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Human monoclonal antibodies with different fine specificity for digoxin derivatives: cloning of heavy and light chain variable region sequences

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SUMMARY

Human–mouse hybridoma cell lines producing human monoclonal antibodies against the cardiac glycoside digoxin were established after *in vitro* immunization or direct immortalization of human peripheral blood lymphocytes with digoxin. Three antibodies, designated MO6, LH92 and LH114, displayed different patterns of fine specificity against digoxin and several digoxin analogues, as elucidated by inhibition ELISA. All three monoclonal antibodies had μ heavy chains, two of them (MO6 and LH114) had κ light chains and one (LH92) λ light chains. DNA encoding the variable regions of both heavy and light chains of the three antibodies were amplified from cDNA using the polymerase chain reaction (PCR). The nucleotide sequences of the amplified DNA were determined after subcloning of PCR fragments in M13 vectors. The deduced amino acid sequences revealed considerable sequence differences in the complementarity determining regions between the three antibodies.

INTRODUCTION

Recently developed procedures for micro-sequencing of proteins as well as improved methods for cloning and nucleotide sequencing of cDNA have facilitated the collection of amino acid sequence data for variable region portions of immunoglobulin polypeptide chains. Comparative studies of variable region sequences for different antibodies directed against the same antigen are of particular interest to elucidate structure–function relationships in antibody–antigen binding. For this purpose, N-terminal protein sequencing of sets of murine monoclonal antibodies directed against well-defined haptens, e.g. digoxin and fluorescein, have been performed.[1,2]

As an alternative to amino acid sequencing to determine immunoglobulin variable region primary structures, we have recently described a procedure that allows rapid cloning and sequence determination of variable region-encoding cDNA fragments.[3] The procedure takes advantage of the polymerase chain reaction (PCR) for specific amplification using a downstream primer, corresponding to a segment in the constant region-encoding part of the immunoglobulin mRNA, together with an oligonucleotide mixture, corresponding to degenerate sequences encoding the immunoglobulin signal peptide, as upstream primer. Optimization of the procedure has allowed cloning and sequence determination from a single hybridoma cell, which further reduces the amount of work required to obtain immunoglobulin sequence data.[4] In this study, we have employed the PCR procedure in order to obtain complete variable region sequence data for heavy and light chains of three human monoclonal antibodies exhibiting small differences in their fine specificity against digoxin and some of its derivatives. Since the approach also allows rapid cloning of DNA fragments encoding the variable regions, the method provides a basis for expression of antibodies with modulated affinity for a given antigen after site-directed mutagenesis.

The human monoclonal antibodies studied in the present investigation were obtained after primary *in vitro* immunizations or a direct immortalization of human peripheral blood lymphocytes[5] with the cardiac glycoside digoxin. The amino acid sequence of the variable regions of both H and L chains of all three antibodies was determined in order to study its relation to antibody fine specificities against digoxin and its derivatives.

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MATERIALS AND METHODS

Antigens

Deslanoside and lanatoside C were kindly donated by Sandoz Ltd (Basel, Switzerland), while other steroids were obtained from Sigma Chem. Co. (St Louis, MO). Digoxin was conjugated to bovine serum albumin (BSA) and human transferrin by a periodate oxidation technique.[6] Conjugations of digitoxin and ouabain to BSA were performed in the same manner.

Production of human monoclonal antibodies

Peripheral blood lymphocytes were obtained after density centrifugation of buffy coats from healthy registered blood

donors, and were then treated with L-leucyl methyl ester hydrochloride or L-leucyl-L-leucine methyl ester hydrobromide to remove lysosome-containing cells.[5] The remaining cell population was immunized *in vitro* with a digoxin–transferrin conjugate or was immediately immortalized. The cells were infected with Epstein–Barr virus (EBV), and the resulting antigen-specific clones were immortalized by fusion with the heteromyeloma K6H6/B5.[7,8] Clones were tested in an antigen-specific ELISA, and positive hybrids were cloned at least twice using limiting dilution.

