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Identification of Monoclonal Antibodies against 2,4-D Herbicide by ELISA and DNA Sequencing

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Seven hybridoma clones, E2/G2, E2/B5, E4/C2, G5/E10, F6/C10, B5/C3, and B7, produced within one fusion experiment in 1991 and the clone E4/C2 originated from 1995 were characterized by sequencing of genes coding for variable domains of the antibodies against 2,4-D herbicide. Amino acid sequences of selected antibodies, deduced from DNA analysis, were confirmed by mass spectrometry. Surprisingly, nucleotide sequence analysis of the clones E2/G2 and E2/B5, producing the most sensitive antibodies, proved to have sequence homology of their variable domains, although the IC_{50} values determined for these antibodies 9 years prior to the DNA analysis were 2.0 and 8.2 ng/mL, respectively. The same findings arose from the comparison of the immunochemical to DNA data established for G5/E10, F6/C10, and B5/C3 clones producing antibodies with IC_{50} values in the range of 26.3−43.1 ng /mL. The clone E4/C2, originating from the later fusion experiment, did not share nucleotide homology with any of the examined clones. Data obtained by ELISA, immunosensor, and DNA analysis within a 9 year period are discussed with respect to hybridoma stability, methodic artifacts, measurement reliability, and other possible factors influencing the result interpretation.

KEYWORDS: Hybridoma technology; monoclonality; antibodies; sequencing; antibody genes; mass spectrometry; artifacts

INTRODUCTION

The wide repertoire of antibody specificities and affinities that can be generated by natural and artificial immune systems provides a rich pool of potential reagents for analytical use. For each antibody, the affinity, kinetic, and antigen recognition properties, expressed most often in terms of the assay sensitivity and cross-reactivity, determine its utility. Although some novel approaches and methods of producing antibodies, such as recombinant or molecular imprinting technologies, have emerged in recent years, monoclonal antibody technology producing antibodies derived from a single cell line (hybridoma) so far has been a strong approach to obtain antibodies with the desired properties. Monoclonal antibodies compared with polyclonals have the obvious advantage of immortality because the hybridoma cell lines that produce the antibodies can be maintained infinitely in vitro. Moreover, monoclonal antibodies can be used more easily in biosensor formats and can also be used as starting material for the preparation of recombinant antibody fragments (*1*). To find the monoclonal antibodies that are best suited for a particular application, a large number of different hybridoma clones have to be screened after cell fusions to recognize the clones with the ability to produce a monoclonal antibody of a given interest. An identity of the isolated hybridoma product is usually tested for monoclonality and antibody isotype class or characterized by immunoassay characteristics (*2*−*4*). Although these data provide usually appropriate information on the cells produced, approaches based on molecular biology may be required to confirm the identity of the new hybridoma species.

Sequencing of genes of antibody variable domains provides fundamental information on the antibody-producing cells and offers an extraordinarily reliable approach to the characterization of monoclonal antibodies. The domains are usually constant among antibodies of the same class, except for at the N-terminal end of the F_{ab} regions, where the amino acid sequence is different in each antibody (*1*). Within the variable domain, there is a framework region, actually fairly constant, and hypervariable loops. Each heavy and light chain contains three hypervariable areas called complementarity-determining regions (CDR) that make up the antibody binding site. The amino acid composition of the CDR determines specific details of the binding interaction with the antigen. In the haptenic (low molecular weight analytes) area, sequence analysis of the CDR has been applied to characterization of a new generation of recombinant antibodies against environmental and food contaminants such as zearalenone (*5*), paraquat (*6*), or PCB (*7*). DNA sequencing has also been used for the analysis of recombinant fragments [e.g., against *p*-azobenzenearsonate (*8*), phosphotyrosine (*9*), trinitrophenyl and fluorescein isothiocyanate (*10*), and musk odorant traseolide (*11*)]. Data obtained from the DNA analysis were employed for manipulating the affinity and the fine specificity of the monoclonal antibody for cortisol (*12*), estradiol (*13*), and testosterone (*14*).

