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Development and Characterization of Antibodies to a Nicked and Hyperglycosylated Form of hCG from a Choriocarcinoma Patient

Generation of Antibodies That Differentiate Between Pregnancy hCG and Choriocarcinoma hCG

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Human chorionic gonadotropin (hCG) exists in blood and urine as a variety of isoforms one of which contains peptide bond cleavages within its β-subunit loop 2 and is referred to as nicked hCG (hCGn). This hCG isoform appears to be more prevalent in the urine of patients with certain malignancies and possibly in some disorders of pregnancy. Until now, only indirect immunoassays could be used to quantify hCGn. We report the development of two monoclonal antibodies (MAbs) to a form of hCGn isolated from a choriocarcinoma patient. This hCG isoform was not only 100% nicked, but also contained 100% tetrasaccharide-core O-linked carbohydrate moieties in its β COOH-terminal region. Two-site immunometric assays have been developed using these new antibodies, B151 and B152. The former exhibits good specificity for hCGn independent of the source of the hCGn, the form excreted by choriocarcinoma patients or the form of hCGn from normal pregnancies. The latter antibody, B152, is sensitive to the carbohydrate moieties and possibly other differences in hCG isoforms, but is not for nicking of the β–subunit. These two immunometric assays provide potential novel diagnostic tools for direct measurement of hCG isoforms which could not be accurately quantified earlier before development of the assays using these newly generated antibodies.

Key Words: Gonadotropins; nicked hCG; monoclonal antibodies; choriocarcinoma; hyperglycosylated hCG.

Introduction

Human chorionic gonadotropin (hCG) is a glycoprotein hormone produced by trophoblast cells of the placenta. The measurement of this hormone in blood or urine is the basis of all pregnancy tests. It is secreted by the trophoblast starting very early in pregnancy and functions to maintain steroid production by the corpus luteum until the placenta takes over that function later in pregnancy *(1)*. There has been much recent interest in measurement of the hormone or its subunits or fragments for purposes other than the diagnosis of pregnancy, such as monitoring of therapy for hCG-secreting malignancies *(1)*, tests for indication of Down's syndrome or other genetically-abnormal pregnancies *(2–11)*, ectopic pregnancies *(12)*, and so forth. HCG appears in urine in a variety of forms, including free subunits, heterodimeric hCG with peptide bond cleavages in loop 2 of its β–subunit (known as nicked hCG), free nicked β–subunits, and the β core fragment *(13–16)*. The nicked form of heterodimeric hCG has been reported to be present in blood as well as urine and is known to have much reduced immunochemical recognition by some antibodies directed to heterodimeric hCG as well as greatly reduced biological activity *(13,16,17)*. There are reported associations between increased nicking of hCG with certain hCG-secreting malignancies *(18,19)*.

There are no satisfactory direct immunoassays for the nicked form of hCG or for the nicked form of free hCG β–subunit. All measurements to date have been conducted with subtractive assay procedures or immunoassays, which include scavenger antibodies *(20–22)*. We report here the development of two antibodies of distinct specificity, using an immunogen the form of hCG produced by a single individual with choriocarcinoma. This particular hCG isoform was 100% nicked and hyperglycosylated both in its *N*- and *O*-linked carbohydrate moieties. One resultant antibody, B151, displays significant preference in recognition for nicked hCG forms. The second antibody, B152, displays distinct preferences toward binding to its choriocarcinoma

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hCG immunogen and does not recognize the nicked characteristic. Immunoassays developed using B152 are of special interest in their enhanced recognition of hCG isoforms more prevalent in preeclampsia and Down syndrome *(23,24)*. B152 also appears to detect an hCG isoform associated with healthy pregnancies as juxtaposed to those pregnancies destined to fail early, which have little of this isoform *(25)*.

Results

Characteristics of Antibodies

A variety of hCG isoforms were employed to characterize the new antibodies described in this article, and the nomenclature and characteristics of each of the reagents employed are summarized in Table 1. The carbohydrate groups in these hCG isoforms as well as the percent nicking were analyzed in an earlier study *(26)* and are directly relevant for defining the nature of these new antibodies described in this article.