Analytical ELISA

Antigen-specific ELISA was performed essentially as described previously,[7] after coating of antigen dissolved in phosphate-buffered saline (PBS) to polystyrene ELISA plates (0·5 μg/well). Antibody-containing samples were diluted in 10 mM sodium phosphate, pH 8·0, 0·5 M sodium chloride and 0·1% Tween 20. Horseradish peroxidase-conjugated antibodies (Zymed Lab. Inc., San Francisco, CA or Dako, Glostrup, Denmark) were used to detect antibody reactivity using ortophenylenediamine or 2,2′-azinobis(3-ethylbenz-thiazoline-sulphonic acid) (ABTS) as chromogen.

Immunoglobulin class and light chain determinations were performed in an antigen-specific assay using heavy and light chain-specific antisera (Dako), followed by horseradish peroxidase-conjugated swine anti-rabbit Ig antisera (Dako).

Quantification of immunoglobulin isotypes was performed using specific antibodies obtained from Zymed, as described elsewhere.[7] A serum-based standard (Dako) was used to obtain quantitative results.

Inhibition assay

Steroids dissolved in ethanol were mixed (at a final ethanol concentration of 5%) with diluted human monoclonal antibody-containing supernatants. After an over-night incubation at room temperature, the samples were assayed for reactivity against digoxin–BSA (0·5 μg/well), using a conventional ELISA with ortophenylene-diamine as enzyme chromogen. The apparent affinity constant was determined as the reciprocal steroid concentration required to inhibit the ELISA reactivity by 50%, as described by Friguet *et al.*[9]

Preparation of RNA

Total RNA was prepared from 10^5 hybridoma cells as described elsewhere.[4] Cells were lysed in 100 μl of guanidine thiocyanate solution and layered on top of 100 μl 5·7 M CsCl solution. The tubes were then centrifuged for 20×10^6 ***g*** × min/cm gradient in a TLA-100 rotor using a Beckman TL-100 bench-top ultracentrifuge. The pelleted RNA was resuspended in 100 μl of diethylpyrocarbonate-treated H_2O and cold ethanol precipitated.

First strand synthesis

First strand DNA was synthesized at 37° for 1 hr in a 10 μl reaction volume with oligo-dT priming using Moloney murine leukaemia virus (MMuLV) reverse transcriptase (Bethesda Research Laboratories Inc., Bethesda, MD). Details regarding the reaction buffer composition have been published elsewhere.[4]

Table 1. Data on the human anti-digoxin monoclonal antibodies

Antibody	Isotype/light chain	K_a (M^{-1})	Ig conc. (μg/ml spent medium)
MO6	μ/κ	$1·7 \times 10^5$	35
LH92	μ/λ	$0·5 \times 10^5$	36
LH114	μ/κ	$0·7 \times 10^5$	50

Amplification of variable region encoding DNA

The primer sequences suitable for amplification of variable region DNA and equipped with 5′ recognition sites for restriction endonucleases (*Hin*d III or *Eco*R I) to facilitate subsequent subcloning, have been reported recently.[3,4] The oligonucleotides were synthesized using an Applied Biosystems 380B DNA Synthesizer, and were used directly after deblocking without further purification. Thirty cycles of amplification (each cycle: denaturation, 94° for 1 min; primer annealing, 50–55° for 2 min; extension, 72° for 3 min) were accomplished using a programmable heating block (Cetus/Perkin-Elmer, Emeryville, CA). The major amplification product was isolated by preparative agarose gel electrophoresis, and ligated between the *Hin*d III and *Eco*R I sites of M13mp18/19.

Nucleotide sequence analysis

Nucleotide sequences were determined by dideoxy sequencing with [α-^{35}S]dATP as labelled deoxynucleotide[10] on subclones in M13mp18 or 19 vectors (Pharmacia, Uppsala, Sweden), using the M13 universal primer and the modified T7 DNA polymerase (Sequenase®, United States Biochemical Corp., Cleveland, OH). The sequence of GC-rich regions resulting in compressed gel bands was solved by substituting ITP for GTP in the sequencing reactions, as described by the manufacturer of the Sequenase kit. For all PCR fragments, the entire nucleotide sequences were determined on both strands and at least three independent M13 subclones were analysed for each strand. Analysis of the sequences was done with computer programs in the GCG package.[11]

