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Analysis of idiotope structure of ovarian cancer antibodies: recognition of the same epitope by two monoclonal antibodies differing mainly in their heavy chain variable sequences

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SUMMARY

Two MoAbs, independently raised against ovarian carcinoma cells and referred to as OV-TL3 and OV-TL16, display an identical reaction pattern with a membrane-associated protein in both normal and malignant ovarian cells. Also, a similar binding affinity constant and a similar number of binding sites per cell indicate that both MoAbs bind to the same antigen. Competition assays reveal that OV-TL16 is able to compete with OV-TL3 for binding to OVCAR-3 cells. Epitope mapping using a filamentous phage hexapeptide epitope library showed that both MoAbs are able to select identical phages, suggesting that their epitopes are identical or at least overlapping. However, purified polyclonal and monoclonal anti-idiotypic antibodies directed against OV-TL3 failed to recognize the OV-TL16 idiotype, indicating that the structure of the antigen-binding regions of both antibodies is distinct. This was corroborated by molecular cloning and sequencing of the variable heavy (V_H) and light (V_L) chain immunoglobulin regions of both MoAbs. The V_H regions of both antibodies were found to be distinct, whereas the V_L regions are almost identical. Computer modelling of the idiotypes suggests that the complementarity determining regions (CDR), with the exception of V_HCDR3, have (almost) identical spatial configurations. Our data indicate that, although structurally different in their V_H regions, OV-TL3 and OV-TL16 are able to bind to identical epitopic regions on the antigen, because differences in primary structure do not exclude the formation of sufficient and similar spatial structures for the interaction with an epitope.

Keywords immunoglobulin variable domains ovarian carcinoma phage display epitope library antibody modelling

INTRODUCTION

For the management of cancer, MoAbs raised against tumour-specific determinants have proven to be of great help. For ovarian carcinomas MoAbs have been generated, among which are OV-TL3 and OV-TL16, that have been raised by different immunization procedures. Both react with a membrane antigen (OA3) [1] expressed in about 90% of the ovarian tumours and only weakly expressed in normal tissue [2]. OV-TL3, raised after immunization with an extract of an endometrioid cancer biopsy, has a reactivity pattern highly restricted to ovarian carcinomas [3,4], and was recently shown to react with a surface glycoprotein by molecular cloning of the antigen [1]. The antibody has been applied in immunoscintigraphy of patients with ovarian cancer [5,6] as well as for drug targeting [7,8]. The other ovarian carcinoma-specific MoAb OV-TL16 was raised after immunization with cyst fluid obtained from a poorly differentiated ovarian carcinoma [9]. The immunohistochemical reactivity patterns of OV-TL16 and OV-TL3 with ovarian carcinomas and some other (neoplastic) tissues are similar [4]. Tumour uptake and biodistribution patterns in nude mice bearing ovarian cancer xenografts as well as binding affinity, binding characteristics and the number of antigenic determinants per cell, were identical for both antibodies [10]. These data suggest that both antibodies, although obtained by completely different immunization protocols and with a time interval of 2 years, might recognize an identical antigen or even the same epitope on ovarian carcinoma cells.

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In this study we show that the epitope of both antibodies is identical or overlapping, as demonstrated by competition assays and by epitope mapping with hexapeptide-displaying filamentous phages. However, the structure of the antigen-binding sites of the MoAbs appears to be different, since anti-idiotypic antibodies interacting with OV-TL3 fail to bind to OV-TL16. This has been confirmed by molecular cloning of the variable heavy and light chain regions. The heavy chains of both antibodies are structurally different, and computer modelling showed that this for the V_HCDR3 results in conformational differences. This appears to be revealed by the anti-idiotypic antibodies, but to have a minor influence on antibody binding. These results support the concept that antibody-binding sites with different primary amino acid sequences can contribute to similar spatial conformations that interact with the same antigenic target.

MATERIALS AND METHODS

Cell lines and antibodies

The human ovarian carcinoma cell line NIH-OVCAR-3 [11], obtained from the American Type Culture Collection (ATCC; Rockville, MD), was used for immunoaffinity studies. The cell line was grown in RPMI 1640-based culture medium (GIBCO, Paisley, UK) supplemented with 15% fetal calf serum (FCS; GIBCO). The following MoAbs directed to ovarian carcinoma-associated antigens were used: OV-TL3 [2], OV-TL16 [9], OV-TL30 [10] and MOv18 [12]; kindly supplied by Dr M. Colnaghi (National Cancer Institute, Milan, Italy). These antibodies were purified by protein A Sepharose affinity chromatography, and adjusted to a concentration of 3–5 mg/ml as determined by the protein quantification method of Lowry *et al.* [13] and PAGE [14]. Preparations were stored at −20°C until use.