Two antibodies designated B151 and B152 were selected by the use of radiolabeled hCG isoforms, chorioCG C5 and pregnancy hCG CR127. Each displayed preferential binding to C5 as compared to CR 127, since this was the selection criterion. However, on performing liquid-phase immunoassays and calculating affinity constants, it was clear that these two antibodies were very different in specificity (Table 2). It was found that antibody B151 had one order of magnitude higher affinity both for C5, which is nicked and hyperglycosylated choriocarcinoma hCG, and for CR127 hCGn (813) as compared to CR 127 hCG or nick-free CR 127 hCG (814) (*see* Table 1 for reagent descriptions). B151 was clearly an antibody with a strong preference for binding to various forms of nicked hCG. Antibody B152 was different in that although it displayed one order of magnitude preference for C5 hCG over CR127 hCG, it recognized the nicked and nonnicked forms of CR 127 hCG, hCG derived from normal pregnancies, to an equal extent.

Liquid Phase Assays

Figure 1 shows potency comparisons of liquid-phase immunoassays of both B151 and B152 antibodies comparing competitors:

1. Standard CR 127 pregnancy hCG (which has a 20% content of nicked hCG).
2. C5 chorio CG (100% nicked and hyperglycosylated).
3. 813, nicked CG made from CR 127 by purification.
4. 814, nonnicked hCG derived from CR 127.

The labeled ligand was C5 chorio CG. It is apparent that B151 (Fig. 1A) shows a preference for nicked forms of hCG. C5 chorio-CG or 813 hCGn bind with similar affinities. The slightly lower potency of 813 hCGn may be ascribed to its 20% contamination with nonnicked hCG. B152 only shows a preference to C5, the hyperglycosylated chorio CG (Fig. 1B). 813 hCGn is no more potent a competitor than nick-free 814 hCG.

Immunometric Two-Site Assays

A variety of two-site antibody formats were tested. Table 3 displays these results. It is apparent that B151 cannot bind simultaneously with antibodies (designated by us as site IV) to the β–subunit and β–subunit core (B201 and B204) or with antibodies directed toward the determinant, which exists in heterodimeric hCG as represented by antibody B109 (site III, to which A109 also belongs) *(27)*. In contrast, a general β antibody that binds to the most common and potent hCG antigenic site previously designated by us as site II (B108 or B207) binds well simultaneously with both B151 and B152 antibodies. B152 binds simultaneously with all antibodies tested except for those to the β COOH-terminal region (CTP) *(28)* in contrast to B151 which binds well with CTP antibodies. B151 may represent a newly revealed hCG epitope, which only exists on nicked hCG as reported in this article.

Using B152 as capture and B207 or B108 as detection antibody produces an assay that measures all normal pregnancy forms of hCG (both intact and nicked and β–subunit) to a similar extent, but prefers binding to the form of hCG or β–subunit from its immunogen, C5. As predicted earlier from the liquid phase studies, this assay does not prefer nicked forms of hCG, but hyperglycosylated forms of hCG such as C5. B152 and CTP 104 as well as several other monoclonal antibodies (MAbs) to the COOH-terminal region of hCG β cannot bind simultaneously to C5, implying that this region is part of or very close to the epitope of B152. Taken together with the apparent B152 preference for hyperglycosylated hCG, these data imply that the carbohydrate of the CTP region may be part of the B152 epitope. Further studies of the behavior of B152 in two-site assays confirm this hypothesis as detailed below.

Characteristics of the B152-B207-I^{125} Radioimmunometric Two-Site Assay

In order to understand better what this assay is measuring, we compared the relative binding potencies of a series of isoforms of hCG shown in Fig. 2 (*also see* Materials and Methods and Table 1):

1. C5, choriocarcinoma hCG
2. 814, nonnicked hCG.
3. 813, nicked hCG (80% nicked).
4. M4 mole–derived hCG, 98% nicked hCG with neglible hyperglycosylation .
5. M1A hCG, nonnicked and not significantly hyperglycosylated but missing 80% of the hCG β COOH-terminus.