In this laboratory, monoclonal antibodies against the herbicide 2,4-D with unique diversity of binding and kinetic characteristics were produced in the previous years (*15*). Some of these anti-

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Table 1. IC_{50} Values of Monoclonal Antibodies against 2,4-D According to Fránek et al. (*15*)

clone marking	E2/G2	E2/B5	E4/C2	F6C10	G5/E10	B5/C3	B7
IC_{50} (ng/mL)	2.0	8.2	5	26.3	36.1	43.1	73.0
ascitic fluid (dilution)	1:10000	1:10000	1:10000	1:20000	1:5000	1:5000	1:1000

bodies have found a wide application in the development of immunoanalytical techniques including immunosensors (*16*−*19*). The unique differences in immunoassay characteristics prompted us to examine nucleotide sequences of variable regions of heavy (V_H) and light (V_L) chains to gain information on the identity of the antibodies and the character of their binding sites. For this purpose, a set of primers that binds to the conserved framework region flanking the CDRs was employed. The V_H and V_L regions were prepared from the respective mRNAs by RT-PCR and cloned in order to establish the nucleotide sequences. Confrontation of the results of the DNA analysis with the respective immunochemical data obtained for corresponding antibodies was a primary aim of this study. Additionally, amino acid sequences of selected antibodies, deduced from DNA analysis, were verified by peptide mass fingerprinting analysis to gain reliable structural information on antibody variable domains.

MATERIALS AND METHODS

Instruments. Sequencing reaction data were read using an ABI prism 7000 capillary electrophoresis analyzer (Applied Biosystems, Foster City, CA). ELISA plates were measured on photometer Labsystem Multiscan MCC (Helsinki, Finland). Mass spectrometry analysis of protein tryptic digests was performed with MALDI-TOF mass spectrometer Reflex IV (Bruker Daltonics, Bremen, Germany).

Buffers and Standards. Coating buffer was 0.05 M carbonate buffer, pH 9.6; PBS was composed of 10 mM phosphate buffer, pH 7.2, and 145 mM NaCl; assay buffer was prepared by mixing 100 mL of PBS with 0.1 mL of Tween 20; TMB solution was composed of 10 mg mL^{-1} TMB in DMSO; acetate buffer was 100 mM sodium acetate/citric acid buffer, pH 5.5; substrate solution (TMB + H_2O_2) was prepared by adding 200 μL of 6% H_2O_2 to 21 mL of acetate buffer; reverse transcription buffer consisted of 10 units of RNAsin, 5 μL of 2× reverse transcription buffer, 1 μL of 10 mM dNTPs, and 5 units of AMV reverse transcriptase (Promega, Madison, WI); the PCR buffer (50 μL) consisted of 2 μL of cDNA template, 5 μL of 10× Pfu reaction buffer, 1 μL of 10 mM dNTPs, 0.5 μL of Pfu DNA polymerase (3 units/μL) (Promega), and 20 pmol of each primer. Vk For 5′-AGGTCCAGCTGCAGSAGTCWGG-3′ and Vk-Back 5′-TGAGGAGACGGTGACCGTGGTCCCTTGGCCCCAG-3′ primers for light chain and Vheavy For and Vheavy Back primers for heavy chain were employed.

Hybridoma clones. Table 1 lists the clones that were employed in this work for the production of antibodies of our interest. The clones were prepared by conventional hybridoma technology in 1991 as described in ref *15*. All mice were immunized with the same, 2,4-D-thyroglobulin, immunogen. Briefly, spleen cells from the immunized mice and myeloma cells were mixed at the ratio of 1:3.55 and fused in 50% PEG 1000. The cells were selected after fusion with HAT (100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine) in D-MEM medium with 15% FCS in microtiter polystyrene plates. After 8 days, the growing hybridoma cells in ELISA positive wells were cloned by limiting dilution according to ref *20* as follows: the cells of each well were counted in a Burker chamber to reach a concentration of 1 cell per 200 μL of medium in the well. After 3−4 days, clones were screened by ELISA on the plates coated with 2,4-D−BSA IV, and the cells from the positive wells were used to repeat the cloning procedure twice more. Monoclonality of the cells in the wells was evaluated by colony morphology, and then the clones were cultured in a 5 mL volume and stored in a liquid nitrogen.

Since 1991, the above hybridoma clones have been used many times for the production of antibodies using repeated cultures. The E2/G2 clone was cultured in at least 20 subsequent runs without further subcloning. After each culture procedure was finished, the clone was always frozen in aliquots and used repeatedly for antibody production. Other clones involved in this work were recultured less often because the extent of their experimental use was relatively lower compared to that of the E2/G2 clone.

Production of Antibodies. The frozen hybridomas were retrieved from liquid nitrogen, rapidly thawed with constant agitation, and cultured in D-MEM medium (Sigma, St. Louis, MO) containing 10% FCS (~10^6/mL of medium). Two female mice (BALB/c, 10 weeks old) were treated with 0.5 mL of pristane 10 days prior to inoculation. The mice were injected intraperitoneally with 10^6 hybridoma cells. The hybridomas grew as ascites tumors in peritoneal space, and ascitic fluids containing antibodies were collected 7 days after the inoculation. The hybridoma pellet obtained after centrifugation of the ascitic fluid at 3000*g* for 10 min was suspended in a medium and reinjected into the four mice treated with pristane as described above. The collected ascitic fluids were pooled, and the fluids or corresponding IgG fractions were stored at 4 °C until further use.