Raising of anti-idiotypic antibodies. Polyclonal anti-idiotypic antibodies were obtained from rabbits immunized with OV-TL3 IgG1. The antibodies were isolated from the rabbit immune serum by affinity chromatography on cyanogen bromide-activated Sepharose 4B to which purified OV-TL3 IgG1 antibodies were coupled according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). The OV-TL3 binding antibodies were eluted at pH 2·5, neutralized to pH 7·5, and passed repeatedly through a mouse IgG Sepharose 4B column to remove anti-isotypic antibodies. In addition, an anti-idiotypic MoAb (JL1) has been developed. It was raised in female Armenian hamsters by a single intrasplenic immunization with 30 μg OV-TL3. After screening with a panel of antibodies and $F(ab')_2$ fragments, this antibody was found to react exclusively with OV-TL3. No reaction was found with mouse MoAbs.

Labelling of monoclonal antibodies

OV-TL3 antibody was labelled with Eu-N′-(*p*-isothiocyanatobenzyl)-diethylene triamine-N^1-N^2-N^3-N^3-tetraacetate, as described [15], purified and assayed as described [16], resulting in an incorporation of 10 atoms of Eu^{3+} per molecule OV-TL3. Labelled antibodies were stored in aliquots at −80°C until use. The antibodies were diluted in assay buffer (100 mM Tris HCl pH 7·8 and 0·5% bovine serum albumin (BSA) from Sigma (St Louis, MO)) to a final concentration of 200 and 400 ng/μl for use in the inhibition assay. Since OV-TL16 immune reactivity is lost even under mild Eu^{3+}-labelling conditions with a substitution rate of only three Eu^{3+} atoms per antibody molecule, all experiments were carried out with Eu^{3+}-labelled OV-TL3, using the other antibodies as non-labelled competitors in a competition-inhibition assay.

Inhibition assay for detection of competitive binding antibodies using time resolved immunofluorometry (TF-IFMA)

NIH-OVCAR-3 cells were grown in 12-well strip plates (Costar, Cambridge, MA) up to 90% confluency (approximately 25 000 cells/well). After fixation in 200 μl 0·25% glutaraldehyde for 10 min at 20°C, cells were washed with PBS and saturated with 200 μl 0·5% BSA in PBS per well for 1 h at 20°C. Subsequently, 20–40 ng Eu-OV-TL3 antibody were added to the coated wells, which appeared to be the optimal amount as determined in previous dose-response experiments. For competition assays, non-labelled antibodies were serially diluted and added in concentrations of 1 ng to 10 μg per well, after which the strips were incubated for 3 h at 20°C. After nine washes with PBS, 200 μl of enhancement solution (1 g/l Triton X100 surfactant, 6·8 mmol potassium hydrogen phthalate, 100 mmol acetic acid, 50 μmol tri-*n*-octylphosphinoxide and 15 μmol 2-naphtoyltrifluoroacetone per litre) were added to each well. Strips are shaken for 5 min on a microshaker (Dynatech, Zug, Switzerland) and after 10 min the fluorescence was measured in an Arcus time-resolved fluorometer model 1230 (Wallac Oy, Turku, Finland) using a 1-s counting time and a 400-μs time delay [17], by which the portion of labelled antibody remaining bound to the cells was assessed. Antibodies OV-TL3, OV-TL16, OV-TL30 and MOv18 were used in the competition assays.