The B152 two-site assay prefers to bind to C5, its immunogen, but shows nearly equal recognition of both 813 and 814, nicked and nonnicked hCG of normal pregnancy. This confirms that B152 does not display significant preference for the nicked form of hCG, but rather displays preference for the form with carbohydrate differences. This is also confirmed by the potency of M4, which is also 100% nicked

Table 1

Characteristics of the Reagents Used to Define Antibody Specificity[a]

Name	Source	*N*-Sialic acid[b]	*O*-Sialic acid[c]	% Triantennary *N*-linked on β	% Tetrasaccharide *O*-linked core	% β nicking
814 hCG	CR 127 hCG[d]	95	66	5	19	19
C5 chorioCG	Choriocarcinoma	95	58	48	100	100
M4 mole CG	Mole pregnancy	120	49	30	20	98
813 hCGn	CR 127 hCG	ND	ND	ND	ND[e]	80
C7 chorioCG	Choriocarcinoma	68	53	48	69	3
P8 hCG	Pregnancy	94	73	21	13	0
M4 mole β	Mole pregnancy	120	49	30	20	98
C5 chorio β	C5 chorioCG	95	58	48	100	100
CR 129 β	CR 129 hCG	96	63	11	17	19
HLH I-1	Pituitary hLH	ND	NA[e]	NA	NA	NA
M1A	Mole pregnancy	98.5	NA	16.5	<15% CTP[f]	24

[a]The peptide and carbohydrate structures of the reagents used were determined earlier ***(26)***. The % nicked β-subunit refers to the proportion of molecules with cleavages (missing peptide bonds) in the region β43 to β48. The % tetrasaccharide core is the proportion of *O*-linked oligosaccharides with tetrasaccharide (vs disaccharide) core structure, and the % sialic acid is the proportion of *O*-linked structures with antennae terminated by sialic acid residues. The proportion of triantennary *N*-linked oligosaccharides on β-subunit is given, as is the corresponding % sialic acid.

[b]% Sialic acid residues per sugar chain, *N*-linked on β.

[c]% Sialic acid residues per sugar chain, *O*-linked on β.

[d]The "CR" series of hCG reference preparations were made at Columbia University and were distributed internationally as reference materials for purified hCG. CR 119 is also known as the 3rd international immunoassay reference preparation for hCG.

[e]ND is not done; NA is not applicable to that reagent.

[f]Fewer than 15% of the hCG molecules have the β COOH-terminal present.

Table 2

Affinity Constants[a] Determined by Liquid-Phase Competition Assays Using C5 as Tracer Ligand

	Competitors			
Antibody	C5 chorioCG	Nicked hCG CR 127 (813)	Parent CR 127 hCG[b]	Nick-free hCG C R127 (814)
B151	4.4×10^8	3.8×10^8	4.2×10^7	1.3×10^7
B152	3.5×10^8	5.4×10^7	4.7×10^7	5×10^7

[a]Ka as L/M.

[b]hCG CR 127 is an NIH-distributed hCG reference preparation produced at Columbia University.

as is C5, but is not hyperglycosylated and displays a potency similar to CR127 hCG whether nicked or non-nicked. M1A is the least potent ligand and is the only one missing most of its β COOH-terminal peptide confirming the role of this region in the B152 epitope.

In order to explore further the nature of the B152 binding site, a commercially available peroxidase-labeled general hCGβ antibody (4001 from Genzyme, San Carlos, CA) was employed as a detection antibody in a two-site enzyme immunometric system. Eight different hCG forms were evaluated in this system illustrated in Fig. 3. Results are analyzed in terms of relative immunopotency (based on the slope of the regression line) in Table 4. Linear regression correlation analysis was performed to compare the relationship of the immunopotencies of preparations 814, C5, M4, C7, and P8 one at a time with the carbohydrate differences (Table 1) as well as nicking differences among the five heterodimeric isoforms of hCG. The correlation results for each comparison are as follows:

1. Tetrasaccharide *O*-linked core: $R^2 = 0.9147$ $P = 0.0108$, significant.
2. Trianntennary branched moeities *N*-linked on β: $R^2 = 0.8853$ $P = 0.0171$, significant.
3. Sialic acid *O*-linked: $R^2 = 0.3062$ $P = 0.3332$, not significant.
4. Sialic acid *N*-linked on β: $R^2 = 0.2289$ $P = 0.4149$, not significant.
5. Percent nicking in β–subunit: $R^2 = 0.0984$ $P = 0.6072$, not significant.