Preparation of IgG. The immunoglobulin fraction was separated from ascitic fluid by using protein A affinity purification kit (Bio-Rad, Hercules, CA). Ascitic fluid was diluted with binding buffer 1:2 (v/v), centrifuged, and filtered through a PF syringe filter, 0.2 μm Acrodisc (Pall Gellman Sciences, Ann Arbor, MI). Nine milliliters of the diluted ascitic fluid was applied to a column filled with 3 mL of sorbent and then allowed to flow through the chromatographic column with immobilized protein A. The column was washed with 10 bed volumes of washing buffer, eluted with 3−4 bed volumes of eluting buffer, and neutralized with Tris-HCl buffer. After dialysis against PBS buffer, the IgG was incorporated into indirect ELISA to establish assay parameters of the respective antibodies.

Indirect ELISA Procedure. Two hundred microliters of 2,4-D−BSA IV conjugate (26.5 mol of 2,4-D/mol of BSA) in the coating buffer (50 ng/mL) was added to each well of a microtiter plate. The plate was incubated overnight at room temperature. After the buffer was removed, the wells were washed three times with 200 μL of PBST. One hundred microliters of standard in the assay buffer and 50 μL of ascitic fluid (or IgG) diluted in PBST was added into the wells. After 1 h of incubation at 4 °C, the unbound compounds were removed by washing (three times) with assay buffer. One hundred and fifty microliters of SwAM-POD (Sevac, Prague, Czech Republic) was added to each well, and the plates were incubated at 4 °C for 1 h. After the solution was removed, each well was washed four times with the washing solution. One hundred microliters of TMB/H_2O_2 was added to the wells, and the detection reaction was stopped by adding 100 μL of 1 mol/L H_2SO_4. The absorbance of the developed color was measured at 450 nm.

Amplification of the Variable Domain Genes. Total RNA was extracted from 5 × 10^6 hybridoma cells using the GIT phenol−chlorophorm method (*21*). cDNA was synthesized in the reaction mixture consisting of 0.5 μg of random hexaoligonucleotides (Promega) and 2 μg of total hybridoma RNA in 10 μL of diethylpyrocarbonate (DEPC)-treated H_2O. The mixture was heated to 75 °C for 10 min and then cooled on ice, and 2 μL of the mixture was added to reverse transcription buffer and transcribed to cDNA. Conditions for PCR were as follows: 2 min initial denaturation (96 °C), 30 cycles of amplification using 40 s for annealing (47 °C), 50 s for elongation (72 °C), and 40 s for denaturation (92 °C). Amplified products were electrophoresed on agarose gels, and PCR products of specific size were gel extracted using a Qiagen kit (Qiagen GmbH, Hilden, Germany). Cloning to the sequencing vector was performed as follows: The A-overhangs were added to the PCR fragments and treated with 1 unit of Taq polymerase (Promega) containing dNTPs in 10 μL of reaction volume. The fragments obtained were used for the ligation reaction with pGEMT

Table 2. Sequence Alignment for Light Chains of Seven Antibodies against 2,4-D[a]

	CDR 1				CDR2				CDR3	
	10	20	30	40	50	60	70	80	90	100
E2/G2	DIELTQSPSS LSASLGERVS LTC**RSSQDIGS SLH**WFQQESD GTIKRLIY				**ATYN LDS**GVPKRFS GSRSGSDYSL TISSLESEDF VDYYC**LQYAS FPYT**FGGGTK LEIK					
E2/B5	DIELTQSPSS LSASLGERVS LTC**RSSQDIGS SLH**WFQQESD GTIKRLIY				**ATYN LDS**GVPKRFS GSRSGSDYSL TISSLESEDF VDYYC**LQYAS FPYT**FGGGTK LEIK					
E4/C2	DIELTQSPSS LSASLGERVS LTC**RASQDIGN SLH**WLQQEPD GTIKRLIY				**ATSG LDS**GVPKRFS GSRSGSDYSL TISSLESEDF VDYYC**LQYAS YPFT**FGGGTK LEIK					
G5/E10	DIELTQSPSS LSASLGERVS LTC**RASQDIGS SLH**WFQQEPD GTIKRLIY				**ATSSL DS**G VPKRFS GSRSGSDYSL TISSLESEDF VDYYC**LQYAS FPYT**FGGGTK LEIK					
F6/C10	DIELTQSPSS LSASLGERVS LTC**RASQDIGS SLH**WFQQEPD GTIKRLIY				**ATSSL DS**GVPKRFS GSRSGSDYSL TISSLESEDF VDYYC**LQYAS FPYT**FGGGTK LEIK					
B5/C3	DIELTQSPSS LSASLGERVS LTC**RASQDIGS SLH**WFQQEPD GTIKRLIY				**ATSSL DS**GVPKRFSG SRSGSDYSL TISSLESEDF VDYYC**LQYAS FPYT**FGGGTK LEIK					
B7	DIELTQSPSS LSASLGERVS LTC**RASQDIGS SSH**WLQQGPD GTIKRLIY				**ATYSL DS**GVPKRFSG SRSGSEYSL TISSLESEDF VDYYC**LQYAS LPYT**FGGGTK LEIK					