Detection and purification of anti-OV-TL3 antibodies

A polyclonal rabbit anti-OV-TL3-id antiserum was produced by four subcutaneous immunizations of a New Zealand rabbit with 300 μg purified OV-TL3 IgG1 in Freund's complete adjuvant (FCA) per site, followed by one intramuscular immunization on day 29 with 100 μg OV-TL3 IgG1 in Freund's incomplete adjuvant (FIA). Subsequent s.c. doses were administered with 100 μg OV-TL3 IgG1 on two sites at 2-week intervals. The assay for the detection of anti-OV-TL3 antibodies was performed as previously published with minor adaptations [16]. The MoAbs OV-TL3 (for homologous sandwich assays), OV-TL16 or OV-TL30 (for heterologous sandwich assays) were coated to polystyrene microtitre wells (Eflab Oy, Helsinki, Finland) by incubating overnight at a concentration of 1 μg per well in Tris-HCl buffer (100 mM Tris-HCl pH 7·8, 154 mM NaCl and 7·5 mM NaN_3). After incubation, the wells were washed five times with a washing buffer (36 mM NaCl, 0·25 g/l Germall-II and 0·05% Tween 20), saturated overnight with 300 μl assay buffer, washed five times with washing buffer and stored in a humidified chamber at 4°C. Two hundred microlitres of five times prediluted rabbit serum were serially diluted with assay buffer and added in duplicate to the coated microtitre wells for overnight incubation at 20°C. The mixture was aspirated and the wells were washed five times with washing solution. Thereafter, 200 μl Eu-OV-TL3 were added to each well and incubated for 3 h at 20°C. After washing nine times with washing solution the wells were further processed for reading in the Arcus fluorometer as

described above. As a negative control, a 200-μl assay buffer was used for zero-dose control, and as positive control an ovarian carcinoma extract was used. In this procedure a positive homologous assay combined with a negative heterologous assay is indicative for anti-idiotypic antibodies.

Molecular cloning of variable heavy and light chain regions

RNA was isolated from OV-TL3 and OV-TL16-producing hybridoma cells according to standard protocols [18]. After cDNA synthesis using an oligo-dT primer and Mo-MuLV-RTase (GIBCO-BRL, Gaithersburg, MD), variable heavy and light chain coding regions were polymerase chain reaction (PCR) amplified using the following degenerate primers:

V_H1: 5'-TCCGATTCGCGGATCCGTG (C/A)A(A/G)CTGCAGGAGTC(A/T)GG-3'

V_H2: 5'-TCTCTGCAGCGGATCCC(A/G)(T/G)GGTCCCTTGGCCCCAG(T/A)-3'

V_L1: 5'-CCTCTGCAGGCATGCGAC(G/A)TTGT(T/G)(C/A)T(C/G)ACCCA(A/G)(T/A)CTCCA-3'

V_L2: 5'-CCTAAGCTTGCATGCTTT(T/G)A(A/G/C/T)(T/C)TCCAGCTTGGT(C/G)CC-3'

DNA fragments of approximately 320 bp were isolated as BamHI (V_H) or SphI (V_L) fragments and cloned into pUC19 according to standard procedures [19]. DNA sequence analysis was performed using the dideoxy chain termination method and T7 DNA polymerase (Pharmacia). At least three DNA clones, obtained from independent cloning procedures, were sequenced in order to eliminate possible artefacts caused by the Taq polymerase.

Computer modelling of V_H and V_L of OVTL-3 and OVTL-16

Modelling of the 3-D structure of the V_H and V_L of OVTL-3 and OVTL-16 was carried out with the UNIX-based computer program AbM (Immunoglobulin Domain Modelling Package; Oxford Molecular Ltd, Oxford, UK). The amino acid sequences of the variable domains of the antibodies have been used as program input for the modelling of the 3-D structures of these domains of OVTL-3 and OVTL-16. The program compared the V_H and V_L sequences of these antibodies with a database of antibodies with known 3-D structures.

The initial step was to produce a model of the antibody framework. The chain sequence to be modelled was assigned the conformation of the chain which compared most favourably on the basis of sequence homology. Once the framework for the antibody structure had been assigned, the program determined the structure of the CDR loops. To do this, it first generated the structure of the loops by using the method of assigning conformations to the loops on the basis of the conformations of canonical forms in the database. The method also indicated which loops can not be built in this way. Then, the remaining loops had to be built using either a database search, a theoretical *ab initio* approach, or a combination of both. The protein structure database was searched for loops which showed structural homology for C-α distances with the antibodies. The middle regions of the loops (the ones most likely to be involved in antibody–antigen interaction) were then deleted and rebuilt by a conformational search procedure [18].

Each of the conformations generated was subjected to energy minimization, using the Eureka forcefield. The graphical visualization of the atomic coordinates was drawn with MolScript [20]. Special attention was paid to the interaction between V_H and V_L and to the position of the CDR attachment.

Epitope mapping using a filamentous phage epitope library

The filamentous phage epitope library used in this study was constructed and generously provided by Dr G. Smith (University of Missouri, Columbia, MO). The library was constructed using a phage fd-derived vector (fUSE5), and consists of 2×10^8 independent phage clones. Each clone expresses a random hexapeptide fused to the phage pIII protein. The library has been estimated to represent 69% of the $6{\cdot}4 \times 10^7$ possible hexapeptides [21]. Phages that reacted with OVTL-3 or OVTL-16 IgG were selected from the epitope library by four rounds of a streptavidin-biotin based panning procedure [22]. The antibody concentration was 100 μg/ml for the first round and 0·1 μg/ml for the subsequent three rounds. From each selection 15 clones were picked. The phage-containing bacteria were grown in liquid cultures, from which single-stranded DNA template was prepared and sequenced.