To summarize this analysis, the immunopotency of hCG isoforms measured with B152 as a capture antibody in a two-site assay correlates best with hyperglycosylated core

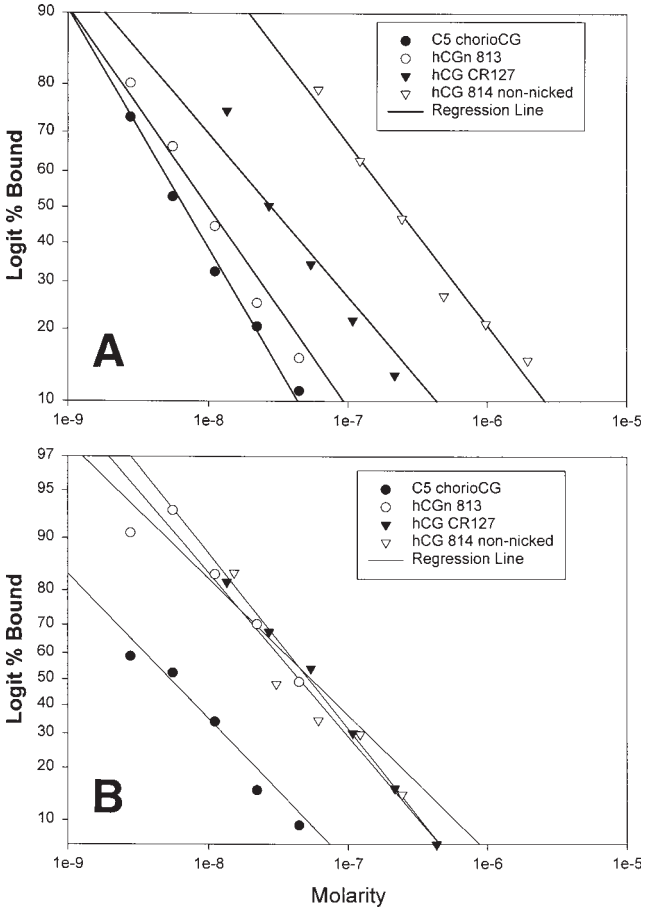

Fig. 1. Liquid-phase radioimmunoassays using antibody B151 (panel **A**) and antibody B152 (panel **B**). Radiolabeled choriocarcinoma hCG C5 was used as tracer, and calibrated (by amino acid analysis) solutions of pregnancy C5 chorioCG, hCG CR 127, hCG CR 127 nonnicked, and hCGn CR 127 were employed as competitors. Nonlinear regression lines were plotted in logit transformed format.

moeities of both *O*- and *N*-linked amino acids, but not with sialic acid residues or with the degree of nicking of the β–subunit.

Discussion

The various isoforms of hCG have received increasing attention during recent years for their potential diagnostic value in problem pregnancies, such as Down's syndrome, preclampsia, trophoblastic diseases, and early pregnancy loss. Nicked hCG at high concentrations has been associated with trophoblastic disease and other abnormal states of hCG production. Although nicked hCG has been measured by a variety of qualitative techniques, such as immunoblotting, as well as by direct isolation and sequence analysis of such hCG isoforms from urine, it is only recently that some investigators have been measuring nicked forms of hCG by a variety of subtractive immunoassays or nonspecific hCG assays with the addition of scavenger antibodies since direct, relatively specific antibodies were not available *(22,29)*. We had earlier shown that our commonly used antibody, B109, to heterodimeric hCG reacted poorly to nicked forms of hCG, and this antibody was employed by other groups in subtractive methods in attempts to quantify the content of nicked hCG in blood and urine *(22,29)*.

