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Production and Characterization of Monoclonal Antibodies Against Urea Derivatives

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ABSTRACT

A panel of monoclonal antibodies was generated against the urea-based hapten *N*-(2-*N*-chloroacetylaminobenzyl)-*N'*-4-chlorophenylurea as a tool for building up sensitive immune assays to detect urea derivatives and to screen them for catalytic antibodies (Abs). Eleven hybridomas were obtained that produced Abs reactive to the hapten. All Abs were of IgG class. Crossreactivities of the Abs to different haptens were examined, especially to a possible transition-state analog. Only four of the hybridomas (R2-DA10/F7, R2-GE7/H2, R2-HC2/A5, R2-HD6/F7) produced Abs crossreactive with the transition-state analog. From the 11 hybridomas, hybridoma B76-BF5 was chosen for further characterization. Compared to the other Abs, B76-BF5 showed the strongest binding and had a rather restricted specificity. These Abs could be used to build up a sensitive enzyme immunoassay for the detection of the hapten. All Abs were screened for crossreactivity with the pesticides monuron and diuron. No reactivity could be detected. In addition, the nucleotide sequences of the variable light and heavy chain genes of the similarly

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reactive Abs B76-BF5, B76-BB3, R2-DA10/F7, and R2-GA6/G3 were determined to clarify whether structure and binding specificity of these Abs showed any correlation.

Index Entries: *N*-(2-*N*-chloroacetylaminobenzyl)-*N'*-4-chlorophenylurea; pesticides; catalytic antibodies; hybridomas; KABAT database.

Nomenclature: BSA, bovine serum albumin; PBS, phosphate buffered saline; NCS, neonatal calf serum; M-MLV-RT, Moloney murine leukemia virus-reverse transcriptase; Ig, immunoglobulin; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl

INTRODUCTION

Pesticides are widely used in agricultural technologies. However, the toxicity of a large number of pesticides makes it necessary to control their concentrations in the environment. Several monoclonal antibodies (MAbs) were developed to detect pesticides by using ELISA *(1–3)*. Recently, single-chain antibodies (Abs) detecting the herbicides atrazine and paraquat were constructed *(4,5)*. The ultimate aim of dealing with pesticides is not only the detection, but also the elimination, of pesticides from the environment by easy-to-use methods. Many of the commonly used pesticides are urea derivatives. Abs that bind and cleave such derivatives would therefore be of special interest to environmental studies.

Already in 1969, Jencks *(6)* postulated that Abs reactive to transition-state analogs of a substrate should have catalytic activity. Several catalytic Abs that catalyze many different reactions, e.g., acyl transfers, pericyclic processes, redox reactions, elimination, and accelerated isomerization, have been described in the past few years. In addition to the production of polyclonal and monoclonal abzymes, the utilization of Ab libraries has become a further possibility to select catalytic Ab fragments *(7)*.

The hapten *N*-(2-*N*-chloroacetylaminobenzyl)-*N'*-4-chlorophenylurea (OC2) (Fig. 1), which is a potential pesticide (G. Kempter and J. Woller, personal communication), was chosen as a model substance for experiments.

Monoclonal antibodies (MAbs) against the hapten will be used to build up a sensitive immunoassay to screen for catalytic Abs. Here are presented the first steps of the project.

MATERIALS AND METHODS

Antigens

The hapten OC2, the similar compounds POCc4, POCc6, POCc9, POCc10, POCc12, and a possible transition-state analog were synthesized by Kempter and Woller (Figs. 1 and 2). The derivatives OC2, POCc4,

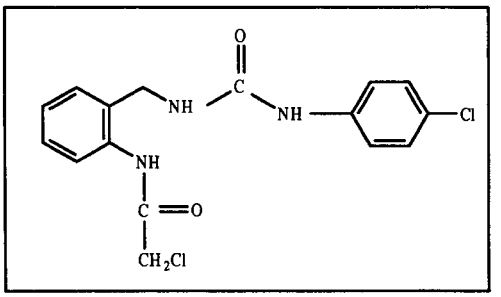

Fig. 1. Hapten OC2: *N*-(2-*N*-chloroacetylaminobenzyl)-*N'*-4-chlorophenylurea used for immunisation.

POCc10, and POCc12 were coupled via the chloracetyl group to iminothiolane-modified carrier proteins bovine serum albumin (BSA), and *Helix pomatia* hemocyanin, using Traut's reagent (Sigma, Deisenhofer, Germany) *(8)*. POCc6 and POCc9 were coupled via the carboxyl group to carrier proteins using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl (EDCI; Sigma) *(9)*, and the transition-state analog was conjugated via the amino group to the carrier proteins using glutaraldehyde *(9)*.