[a] Positions of CDR are indicated by boldface underlined symbols. Amino acid changes out of the CDR are marked by boldface symbols. Amino acid residues are numbered according to the Kabat scheme (*28*).

easy vector (Promega) by using the molar ratios 1:1 and 5:1 (PCR product /vector). The ligation was performed in 10 μL of the reaction mixture (4 °C) overnight. XL-1 blue *Escherichia coli* strain was electroporated with 1 μL of the ligation mixture. The bacterial colonies were cultured on blue/white selection medium, and white colonies were screened by PCR, which was performed with untreated *E.coli* to confirm the presence of the specific insert in the plasmid.

Variable Domain Sequence Determination. Plasmids were isolated from *E. coli* Xl-1 using a Wizzard plasmid miniprep kit (Promega) according to the manufacturer's instructions. The DNA sequence was determined via an ABI PRISM BigDye TerminatorCycle sequencing kit. The procedure was carried out in a 20 μL Ready Reaction mixture consisting of 100−250 nmol (200−500 ng) of circular plasmid and 10 pmol of the sequencing primers: M13 REVERSE, 5′-AGCGGATAACAATTTCACAC, and M13 FORWARD (−21), 5′-TGTAAAACGACGGCCAGT. The mixture was subjected to 25 cycles consisting of 96 °C for 15 s, 47 °C for 15 s, and 60 °C for 4 min. The analysis of the sequencing reaction products was done by capillary electrophoresis analyzer. The two coding plasmid inserts per each variable domain were sequenced from both 5′ and 3′ end directions. Nucleic acid sequence data were analyzed and converted to amino acid sequence using BioEdit sequence alignment software (*22*).

Mass Spectrometry Analysis. Antibody chains were separated by discontinuous SDS-PAGE (10%) under reducing conditions. Separated bands of heavy and light chains were cut from the gel and subjected to proteolytic in-gel digestion with trypsin at 37 °C for 16 h. After digestion, the formed peptides were extracted from the gel plugs, and the extract aliquots were mixed with matrix solution (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile). The peptide maps obtained by MALDI-TOF MS analysis for particulate chains (accuracy = 50 ppm) were compared with theoretical peptide chains derived from nucleic acid sequence data (*23*).

RESULTS AND DISCUSSION

Immunochemical Characterization. Of 12 hybridoma clones produced by two fusions in 1991, 6 were selected for sequence analysis of the variable domain in 2000. Additional clone E4/C2, produced by a fusion experiment in 1995, provided antibodies having assay characteristics comparable with those shown in **Table 1**. The IC_{50} values of the 7 studied antibodies ranged from 2.0 to 73.0 ng/mL at the working ascitic fluid dilution of 1:1000−1:20000 (v/v) in the optimized indirect assay system. In this study, the starting working hypothesis was based on a premise that relatively small but distinguishable differences in assay parameters determined for selected monoclonal antibodies will reflect adequate changes in sequence pattern. In this context, the IC_{50} values established for antibodies are assumed to be reliable analytical characteristics determined with a high precision for a given immunoassay system. The specificity of the antibody was quantitated as the reciprocal cross-reactivity (CR), where the CR (percent) values are calculated as follows: (IC_{50} for a target analyte/IC_{50} for a structural analogue) × 100 (*24*). Generally, the assay characteristics are understood as parameters typical for each antibody, thus making its identification possible.

DNA Analysis and Assessment of New Antibody Characteristics. Whereas measurement of immunochemical characteristics of antibodies had been carried out at the early stages of the clone existence, the technique allowing analysis of the antibody genes was available in this laboratory only several years later. Actually, there was a 9-year gap between the performances of immunochemical and DNA analyses. The antibodies produced by clones E2/G2, E2/B5, and E4/C2 were chosen first for the nucleotide sequence investigation because small differences in their assay and kinetic parameters postulated that interesting changes would appear in the variable regions of their heavy and light chains. It can be noted that the antibody E2/G2 was supposed to be most sensitive toward the 2,4-D target in the long term and therefore was most often used for the development of various immunoanalytical formats beginning in 1994.