In this article, we describe the development of two antibodies produced using choriocarcinoma-derived nicked hCG as an immunogen. This led to a direct assay for nicked hCG. By employing a nicked, hyperglycosylated form of hCG from a single choriocarcinoma patient, we have developed two antibodies with distinct specificities. One antibody (B151) prefers binding to nicked forms of hCG, regardless of the origin of the hCG molecule (normal pregnancy or choriocarcinoma). Nick-free CR127 hCG has the lowest affinity to this antibody as would be expected for a nick-directed antibody. Although B151 was directed toward an epitope dependent on peptide bond cleavages in β loop 2, B152 binding was not affected by peptide bond cleavages within this loop. Because the main difference between C5 hCG and CR 127 hCGn was the hyperglycosylation of the former, it was inferred that B152 was chiefly a carbohydrate-directed antibody. When B151 is used as capture antibody and virtually any general beta antibody as detection antibody in a two-site assay (*see* Table 3), little crossreaction with hLH is observed. B151 cannot bind simultaneously with antibodies that are directed chiefly toward the hCG β core region (site IV, such as B201 and B204) *(28)*, nor can the antibody bind with site III antibodies directed to heterodimeric hCG, such as B109 or A109. However, B151 can bind at the same time as antibodies to the β COOH-region. B151 may represent a new hCG epitope revealed after nicking of β loop 2. The creation of new epitopes by nicking of the hCG molecule has been reported by others *(30)*.

The second antibody, B152, preferentially binds hCG with the type of carbohydrate modifications prevalent in choriocarcinoma CG C5 regardless of the state of the nicking of the polypeptide chain. This was shown by the measurement of the relative immunopotencies of several well-characterized hCG isoforms, as compared to their contents of *N*- and *O*-linked carbohydrate moieties, sialic acid, and percentages of nicking. A significant correlation of B152 binding to hyperglycosylated hCG isoforms, but not to those with sialic acid or nicking differences was demonstrated. Antibody B152 appears to have at least partial specificity toward the hCG β COOH-terminal region. This is shown by failure of an MAb to bind simultaneously with β COOH terminal antibodies, such as CTP 104. In studies of B152 as a two-site assay, it was shown that hCG isoform M1A, which is missing most of its COOH-terminal region, binds poorly to B152.

Each antibody has a different application in accordance with its specificity. Up to the present time, only indirect assays were available to quantify nicked hCG, such as subtractive assays or assays with scavenger antibodies added.

Table 3

Matrices of Data for Binding Characteristics of Different Pairs of Detection Antibodies Using B151 or B152 as Capture Antibody[a]

	Relative crossreactivities of two-site assay using B151 as capture antibody							Relative crossreactivities of two-site assay using B152 as capture antibody						
Ligand	B207[b,c]	B204[b]	B201[b]	B108[b]	B109[b]	A109[b]	CTP104[b]	B207[b,d]	B204[b]	B201[b]	B108[b]	B109[b]	A109[b]	CTP104[b]
C5	26%	<[c]	<	100%	<	<	100	100%	100%	94%	42%	53%	100%	<
813 CR 127 hCGn	100%	<	<	100%	<	<	47%	10%	<[c]	<	15%	32%	64%	<
814 CR127 hCG	12%	<	<	37%	<	<	14%	7%	<	<	30%	100%	26%	<
HCGβ	2%	<	<	2%	<	<	<	6%	20%	19%	11%	<	<	<
C5β	5%	<	<		<	<	<	190%	100%	100%	100%	<	<	<
HCGβ core	<	<	<		<	<	<	<	<	<	<	<	<	<
HLH	2%	<	<	3%	<	<	<	<	<	<	<	<	<	<
HLHβ	5%	<	<		<	<	<	<	<	<	<	<	<	<
HCGα	<	<	<	3%	<	<	<	<	<	<	<	<	<	<
Maximum binding[c]	50%	0%	0%	13%	0%	0%	83%	64%	2%	44%	80%	2%	14%	0%

[a]The molar quantity of ligand required to produce binding equal to 50% of the maximum binding achieved by C5 was determined. Crossreactivity shown in this table as a percentage is calculated by dividing the molar quantity of the standard by the molar quantity of the other ligand at 50% maximum binding dose.

