subunit of hCG (720%), which is not reached by most commercially available hCG (+hCG-β) kits (14). In that respect, we could previously demonstrate that the use of such an assay yielded a higher sensitivity in certain types of cancer, such as hCG-positive seminoma, in which free hCG-β subunit secretion by the tumor occurs in the absence of holo-hCG production(13, 14).

In addition to measuring total hCG-related immunoreactivity, we were particularly interested in further elucidating the nature of this material. To this end, ascites samples containing large amounts of hCG (+hCG-β) immunoreactive material were subjected to gel chromatography. The material showed a pattern of elution very similar to that of the free β subunit of hCG as opposed to holo-hCG. Furthermore, it exhibited a strong immunoreactivity in our IRMA that recognizes specifically the free β subunit of hCG and does not appreciably cross-react with holo-hCG; whereas there was no activity in an IRMA specific for holo-hCG. These findings suggested that free hCG-β activity was more abundant in malignant ascites than holo-hCG.

All ascites samples were then directly measured by the two IRMAs specific for free hCG-β and for holo-hCG. Reference values for each assay were defined on the basis of the results obtained in cirrhotic ascites samples. Cutoff values of 0.2 IU/liter for free hCG-β and 5 IU/liter for holo-hCG yielded negative results in all 20 cirrhotic ascites samples. Using these reference values, we found that free hCG-β activity was elevated in 80% of malignant ascites samples. In contrast, holo-hCG immunoactivity was negative in all but one of the malignant ascites samples tested.

Presence of free hCG-β in ascites is unlikely to be due to release from the holo-hCG molecule, as indicated by the following observations: (*a*) incubation experiments with purified holo-hCG failed to detect a meaningful subunit dissociation in ascites, and (*b*) more important, in ascites samples obtained from women who had received hCG injections for therapeutic reasons and who developed ascites due to ovarian overstimulation, representing a model of *in vivo* incubation of hCG, holo-hCG

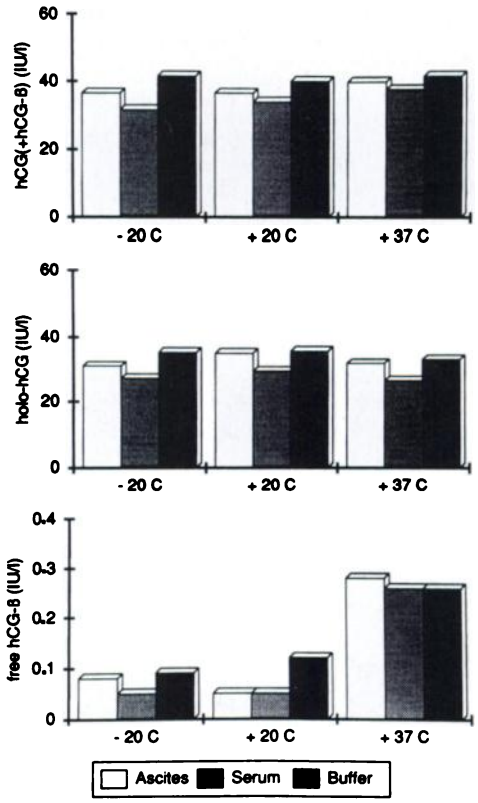

Fig. 5. Incubation of holo-hCG in ascites, serum, and IRMA buffer. A defined amount of purified holo-hCG (NIH CR121) was incubated in the various media for 24 h at −20, 20, or 37°C, and the samples were subsequently measured by IRMAs specific for hCG (+hCG-β), holo-hCG, and free hCG-β. The data show a small increase in free hCG-β activity in all three media at 37°C incubation but no substantial dissociation of holo-hCG into its subunits under any condition.

(not free hCG-β) was preferentially detectable. The prevalence of free hCG-β in malignant ascites, on the other hand, was consistent with the pattern in the sera of our tumor patients in which free hCG-β was detectable more often than holo-hCG. For those reasons, we assume that secretion by the tumor cells of free β subunit may occur independently of holo-hCG, as has been reported for seminoma and other solid tumors (13, 14, 26, 27).

In conclusion, the present data indicate that hCG-β immunoactivity is frequently elevated in malignancy-related ascites, and, with respect to the nature of the material, free hCG-β subunit rather than holo-hCG is abundant. Remarkably, hCG-β concentrations were higher in the ascites than in the serum of tumor patients, and hCG-β determination in ascites was superior to serum measurement in discriminating between cirrhosis and tumor.

As for the clinical implications of this study, hCG-β could possibly be a marker of malignancy-related ascites. In this respect, our preliminary data seem to be promising but need to be confirmed in another larger prospective study which should clarify the role of hCG-β in comparison with other well-established parameters, such as cytological examination or measurements of protein, cholesterol, and fibronectin (10, 11, 28, 29). The sensitivity of this marker, however, may depend on the method used for hCG determination, and, according to our results, only assays that exhibit a high sensitivity for the free β subunit of hCG, but not assays that specifically recognize the holo-hCG molecule, can be recommended.

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