The results of the sequence analysis are presented in **Tables 2** and **3**. The sequences are expressed in terms of deduced amino acid sequences. Surprisingly, variable regions of the Vh and Vl chains, established for the E2/G2 and E2/B5 clones, shared a total nucleotide identity, although the IC_{50} data found for these antibodies in the early production period showed a 4.1-fold difference (**Table 1**). On the other hand, the nucleotide sequence of the antibody E4/C2, having a similar assay sensitivity, differed dramatically from those antibodies as shown in **Table 4**. Taking into consideration the fact that this clone originated from the later independent fusion experiment, the observed difference between the clones was not surprising because of the random character of the variable subgene selection process (*25*).

The finding that DNA characteristics were identical for two antibodies with different IC_{50} and kinetic values prompted us to re-examine the validity of the results for the E2/G2 and E2/B5 antibody species from the early period of their production. Thus, new antibodies were prepared through the corresponding hybridoma clones and incorporated into competitive ELISA to confront the newly established data with the previous assay results. To retain the same assay conditions for indirect ELISA performance, the measurement was carried out according to the standard protocol described in our previous work (*15*). A factor

Table 3. Sequence Alignment for Heavy Chains of Seven Antibodies against 2,4-D[a]

		CDR1			CDR2				CDR3			
	1 10	20	30	40	50	60	70	80	90	100	110	
E2/G2	QVQLQQSGPE **L**VKPG**T**SVKM SCKAS**GHTFT VYVIS**WVKQR **T**GQGLE WIGE **IYPGRGSIY** Y NEKFRDKATL **TADISSNIAH** MQLSSLT**SDD** SAVY**I** C**AAYN RVGAY**WGQGTT VTVSS											
E2/B5	QVQLQQSGPE **L**VKPG**T**SVKM SCKAS**GHTFT VYVIS**WVKQR **T**GQGLE WIGE **IYPGRGSIY** Y NEKFRDKATL **TADISSNIAH** MQLSSLT**SDD** SAVY**I** C**AAYN RVGAY**WGQGTT VTVSS											
E4/C2	QVQLQQSGPE **L**VKPG**D**SVKM SCKAS**GITLA YFILS**WVKQR **S**GQGLE WIGE **IYPGSGRVF**Y NENFRGKATL **TVDKS**STTVY MQLSSLT**SED** SAVY**F** C**VTYG YAFAY**WGQGTT VTVSS											
G5/E10	QVQLQQSGPE **L**VKPGASVKM SCKAS**GYRVT DFLIS**WVKQR **T**GQGLE WIGE **IYPGSGTTY**Y NENFKGKATL **TADKS**SNTA**Y** MQLSSLT**SED** SAVY**F** C**ASYG YAFAY**WGQGTT VTVSS											
F6/C10	QVQLQQSGPE **L**VKPGASVKM SCKAS**GYRVT DFLIS**WVKQR **T**GQGLE WIGE **IYPGSGTTY**Y NENFKGKATL **TADKS**SNTA**Y** MQLSSLT**SED** SAVY**F** C**ASYG YAFAY**WGQGTT VTVSS											
B5/C3	QVQLQQSGPE **L**VKPGASVKM SCKAS**GYRVT DFLIS**WVKQR **T**GQGLE WIGE **IYPGSGTTY**Y NENFKGKATL **TADKS**SNTA**Y** MQLSSLT**SED** SAVY**F** C**ASYG YAFAY**WGQGTT VTVSS											
B7	QVQLQQSGPE **R**VKPGASVKM SCKAS**GHTFT VYVIS**WVKQR **T**GQGLE WIGE **IYPGSGSNF**Y NEKFKDKATL **TADKS**SNTA**Y** MQLSSLT**SED** SAVY**F**C**VTYG YAFAY**WGQGTT VTVSS											

[a] Positions of CDR are indicated by boldface underlined symbols. Amino acid changes out of the CDR are marked by boldface symbols. Amino acid residues are numbered according to the Kabat scheme (*28*).

that was particularly considered with respect to the assay performance was the composition of the ascitic fluids collected from a large number of mice. For that reason, both diluted ascitic fluids and IgG isolated from the fluids were employed for the competitive reactions to establish any influence of the ascitic matrix on the assay system.