[b]Labeled detection antibodies.

[c]< out of low range detection.

[d]Maximum binding represents the total quantity of radiolabeled detection antibody which can bind to the plate in the system described.

[e]This particular assay format was applied in O'Connor et al. *(25)*.

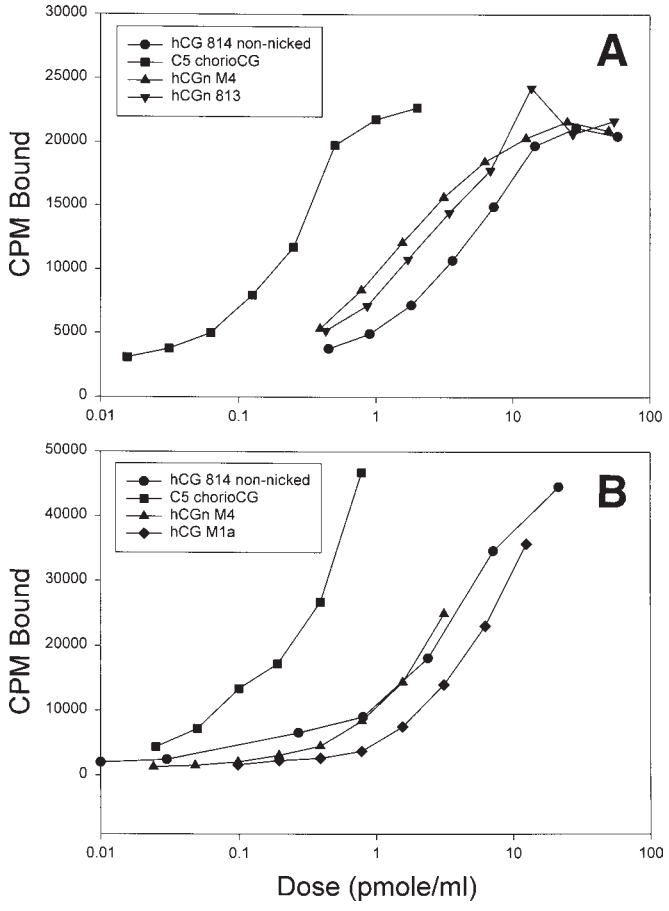

Fig. 2. Radio-immunometric (two-site) assay using antibody B152 as capture and B207 as radiolabeled detection reagent. Binding curves are shown for various competitors as detailed in Materials and Methods and Results. Each panel represents a separate assay in which all ligands were introduced in the same assay. Points were connected by straight lines, although regression analysis (four-parameter logistic) indicated excellent fit to logistic or sigmoidal curve-shape model. Panels **A** and **B** represent two distinct assays with similar results. It is clear that this assay has greatest recognition of the nicked, choriocarcinoma hCG immunogen, which is hyperglycosylated and binds similarly to nicked and nonnicked forms of hCG which contain the usual quantities of sugars. Reagent M1A is missing most of its β COOH-terminal region, supporting a role of this region in the binding site of B152 (*see* panel B and discussion in text).