RESULTS

Establishment of anti-digoxin-secreting human hybridomas

Human peripheral blood lymphocytes were *in vitro* immunized using digoxin–transferrin conjugates as immunogen. These cells were then infected by EBV, and resulting positive clones were immortalized by somatic cell fusion to a heteromyeloma. Positive clones were identified by reactivity against a digoxin–BSA conjugate in an ELISA. Three different anti-digoxin antibody-secreting cell lines (MO6, LH92 and LH114), originating from different donors, were established and characterized (Table 1). MO6 was established by direct EBV infection of peripheral lymphocytes, omitting the *in vitro* immunization step. The heavy chains were all of the μ isotype, and two antibodies (MO6, LH114) had κ light chains, whereas the antibody LH92 had a λ light chain. The apparent affinity constants (K_a) for binding to digoxin were, for all three antibodies, approximately 10^5 M^{-1}. None of the antibodies reacted with BSA or other irrelevant antigens (ovalbumin, HSA, DNP-HSA, transferrin, histones, thyroglobulin, insulin,

Table 2. Structural differences between a number of glycosides and their reactivity to human anti-digoxin monoclonal antibodies, as determined by inhibition ELISA and direct reactivity of the antibodies to protein conjugated glycosides, are shown. Only substituents that differ between the described steroids are shown in the table. None of the antibodies reacted with digitonin, as determined by inhibition ELISA

	Substitution						Cross-reactivity (%) as determined by inhibition ELISA*			Antibody reactivity to protein-conjugated steroids as determined by ELISA†		
Glysoside	1β	3β	5β	11α	12β	19	MO6	LH92	LH114	MO6	LH92	LH114
Digoxin	H	a	H	H	OH	H	100	100	100	0·02	0·03	0·02
Deslanoside	H	a′	H	H	OH	H	90	80	80	ND	ND	ND
Lanatoside C	H	a″	H	H	OH	H	100	70	70	ND	ND	ND
Digitoxin	H	a	H	H	H	H	70	<10	150	1	>3	0·03
Acovenoside A	OH	b	H	H	H	H	<10	<10	770	ND	ND	ND
Ouabain	OH	c	OH	OH	H	OH	<10	<10	50	>3	>3	0·02
Convallatoxin	H	c	OH	H	H	one	<10	<10	120	ND	ND	ND
Helveticoside	H	d	OH	H	H	one	<10	<10	100	ND	ND	ND

* Determined as:

$$\frac{\text{(concentration of digoxin required to inhibit antibody binding by 50\%)}}{\text{(concentration of test steroid required to inhibit antibody binding by 50\%)}} \times 100.$$

† Determined as concentration of antibody (μg/ml) required to obtain A_{492}nm = 1·0 in ELISA using a steroid–BSA conjugate. Antibodies that did not bind showed A_{492}nm < 0·1 at the highest antibody concentration tested (3 μg/ml).

ND, not determined.

a, digitoxose-digitoxose-digitoxose; a′, digitoxose-digitoxose-digitoxose-glucose; a″, digitoxose-digitoxose-acetyldigitoxose-glucose; b, 6-deoxy-3-O-methyl-α-L-talopyranosyl; c, 6-deoxy-α-L-mannopyranosyl; d, digitoxoside; H, hydrogen-atom; OH, hydroxyl-group; one, aldehyde moiety.

acid-soluble collagen, RNA, dsDNA, ssDNA and a cell homogenate of the HT29 adenocarcinoma cell line) (data not shown). The productivity of the MO6 cell line was 30 μg/(10^6 cells × 24 hr).

Inhibition studies using digoxin derivatives

All antibodies bound to free digoxin and digoxin analogues that were only modified in the carbohydrate-moiety (deslanoside, lanatoside C), but showed different reactivities towards related cardiac steroids, as outlined in Table 2. Antibody LH92 did not cross-react with digitoxin, which differs from digoxin only by the lack of a hydroxyl group in position 12β of the steroid backbone, while MO6 and LH114 cross-reacted with this analogue. Antibody LH114 did, furthermore, cross-react with ouabain, whereas none of the antibodies showed any reactivity against digitonin (Fig. 1). Similar specificities were obtained when testing the antibodies in direct ELISA against digitoxin–BSA and ouabain–BSA conjugates.