The comparison of indirect ELISA curves for three ascitic IgG reagents and 2,4-D, MCPA, and 2,3,5-T as standard calibrators is presented in **Figure 1**. Identical characters of the curves within the whole calibration range are apparent for the antibodies E2/G2 and E2/B5, whereas the curve for the antibody E4C2 followed a rather distinct slope (**Figure 1a**). The respective IC_{50} values, that is, the detection regions with the highest assay precision, were reached for E2/G2 and E2/B5 at 3.1 ng mL^{-1} and for E4C2 at 8.2 ng mL^{-1}. Apparently, the immunoassay results for the E2/G2 and E2/B5 antibodies are in good agreement with the sequence analysis data but do not correlate with the original IC_{50} values presented in **Table 1**. Dose responses to MCPA and 2,4,5-T herbicides were examined in the same assay system. The curves depicted in **Figure 1c** show an almost identical slope throughout all the calibration curve for 2,4,5-T competitor, whereas certain differences can be observed when MCPA was employed as a standard in the same assay system (**Figure 1b**). The same response of the three antibodies to 2,4,5-T in **Figure 1c** represents rather a random phenomenon that is not typical for monoclonal antibodies originating from different fusions. The cross-reactivity values to the above herbicides were also tested with the new ascitic fluid preparations. Generally, the character of the ELISA curves obtained using these ascitic fluids was the same as in the curves using purified IgG (unpublished data). It was concluded that the influence of the matrix on the assay reaction was negligible.

In the following experimental phase, the clones producing less sensitive antibodies were taken for DNA analysis. As shown in **Tables 2** and **3**, the sequences of variable regions of the clones G5/E10, F6/C10, and B5/C3 indicate 100% sequence homology, although the respective IC_{50} values determined in the early stage of the antibody characterization ranged between 26.3 and 43.1 ng mL^{-1} (**Table 2**). On the basis of the results, reanalyzis of the immunochemical data for the newly prepared antibodies was carried out to compare the results of the two analytical approaches. It appeared from the results that the ELISA curves had practically identical character as in the case of the E2/G2 and E2/B5 antibodies.

Analysis of Variable Domain by MALDI-TOF Peptide Mass Fingerprinting. To prove the presence of amino acid sequences in variable antibody regions (**Tables 2** and **3**), MALDI-TOF MS analysis of tryptic digests of the selected antibody chains was done to find peptides corresponding to the deduced primary amino acid structure in the CDR. The results of MALDI-TOF MS analysis of light and heavy chains for four selected monoclonal antibodies are summarized in **Table 5**. The peptides covering corresponding CDR (except CDR3 of heavy chains) were determined in mass spectra. The total sequence coverage of the analyzed proteins was >70%. To confirm the reliability of peptide mass fingerprinting results, post source decay analysis of two selected peptides (covering CDR1 and CDR2 of E2G2 light chain) was performed. As shown in **Table 5**, the amino acid sequences of CDR were proved by this method. However, the CDR3 of the heavy chain was not accessible to MALDI-TOF analysis. The lack of success can be explained at least by two reasons. First, the CDR3 is located within a relatively long sequence without any trypsin cleavage site. This did not allow quantitation of the unsplit peptide with

Table 4. Amino Acid Differences in Variable Antibody Domains

	light chain						heavy chain						total
	CDR1	CDR2	CDR3	CDRs	Fw	total	CDR1	CDR2	CDR3	CDRs	Fw	total	
E2/G2	0	0	0	0	0	0	0	0	0	0	0	0	0
E2/B5	0	0	0	0	0	0	0	0	0	0	0	0	0
E4/C2	2	2	2	6	1	7	7	4	4	15	9	24	31
G5/E10	1	2	0	3	0	3	6	3	4	13	6	19	22
F6/C10	1	2	0	3	0	3	6	3	4	13	6	19	22
B5/C3	1	2	0	3	0	3	6	3	4	13	6	19	22
B7	2	1	1	4	3	7	0	3	4	7	8	15	22