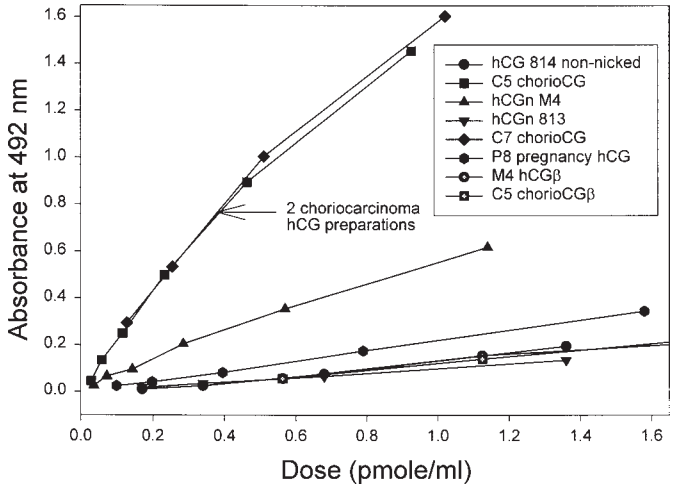

Fig. 3. Enzymic-immunometric (two-site) assay using antibody B152 as capture and peroxidase-labeled B4001 as detection antibody. A linear–linear plot of molar quantities of ligand added is plotted vs absorbance at 492 nm, which is the response factor from the peroxidase detection system. Eight different hormone forms were measured as ligands within the same assay as indicated in the legend and described in Tables 1 and 4. The two hyperglycosylated choriocarcinoma-derived hCG isoforms are both the most potent ligands (Table 4). Potency correlated well with hyperglycosylation of ligand (*see* Tables 1 and 4 and text).

Table 4

Immunoreactivity of Antigens in the B152 Immunoradiometric Assay[a]

Reagent	Slope[b]	S.E.	R^2	Relative potency
814 hCG	0.1588	0.0098	0.992	10.2%
C5 chorioCG	1.5603	0.1015	0.983	100%
M4 mole hCGn	0.5317	0.0240	0.992	34%
813 hCGn	0.0986	0.0021	0.999	6.3%
C7 chorioCG	1.4515	0.1246	0.985	93.0%
P8 hCG	0.2192	0.0031	0.999	14.0%
M4 mole hCGβ	0.1038	0.0069	0.991	6.67%
C5 chorioCGβ	0.1286	0.0042	0.998	8.24%
CR 129 hCGβ	Only 2 points			
HLH batch I-1	No response			

[a]The dose-response curves used to provide data for this table are shown in Fig. 3. Each curve was fitted with four to five points. Slope and coefficient of determination (R^2) were determined using a nonlinear regression algorithm. Slopes were used as an indicator of antigen affinity. Relative affinity was estimated as the slope of antigens relative to the slope of C5 choriocarcarcinoma hCG (the immunogen).

[b]Slope are from Fig. 3 as calculated in Sigmaplot 4.01 by linear regression analysis. Units of slope are pmol/mL absorbance at 492 nm.

Development of B151 permitted formulation of direct assays for nicked hCG, which have been applied in studies of early pregnancy *(25,31)*. These measurements may have diagnostic applications for certain cancers *(18,19)* and in the detection of Down's syndrome *(22)*. Application of the B152 antibody resulted in development of assays that can detect differences in the carbohydrate portion of hCG. Major potential applications of this antibody include detection of Down's syndrome and recognition of pregnancies destined for early pregnancy loss *(25,32)*. This antibody is unusual, since it is rare for carbohydrate-discriminating antibodies to be developed to hCG. The only earlier such development was antibodies to the hCG β COOH-terminal region by the use of the isolated peptide bound to carrier *(28,33)*. It is of interest that at least part of the epitope of B152 is also directed toward the β COOH-terminal region.

As reported earlier, the C5 choriocarcinoma form of hCG was the only such hCG displaying 100% hexasaccharide structure on its *O*-serine-linked carbohydrate moieties in the β COOH-terminal region. This may have resulted in production of this rare antibody. A second choriocarcinoma-derived hCG isoform, C7, displayed 69% of this same *O*-linked core hexasaccharide structure and proved to be of similar potency as C5 with the B152 epitope.

In conclusion, we have produced two novel MAbs, each with potential clinical utility: B151, which can be used to measure nicked forms of hCG with better specificity than any antibody reported, and B152, which is a unique antibody to a choriocarcinoma form of hCG and can discriminate hyperglycosylated from standard pregnancy hCG.