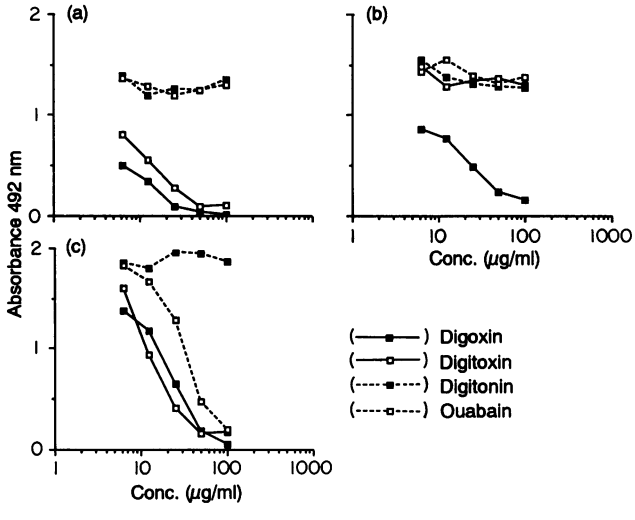

Figure 1. Inhibition studies with the antibodies (a) MO6, (b) LH92 and (c) LH114. Diluted cell culture supernatant was incubated over night with various concentrations of each tested digoxin analogue. The inhibition was determined using a standard antigen-specific ELISA against digoxin coupled to BSA.

Cloning and determination of nucleotide sequences for variable regions

Total RNA isolated from the three different hybridoma lines was used for cDNA synthesis, and the DNA segments encoding the immunoglobulin variable regions of both heavy and light chains were amplified enzymatically using the PCR technique. Amplified DNA fragments were then subcloned into M13 sequencing vectors, and their nucleotide sequences were determined. The amino acid sequences deduced from the nucleotide sequences are aligned in Fig. 2.

The deduced framework region sequences show that the heavy chains of antibodies MO6, LH92 and LH114 belong to V_H subfamilies III, III and IV, respectively. The light chain families are κI1, λI and κIII, respectively. Comparison of the DNA sequences with those of the GenBank/EMBL databases (December 1990) revealed that the MO6 κ chain is 98% identical to the rearranged V-region of GM 607;[13] both are using the Jκ1 segment. The LH114 heavy chain V region gene is similar to a number of germ-line sequences belonging to the V_{HIV} family, in particular the $V_{2\text{-}1}$ gene[14] with a 99% identity in nucleotides. In

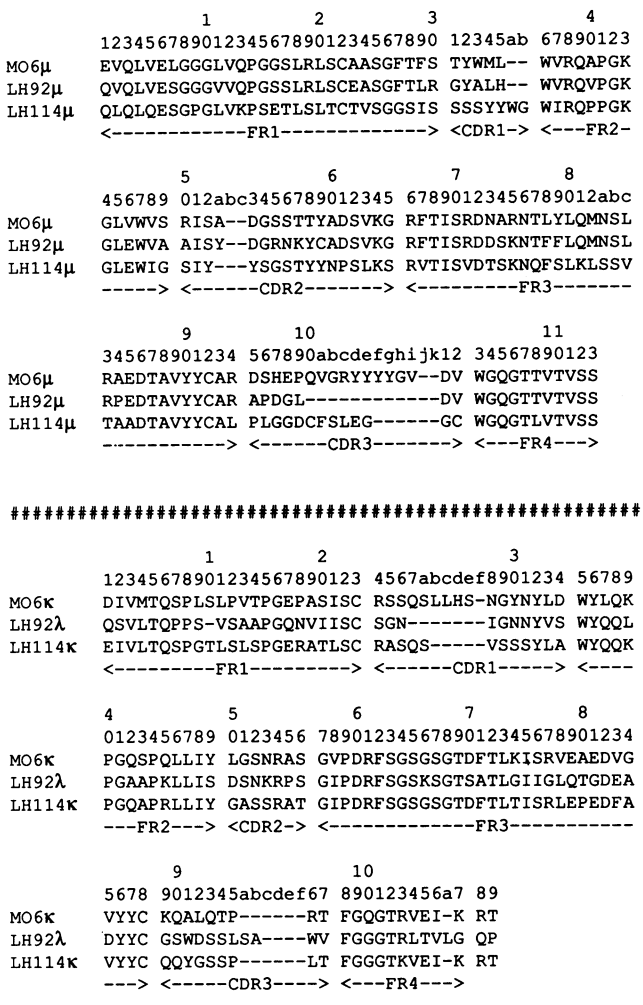

Figure 2. Deduced amino acid sequences of anti-digoxin antibodies. The amino acid residue numbering is according to Kabat *et al.*[12]