Immunization

Balb/c mice were immunized with OC2–hemocyanin conjugates. Four days later the booster-injection mice were killed and spleen cells were isolated. A total of 1×10^8 spleen cells was then fused with 2×10^7 myeloma cells X 63-Ag 8.653 *(10)* using polyethylene glycol 1550 *(11)*.

The fused cells were distributed on mouse peritoneal feeder cells grown in hypoxanthine/azaserine/thymidine (HAT)-medium. After 1 wk, hybridomas were screened for the production of Abs against the urea-based hapten OC2 conjugated to BSA, using an enzyme immunoassay. Cells from positive wells were selected and recloned.

Enzyme Immunoassay

Screening for Abs in culture supernatants was performed using the following solid-phase enzyme immunoassay. The antigen solution, hapten–BSA conjugates diluted to 2.5 or 5 μg/mL in phosphate-buffered saline (PBS), were added to the wells of microtiter plates for overnight adsorption at 4°C. The wells were successively incubated with PBS/5% neonatal calf serum (NCS), Ab containing culture fluid, and peroxidase-labeled antimouse-Ig-Abs. These incubations were performed, in general, for 1 h at room temperature. For the detection of peroxidase activity, 1 mg/mL o-phenylenediamine and 0.01% H_2O_2 in 0.1 *M* citrate buffer, pH 5.0, were used as substrates *(12)*. The reaction was stopped by the addition