Materials and Methods

Hormones

Nick-free hCG (814) and nicked hCG (813) were prepared from pooled urine standard hCG (batch CR127) by hydrophobic chromatography, as described previously *(14)*. C8 hCG was purified from an individual with normal pregnancy, M1 and M4 hCG were both purified from individuals with gestational trophoblastic disease (hydatidiform mole), and C5 and C7 hCG from an individual with malignant trophoblastic disease (choriocarcinoma), as described elsewhere *(16,26)*. The *N*-terminal peptide sequences of the separated α- and β-subunits of CR127, P8, M1, M4, C5, and C7 hCG and complete *N*- and *O*-linked oligosaccharide structures have recently been published *(26)*. Two peaks were observed by Sephacryl S100 HR chromatography during purification of hCG preparation M1. The peak eluting in the position of standard hCG (M1) and the peak eluting later (M1A) were purified separately. M1 and M4 hCG were both purified from individuals with hydatidiform mole. They were isolated as described earlier *(16)*.

Immunogens

Choriocarcinoma hCG designated C5 was isolated from a single patient as described earlier *(16)*, and its complete carbohydrate analysis has recently appeared *(26)*.

Immunization of Mice

Mice were immunized intraperitoneally with choriocarcinoma hCG preparation C5 diluted in saline (600 μg /mL) and mixed 1:1 with Freund's adjuvant. After three consecutive immunizations (50 μg/mouse) at 3–4-wk intervals, animals were rested for 3 mo and then given an ip booster immunization *(34)*. Ten days after the last booster, the sera from the mice were tested for binding (in liquid phase radioimmunoassays) to both iodinated CR 127 hCG and to iodinated C5 hCG.

Liquid-Phase Assays

Liquid phase assays were performed using a solution of 80 μL 0.3 *M* phosphate-buffered saline (PBS) with 0.02% sodium azide, 50 μL tracer, 20 μL normal mouse serum, 50 μL 1% horse serum free of γ–globulin for titrations (when competition studies were performed, 50 μL of competitor solution were substituted to generate dose-response or Scatchard curves), and 100 μL of diluted sera followed by incubation for 1 h at 37°C and then overnight at 4C. On the next day, 100 μL rabbit antimouse sera were added, incubated for 10 min at 37°C, and then for 1 h at ambient temperature. Then they were centrifuged, supernatant was aspirated, and pellets were counted. The mouse whose antisera had the greatest discrimination between binding of radiolabeled C5 and radiolabeled CR 127 hCG was sacrificed and its spleen was used for hybridoma production. The methods and materials used for the fusion work were described earlier *(27,34)*.

Characterization of Antibodies

The cloned MAbs, B151 and B152, were each studied in liquid-phase competitive radioimmunoassays using C5 as radiolabeled ligand and C5 and CR127 hCG as competitors. The method is as described under Liquid-Phase Assays. Affinity constants were calculated by Scatchard plots as described earlier *(35–37)*.

Two-Site Assays

Two-site assay testing was conducted at Yale University (enzyme immunometric) and Columbia University (radioimmunometric) as described earlier *(24,37)*. Briefly, Immulon microtiter wells are coated with capture antibody at a titer determined to provide the best combination of sensitivity and range. The plates were then washed and then blocked with 1% bovine serum albumin (BSA) in PBS. After further wash standards, clinical samples and controls were added to the coated wells (200 μL/well). Plates are incubated, then samples are removed, and plates are washed. Labeled antibody (either radiolabeled or peroxidase-labeled) is then added, and plates incubated further. The response variable was generated by a γ–counter (Packard Instruments Cobra, Meriden, CT) or by absorption spectrophotometry as appropriate. A cubic spline curve is generated for standard values, and sample values are read from this curve. Regression lines and all graphs were created using Sigmaplot 4.01 from SPSS software, Chicago, IL. Linear regression analysis of immunopotency (Table 3) as compared to each of the carbohydrate differences and nicking differences of the hCG isoforms (Table 1) was accomplished with Instat 1998, GraphPad Software, Inc., San Diego CA (copyright 1992–1998 GraphPad Software Inc. [www.graphpad.com]).

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