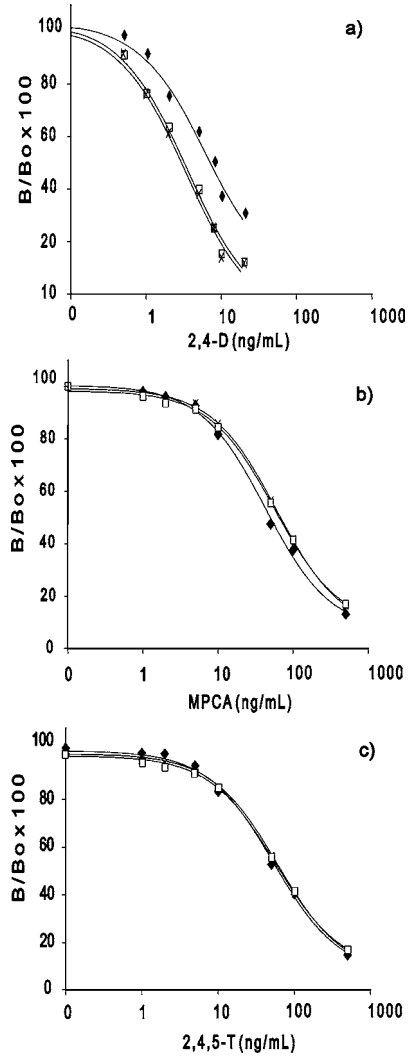

Figure 1. Comparison of indirect ELISA curves for E2G2 (□), E2B5 (×), and E4C2 (◆) IgG antibody reagents. The coating of wells in microtiter plates was carried out with 2,4-D–BSA IV at a concentration of 200 ng/mL for the E2G2 and E2B5 assay systems and at 100 ng/mL for the E4C2 assay system. B/B_0 is the ratio of the absorbance at the measured concentration of analyte (B) to the absorbance obtained with no added analyte (B_0).

the desired accuracy. Another factor to be considered is the primer used on the downstream end of the analyzed PCR product. In this case, the primer could induce a nucleotide change in the priming site, changing subsequently the output of the amino acid sequence. Thus, unspecified change in peptide mass or concealment of trypsin cleavage sites could make the analysis impossible. Despite this limitation, we can conclude that the deduced amino acid sequences are in good agreement with the data measured by the MALDI-TOF technique.

Table 5. Confirmation of Amino Acid Sequences Presented in **Tables 2** and **3**

antibody chain	peptides covering CDR	confirmed amino acid sequence of CDR1 CDR2 CDR3
E2G2 light	19–24, 25–45, and 25–46 47–60 and 46–60 (resp 47–61) 67–103	24–34 **RSSQDIGSSLH** 50–56 **ATYNLDS** 89–97 **LQYASFPYT**
E2B5 light	19–24, 25–45, and 25–46 47–60 a 46–60 (resp 47–61) 67–103	24–34 **RSSQDIGSSLH** 50–56 **ATYNLDS** 89–97 **LQYASFPYT**
E4C2 light	19–24, 25–45, and 25–46 47–60 and 46–60 (resp 47–61) 67–103	24–34 **RASQDIGNSLH** 50–56 **ATSGLDS** 89–97 **LQYASYPFT**
F6C10 light	19–24, 25–45, and 25–46 47–60 and 46–60 (resp 47–61) 67–103	24–34 **RASQDIGSSLH** 50–56 **ATSSLDS** 89–97 **LQYASFPYT**
E2G2 heavy	24–38 41–55 (EIYPGR) not analyzed	26–35 **GHTFTVYVIS** 50–59 **EIYPGRGSIY** 99–105 **YNRYGAY**
E2B5 heavy	24–38 and 24–40 41–55 and 56–63 not analyzed	26–35 **GHTFTVYVIS** 50–59 **EIYPGRGSIY** 99–105 **YNRYGAY**
E4C2 heavy	24–38 41–57 and 58–65 not analyzed	26–35 **GITLAYFILS** 50–59 **EIYPGSGRVF** 99–105 **YGYAFAY**
F6C10 heavy	24–38 and 29–38 41–65 and 41–67 not analyzed	26–35 **GYRVTDFLIS** 50–59 **EIYPGSGTTY** 99–105 **YGYAFAY**

Comparison of Primary Structure of Antibodies. Table 4 summarizes in detail the amino acid differences in the structure of seven examined clones. The number of amino acid differences in a given sequence is related to the structure of E2/G2 and E2/B5 species having the highest assay sensitivity (**Tables 2** and **3**). The clone E4/C2 showed a total of 31 differences, of which 6 and 15 are indicated in the CDR of the light and heavy chain areas, respectively. Additional 10 differences in amino acid sequence (1 + 9) are found in the Fw part of the variable area. Substantial differences in amino acid sequences are evident also in the G5/E10, F6/C10, and B5/C3 clones; however, identical structures for the three monoclonal antibodies are apparent from the presented data. Of the total 22 differences, 19 were found in the heavy chain, whereas only 3 amino acid differences are ascribed to the light chain. Interestingly enough, 6 changes occurred in the conservative Fw area of the heavy chain, but no difference is indicated in the corresponding Fw part of the light chain. Finally, the B7 clone producing the least sensitive antibody exhibited 22 total differences, which is the same frequency rate as in the above clones, but the distribution of the amino acid distinction in the primary structure is different.