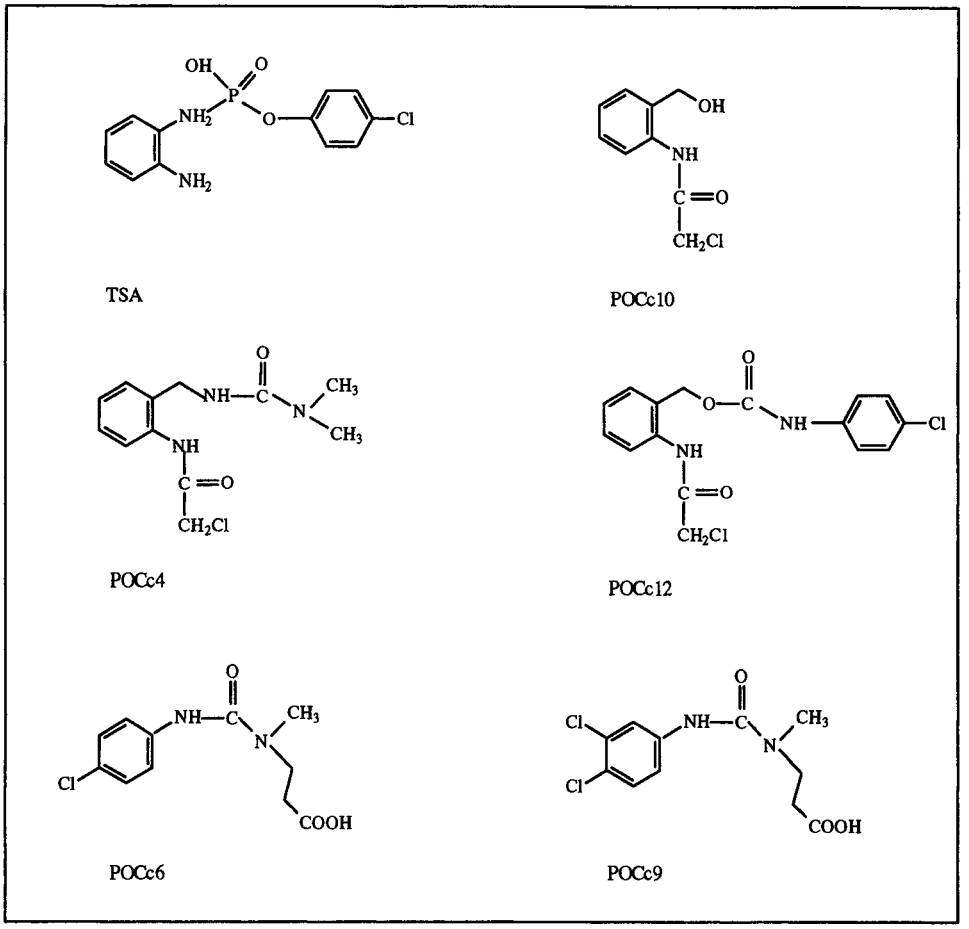

Fig. 2. Compounds used to detect cross-reactivities of monoclonal antibodies against OC2: *N*-(2-*N*-chloroacetylaminobenzyl)-*N'*-4-chlorophenylurea
TSA: Transition state analogue (2-aminophenyl)-phosphoramidicacid-mono (4-chlorophenyl)ester
POCc4: 2-chloro-N-[2-(3,3-dimethyl-ureido)-phenyl]-acetamide
POCc6: 3-[3-(chloro-phenyl)-1-methyl-ureido]-propionacid
POCc9: 3-[3-(3,4-dichloro-phenyl)-1-methyl-ureido]-propionacid
POCc10: 2-chloro-N-(2-hydroxymethyl-phenyl)-acetamide
POCc12: N-(4-chloro-phenyl)-[2-(2-chloracetylamino)]-benzylcarbamate

of 2 *N* H_2SO_4 supplemented with 50 m*M* Na_2SO_3, and the tests were evaluated by measuring the absorbance at 492 nm.

Determination of Antibody Class and Subclass

The class and subclass of the MAbs were determined by an enzyme immunoassay using biotin labeled antimouse-Ig class- and subclass-specific Ab (Serva, Heidelberg) *(13)*.

Primers for the variable light genes:

Bam HI Not I
FOR-primer K: 5' GAAGATGGATCCAGCGGCCGCAGCATCAGC 3'

BACK-primers: Mlu I Eco RV
L1 5' GAAGCACGCGTAGATATCKTGMTSACCCAAWCTCCA 3'
L2 5' GAAGCACGCGTAGATATCGTGATRACMCARGATGAACTCTC 3'
L3 5' GAAGCACGCGTAGATATCWTGMTGACCCAAWCTCCACTCTC 3'
L4 5' GAAGCACGCGTAGATATCGTKCTCACYCAGTCTCCAGCAAT 3'

Primers for the variable heavy genes:

Hind III
FOR-primer G: 5' ACCAGGGGCCAGTGGATAGACAAGCTTGGGTGTCGTTTT 3'

BACK-primers: Nco I Pvu II/PstI
H1 5' CAGCCGGCCATGGCGCAGGTSCAGCTGCAGSAG 3'
H2 5' CAGCCGGCCATGGCGGAGGTGAAGCTGCAGGAGTCAGGACCTAGCCTGGTG 3'
H3 5' CAGCCGGCCATGGCGCAGGTSMARCTGCAGCARTCWGG 3'

Fig. 3. Oligonucleotides used for mouse-Ig PCR reactions *(11)*. Bold letters and underlined nucleotides represent the restriction sites for the restriction enzymes.

Degenerate Nucleotides are S = C or G, M = A or C, W = A or T, R = A or G, K = G or T, Y = C or T.

Sequence Analysis of Variable Antibody Regions

Total RNA was isolated from hybridoma cells using the RNAzol method *(14)* and a Li-based RNA-extraction protocol *(15,16)* in parallel. First-strand cDNA was synthesized using either mouse Ig-specific primers, indicated in Fig. 3, plus InViScript (InViTek GmbH, Berlin) in the presence of Mn^{2+} or oligo(dT)14/random hexamer primers plus M-MLV-RT (Gibco-BRL, Gaithersburg, MD) under conditions recommended by the manufacturer. PCR was carried out beginning with a delay of 1 min at 94°C, 30 cycles of 30 s at 94°C, 20 s at 56.5°C, 10 s at 72°C (plus an additional second for each cycle), using CombiPol-Polymerase (InViTek GmbH) in recommended buffer. Primers used were complementary to the beginning of framework I of heavy (H) or light (L) chain genes (H1–3, L1–4) and to the beginning of H- and L-chain constant regions, respectively (Fig. 3; *17*). PCR products were purified and then sequenced by the chain termination method, using fluorescent dideoxinucleotid triposphate derivatives and amplification primers, without further cloning. Monitored sequences were compared with the KABAT-database *(18)* using FASTA *(19)* and BLAST *(20)* algorithms, and the framework- and complementarity determining regions were deduced.