RESULTS

Competition assays of OV-TL3 and OV-TL16 antibodies

Time-resolved immunofluorometric assay (TR-IFMA) competition assays, using OVCAR-3 cells and Eu-labelled OV-TL3 as a tracer, showed that both OV-TL3 and OV-TL16 antibody interfered with the binding of labelled OV-TL3 to the tumour cells (Fig. 1). The inhibition characteristics of OV-TL16 and OV-TL3 showed that they are capable of competing with each other with efficiencies depending on the concentration of the competing antibody. As a control, two unrelated antibodies, OV-TL30 and MOv18, were analysed and did not show any effect on the binding of OV-TL3 to OVCAR-3 cells. This suggests that both antibodies bind to the same antigen. Whether they bind to the same epitope or overlapping epitopes can not be deduced from these data.

Epitope mapping of OV-TL3 and OV-TL16 using the phage display system

A filamentous phage epitope library was used to compare the epitopes of the two MoAbs. Phages of the library display on

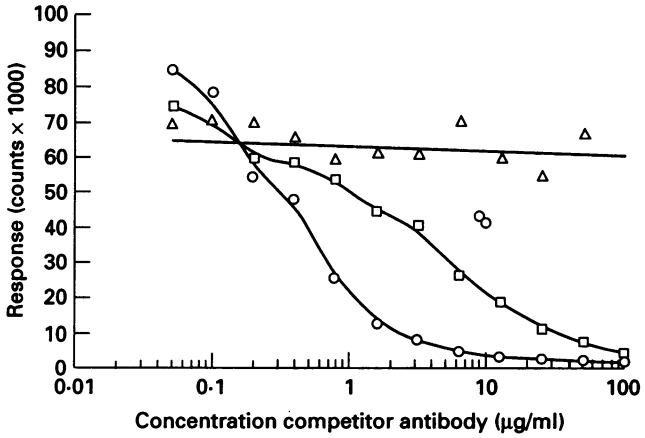

Fig. 1. Competition assay of Eu-labelled OV-TL3 with OV-TL16. Eu-labelled OV-TL3 (○) was incubated with OVCAR-3 cells in the presence of different concentrations of competitor antibodies, as indicated. Both OV-TL3 and OV-TL16 (□) competitor antibodies were able to interfere with the binding of Eu-labelled OV-TL3 on OVCAR-3 cells. Control antibodies OV-TL30 and MOv18 (△) showed no competition effects.

their surface hexapeptide sequences fused to a coat protein. These hexapeptides are available for binding to the antibody. The library contains about $6{\cdot}4 \times 10^7$ different hexapeptides. After four rounds of biopanning 15 clones were picked for OV-TL3 and 15 for OV-TL16. Phage clones were considered to react specifically with an antibody if they made up 20% or more of the picked clones. Two groups, four and three clones respectively, isolated from the filamentous phage epitope library by biopanning against OV-TL3 or OV-TL16, displayed homologous hexapeptides at their surfaces (Fig. 2). The other eight clones did not show any similarity, and probably represent non-specifically binding phages. Cross-reactivity was found for the groups of phages that reacted with both antibodies, but not for the group of non-specific 'binders'. From these experiments we conclude that both antibodies were able to select identical hexapeptide sequences at a significant rate and, therefore, can bind to the same epitope on the antigen.

Comparison of the idiotopes of OV-TL3 and OV-TL16

To compare the idiotopes of OV-TL3 and OV-TL16 a rabbit polyclonal anti-idiotypic antibody was raised to OV-TL3. Unpurified rabbit serum obtained 197 days after the start of the immunization procedure showed an anti-idiotype titre of 1/1280 at 50% inhibition level assayed in the competitive TR-IFMA on OVCAR-3 cells, while preimmune serum had no detectable anti-idiotype titre. In order to remove anti-isotype/allotype antibodies from the immune serum, the serum was passed through an OV-TL3-Sepharose 4B column several times. The eluted antibodies were then passed through a second column containing polyclonal BALB/c IgG-Sepharose several times, until no antibodies bound to an OV-TL3-isotype matched MoAb (OV-TL30) in a heterologous TR-IFMA assay. To substantiate that this serum contained anti-idiotype antibodies to OV-TL3, it was tested for its ability to displace Eu-labelled OV-TL3 on NIH:OVCAR-3 cells in a competitive