Interpretation of the Results and Concluding Remarks. The wells containing growing hybridomas were cloned by limiting dilution to ensure that the antibody will be produced from one hybridoma clone. In the procedure, hybridoma cells are diluted across a plate to a theoretical concentration of one cell per well. The number of cells in the wells is determined by the density function of the Poisson distribution. Thus, most wells contain no cells or one cell, whereas the rest of the wells may contain two or more cells. The growing hybridomas are used to repeat the cloning procedure twice more. Following this step it is highly statistically probable that the cells will originate from one cell line and that the antibody produced is monoclonal. Apparently, the real result of the cloning procedure can be influenced by various random factors such as precision of the cell dilution, cell viability, and mutual cell adherence. Considering the above factors, the desired monoclonality may not be always achieved. Methods such as colony morphology, antibody isotyping, or chromosomal count do not provide a definite response as to whether monoclonality was achieved and, therefore, more definite techniques are needed.

Comparison of the DNA data with the immunoassay characteristics obtained in the early and recent period showed that contamination of the earliest clones by another antibody-secreting cell line(s) might exist within the early period of the antibody production. Considering the above points, one can presume that the foreign lines could contribute to antibody formation with a certain proportion for a certain time. Additionally, one can hypothesize that the minor line disappeared from the medium during repetitive cell cultures, whereas major antibody-secreted clones survived as one stable production line. It should be also taken into consideration that the selection or differentiation of the original clone E2/B5 could have evoked the alteration in immunochemical performance upon the repeated cell passage. Experimental verification of these hypotheses could not be carried out due to the lack of the antibody E2/B5 from the early production time. Interestingly, the same findings are observed in three additional clones with the same sequence pattern. It is believed that explanation of the phenomenon may be made on the same basis as suggested above.

Generally, the stability of the production clones is retained in some cases only within a short-term period. In this laboratory, clones producing unique generic antibodies against sulfonylurea herbicides (*27*) lost substantially their potential to produce antibodies in the course of the second year after fusion. Moreover, these antibodies exhibited lower assay sensitivity in comparison to the antibody batches produced at the early production stage.

Methodical artifacts caused by additional immunochemical and biological factors such as quality of microtiter plates, coating performance, washing steps, or variability in antibody amount, including binding components in ascitic fluids, can also be considered as a possible source of the result inaccuracies, although enzyme immunoassays provide quantitative and reproducible data under the standard assay conditions. To exclude any errors from interassay performance, measurement of the competitive characteristics has always been performed on one microtiter plate for several antibodies in question. Good agreement between ELISA and DNA data was achieved using the newly prepared antibody batches. The results suggest that the effects of these factors on the assay precision are negligible.

This work demonstrates the significance of nucleotide analysis for the identification of monoclonal antibodies with respect to their hybridoma origin. It should be emphasized that nucleotide/amino acid sequences of variable domains (CDR and Fw areas) are considered to be relevant structural characteristics for specific binding interactions, but they do not provide definitive information about the monoclonality of hybridomas and their products—monoclonal antibodies. Gene analysis enables monitoring of changes in antibody-secreting cells in the course of a long-term production when the starting data are available. Although the monitoring can be done also by immunoassay techniques through antibody analysis, the DNA approach is absolutely specific, allowing changes even at one point mutation level to be recognized. Finally, amino acid sequences of variable domains confirmed by MS analysis are presented in this paper. Thus, the sequences of the variable antibody regions, presented in **Tables 3** and **4**, actually correspond to the sequences of the antibodies that are secreted by the hybridoma clones.

ABBREVIATIONS USED

2,4-D, 2,4-dichlorophenoxyacetic acid; MCPA, 2-methyl-4-chlorophenoxyacetic acid; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; BSA, bovine serum albumin; CDR, complementarity determining regions; cDNA, complementary deoxyribonucleic acid; dNTPs, deoxyribonucleotide triphosphates; ELISA, enzyme-linked imunosorbent assay; TMB, tetramethyl benzidine; PBS, phosphate-buffered saline; IC_{50}, analyte concentration at 50% binding inhibition; Fw, framework region of antibody domain; GIT, guanidinium isothiocyanate; FCS, fetal calf serum; IgG, immunoglobulin class G; RT-PCR, reverse transcription Polymerase Chain Reaction; SwAM-POD, swine anti-mouse peroxidase-labeled antibody; V_H, variable domain of heavy chain; V_L, variable domain of light chain; Pfu, *Pyrrococcus furiosus*; AMV, avian myeloblastosis virus; D-MEM, Dulbecco's Modified Eagle medium.

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