RESULTS AND DISCUSSION

Eleven hybridomas producing MAbs were generated against the hapten OC2. All hybridomas secreted Abs of IgG subclass (Table 1). The Abs

Table 1
Subclasses and Crossreactivities of MAbs Against OC2 Reactivity with Different Compounds Tested Using Enzyme Immunoassays

Hybridoma	Ig class	OC2	TSA	POCc4	POCc6	POCc9	POCc10	POCc12	PHOX
B76-BF5	IgG1	+	−	−	−	−	−	+	−
B76-BB3	IgG1	+	−	−	−	−	−	−	−
R2-AG1/C5	IgG2b	+	−	−	−	−	−	+	−
R2-DA10/F7	IgG1	+	+	−	−	−	−	+	−
R2-FD1/H4	IgG1	+	−	−	−	−	−	+	−
R2-GA6/G3	IgG1	+	−	−	−	−	−	−	−
R2-GB9/E9	IgG2b	+	−	−	−	−	−	−	−
R2-GE7/H2	IgG1	+	+	−	−	−	−	+	−
R2-GF2/D9	IgG1	+	−	−	−	−	−	−	−
R2-HC2/A5	IgG2a	+	+	−	−	−	−	+	−
R2-HD6/A5	IgG1	+	+	−	−	−	−	+	−

Crossreactivity was deduced from the OD values obtained; − indicates binding to the corresponding hapten-conjugated BSA at the same magnitude as to nonconjugated BSA; + indicates binding to the corresponding hapten-conjugated BSA at a magnitude of at least 35% of the binding to OC2-conjugated BSA.

OC2, *N*-(2-*N*-chloroacetylaminobenzyl)-*N'*-4-chlorophenylurea; TSA, transition-state analog (2-aminophenyl)-phosphoramidicacid-mono(4-chlorophenyl)ester; POCc4, 2-chloro-N-(2-[3, 3-dimethyl-ureido]-phenyl)-acetamide; POCc6, 3-(3-[chloro-phenyl]-1-methyl-ureido)-propionacid; POCc9, 3-(3-[3,4-dichloro-phenyl]-1-methyl-ureido)-propionacid; POCc10, 2-chloro-N-(2-hydroxymethyl-phenyl)-acetamide; POCc12, N-(4-chloro-phenyl)-(2-[2-chloracetylamino])-benzylcarbamate; PHOX, 4-phenyl-2-oxayolone.

were examined for crossreactivities with other substances, e.g., possible cleavage product analog POCc10 (Fig. 2), transition-state analog (Fig. 2), urea-based pesticide analog POCc6 (monuron analog) and POCc9 (diuron analog) (Fig. 2), and possible other substrates POCc4 and POCc12 (Fig. 2). Four of the hybridomas (R2-DA10/F7, R2-GE7/H2, R2-HC2/A5, R2-HD6/A5) produced Abs that also bound the possible transition-state analog and compound POCc12. Three of the hybridomas (B76-BF5, R2-FD1/H4, R2-AG1/C5) produced Abs specific for the urea-based haptens OC2 and POCc12 (Table 1), and four other Abs (B76-BB3, R2-GA6/G3, R2-GF2/D9, R2-GB9/E9) produced Abs exclusively reactive with OC2. The highest binding affinity was detected with Ab B76-BF5. Using a BIOCORE, a $K_D = 1.1 \times 10^{-9}$ mol/L for immobilized hapten was determined *(27)*. It should therefore be useful to build up a sensitive immunoassay to screen for catalytic Abs. The product analogs POCc10 and the pesticide analogs POCc6 and POCc9 were not bound by Ab B76-BF5.

The comparison of the nucleotide sequences of the variable light (V_L)- and heavy (V_H)-chain genes of the Abs B76-BF5, B76-BB3, R2-GA6/G3, and

Light chains

Clone	FR1	CD1	FR2
B76-BF5	DIVLTQSPIQASLAVSLGQRATISC	**RAS-ESVDSFGNSFMH**	WHQQKPGQPPKLLIY
B76-BB3	DILMTQSPL--SLPVSLGDQASISC	**RSSQNIVHSNGNTYLE**	WYLQKPGQSPKPLIY
R2-GA6/G3	DILITQXPL--SLPVSLGDQASISC	**RSSQSLVHSNGNTYLH**	WYLQKPGQSPKLLIY
R2-DA10/F7	DILITQSPT--TMAASPGEKITITC	**SASSSINSN----YLR**	WYQQKPGFSPKLLIY

Clone	CD2	FR3	CD3	FR4
B76-BF5	**LASNLGS**	GVPARFSGSGSRTDFTLSIDPVEADDAATYYC	**QQNNEDPPT**	FGAGTKLELR
B76-BB3	**KVSNRFS**	GVPDRFSGSGSGTDFTLKISRVEAEDLGVYYC	**FQGSHVPYT**	FGGGTKLEIN
R2-GA6/G3	**KVSNRFS**	GVPDRFSGSGSGTDFTLKISRVEAEDLGVYFC	**SQSTHVPLT**	FGAGTKLELK
R2-DA10/F7	**ATSNLAS**	GVPARFSGSGSGTSYSLTIGTMEAEDVATYYC	**QQGSSMPIT**	FGSGTKLEIK

Fig. 4. Deduced amino acid sequences of the light chains of monoclonal antibodies against OC2 FR, framework region; CDR, complementarity-determining region.