addition, the LH114 light chain uses the V_k germ-line gene termed *Hum*kv325,[15] in unmutated configuration. The complementarity determing regions of the three antibodies showed considerable differences, both in amino acid sequence and in length.

DISCUSSION

The polymerase chain reaction has greatly facilitated the cloning of DNA encoding heavy and light chain variable regions, which has been a rate limiting step in antibody engineering. To obtain PCR amplification of a variable region of choice we utilized the leader sequence as 5′ priming site using degenerate primers, following a recently designed procedure for cloning of any variable region encoding sequence.[3,4] This approach will greatly facilitate comparative studies on immunoglobulin structures since the simplicity and rapidity of the method enables large numbers of recombined antibody genes to be sequenced. If cloning is not desired (e.g. for subsequent expression and site-directed mutagenesis), the time for data collection can be reduced further if nucleotide sequence determinations are performed directly on the PCR product.[16] Here we have used the PCR method to study the relation between antibody specificity and amino acid sequence of complementarity determining regions 1–3 in both heavy and light chains of human monoclonal antibodies, to investigate if there is a particular V_H and V_L-gene segment utilization in the human primary immune response against digoxin. This type of restricted use of particular V-gene segments is not uncommon in the murine immune response to smaller haptens, which Crews *et al.*[17] demonstrated using monoclonal antibodies against phosphorylcholine. Mouse monoclonal antibodies against digoxin have also been shown to contain homologous variable regions where differences in fine specificity were due to point mutations in $V_{H/L}$.[18]

Murine monoclonal antibodies against digoxin have been studied extensively on a structural level because digoxin: (i) is a small molecule with a size that approximates the size of an epitope;[19] (ii) has a well-defined three-dimensional structure;[20] and (iii) has many structurally well-defined analogues which differ in substitutions on the steroid rings and/or the carbohydrate moieties. This makes these glycosides well suited as a model system for the study of structure–function relationships of antibodies. We have used *in vitro* immunization (reviewed in ref. 21) of apparently non-sensitized human peripheral blood lymphocytes from healthy donors and produced a panel of human monoclonal antibodies against digoxin. Even though this humoral immune response is of a primary nature, with no affinity maturation of the antibodies, it gave rise to very specific, although low affinity, IgM antibodies. The specificity data showed that the monoclonal antibodies were not so-called broad-reactive natural antibodies. The low affinities also make the antibodies well suited for site-directed mutagenesis studies since improvements in affinities should be possible to obtain by several different amino acid substitutions. Three antibodies reacting with the steroid residue of digoxin and its derivatives were selected on the basis of their similarities in fine specificity. The specificity of LH92 and MO6 is of particular interest, since LH92 reacts very specifically with digoxin but does not react with digitoxin where the 12β-hydroxyl group has been removed. MO6 reacts with both digoxin and digitoxin. Site-directed mutagenesis studies of these antibodies in parallel could therefore also be important to identify amino acid residues critical for specificity. The amino acid sequences reported here are the first for variable regions of human anti-digoxin antibodies, although several mouse monoclonal antibodies have been studied using protein sequencing[1,22,23] and recently also cloning of cDNA.[18] These murine data support the results of the present investigation, in that the mouse immune response to digoxin also seemed not to be restricted to the use of a particular set of V gene segments.

In summary, despite the similar reactivity of the three antibodies against digoxin we found significant variations in the amino acid sequences of the variable regions. Thus, our results demonstrate that the binding specificities is not a consequence of an apparent restriction of particular V_H, D_H and V_L gene segments in the primary human immune response against digoxin.

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