Heavy chain

Clone	FR1	CDR1	FR2
B76-BF5	QVQLQESGGGLVQPGGSMKLSCIGSGFTFS	**SYWMS**	WVRQSPERGLEWVA
B76-BB3	QVQLQQSGAELAKPGASVKMSCKASGYSFT	**IYRMH**	WAKQRPGQGLEWIG
R2-GA6/G3	QVQLQQSGAELVRPGASVTLSCKASGYTFT	**DYEMH**	WVKQTPVHGLEWIG
R2-DA10/F7	AGQTAGVWTELEKPGASVNISCKASGYSFT	**GYNMN**	WVKQGNGKSLEWIG

Clone	CDR2	FR3
B76-BF5	**EIRLKSANFATHYAESVKG**	RFTISRDVSKSRLYLQMNSLRAEDTGIYYCTA
B76-BB3	**YSYPSTG--YSEYNQKFKD**	KATLTADTSSSTAYMQLTSLTSEDSAVYYCVR
R2-GA6/G3	**AIDPETG--GTAYNQKFKG**	KATLTADKSSSTAYMELRSLTSEDSAVYYCTD
R2-DA10/F7	**NINHYYQ--GATYNQKFKD**	KATLTVDKSSNIAYMRLKSLTSEDSAVYYCAG

Clone	CDR3	FR4
B76-BF5	**LYFSGSKFEVH**	WGQGTTLTVSS
B76-BB3	**-----SGGDY**	WGQGTSVIVSS
R2-GA6/G3	**GNYL-AWFAY**	WGQGTTVTVSS
R2-DA10/F7	**STMTSTSFTY**	WGQGTLVTVSA

Fig. 5. Deduced amino acid sequences of the heavy chains of monoclonal antibodies against OC2 FR, framework region; CDR, complementarity-determining region.

R2-DA10/F7 showed that there is no correlation between binding specificity and amino acid sequence. The deduced amino acid sequences of the V_L and V_H regions are shown in Figs. 4 and 5. Analysis of Ab sequences using the immunological database of Kabat et al. *(18)* showed that the H chain of B76-BF5 is a member of the mouse H-chain subgroup III (C) (Table 2); H chains of the other Abs belong to the mouse H-chain subgroup II. The Abs R2-DA10/F7 and R2-GA6/G3 are of subgroup II (A) and the Ab B76-BB3 belongs to subgroup II (B). CDR3 of B76-BB3 was extremely short: Only about 7% of the immunoglobulins of subgroup II (B) have such a short region of only five amino acids. All L chains were κ-chains. B76-BF5 L chain was a member of mouse κ-chain subgroup III, B76-BB3 and R2-GA6/G3 L chains of subgroup II and R2-DA10/F7 L chain of subgroup IV. The sequences of B76-BF5 showed homology with some published Ab sequences as, e.g., anti-human interferon-β receptor-Ab (V_H, 92%) *(21)* or anti-DNA-Ab (V_L, 94%) *(22)*. The sequences of the V_H of the other Abs showed homology with various murine anti-2-phenyloxazolone-Abs *(23)*. The V_L sequences of the Abs

Table 2
Subgroups of V_L and V_H

Hybridom	V_L κ chain	V_H
B76-BF5	III	III (C)
B76-BB3	II	II (B)
R2-DA10/F7	IV	II (A)
R2-GA6/G3	II	II (A)

Subgroup assignment of the variable L- and H-chain genes of MAbs to OC2, according to the KABAT database.

B76-BB3 and R2-GA6/G3 showed homology to an anti-*Escherichia coli*-Ab (99%) *(24)*, and that of R2-DA10/F7 to Abs of 28C13-hybridomas (carcinogen-induced murine B-cell lymphomas) (97%) *(25)*.

Because the sequences of V_H of three Abs showed homology to Abs against 2-phenyl oxazolone, the authors checked Abs for crossreactivity to this hapten, but no significant crossreactivity was detected (Table 1).

Further experiments are in progress to apply B76-BF5-based immunoassays to screen Abs to the possible transition-state analog for catalytic activity using OC2 as substrate.

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