GROUP 1	gca agg ctg tct agg aag	gct agg ctt tct agc aag
	ALA ARG LEU SER ARG LYS	ALA ARG LEU SER ARG LYS
	gca agg ctg tct agg aag	gca agg ctg tct agg aag
	ALA ARG LEU SER ARG LYS	ALA ARG LEU SER ARG LYS
	--t agg ctg tct agg aag	gcg aag ctt tct agg aag
	ARG LEU SER ARG LYS	ALA LYS LEU SER ARG LYS
	cta agg ctg tct agg aag	gcg aac ctt tct aga aag
	LEU ARG LEU SER ARG LYS	ALA ASN LEU SER ARG LYS
GROUP 2	ggg ccg ttg cgt tct atg	ggg ccg ttc cgt tct atg
	GLY PRO LEU ARG SER MET	GLY PRO PHE ARG SER MET
	cgt cag ttg cgt tct atg	cgg cag ttg cgt tct atg
	ARG GLN LEU ARG SER MET	ARG GLN LEU ARG SER MET
	--- -ag ttg cgt tct atg	gtt tca cgg cat tct atg
	LEU ARG SER MET	VAL SER ARG HIS SER MET
GROUP 3	cgt ggt atg act ctg tct	cgt aag ttg cta cgt acg
	ARG GLY MET THR LEU SER	ARG LYS LEU LEU ARG THR
	ctt cat atg atc ctg tct	caa tgc gtt tca atc atg
	LEU HIS MET THR LEU SER	GLN CYS VAL SER ILE MET
	ctt gtg gct gtt cct act	agc agt ccg atc gta tct
	LEU VAL ALA VAL PRO THR	SER SER PRO ILE VAL SER
	ccg tgt agc gtt cat act	aca gag ccg atc gta tct
	PRO CYS SER VAL HIS THR	THR GLU PRO ILE VAL SER
	gct ttg gct ggt cat tct	acg agt ctg gac agt att
	ALA LEU ALA GLY HIS SER	THR SER LEU ASP ARG ILE
	cag tgc gga gac gta aag	ggt gcg tat cat acg gtt
	GLN CYS GLY ASP VAL LYS	GLY ALA TYR HIS THR VAL
	cct gtg ctg ttc ata act	gct agt tgc ata ctg tct
	PRO VAL LEU CYS ILE THR	ALA SER CYS ILE LEU SER
	gct tgt tgt tgc ctt aat	tct aga tat atc ggg tct
	ALA CYS CYS CYS LEU ASN	SER ARG TYR ILE GLY SER

Fig. 2. Sequences of hexapeptides selected by OV-TL3 and OV-TL16 from the phage display epitope library. Hexapeptide inserts of the pIII gene of filamentous phages selected by OV-TL3 (left) and OV-TL16 (right) were sequenced with the use of specific primers. Group 1 is composed of homologous hexapeptide sequences selected by both antibodies, group 2 of sequences that are similar. Group 3 consists of hexapeptide-displaying phages selected by only one antibody, that have no similarity with hexapeptide sequences of either group 1 or group 2.

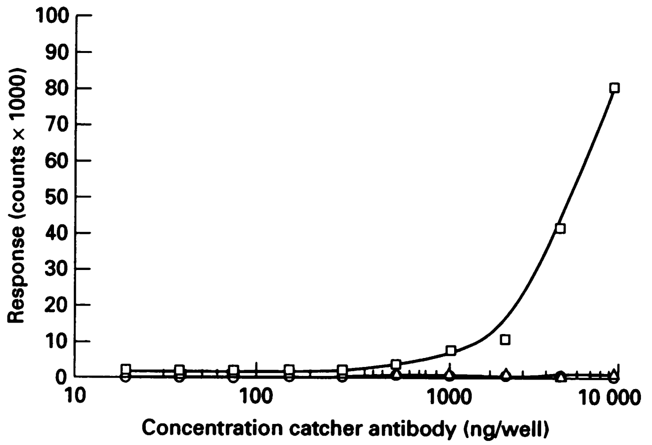

Fig. 3. Reaction of anti-idiotypic polyclonal antibodies to OV-TL3 with OV-TL3 and OV-TL16. Anti-idiotypic antibodies were incubated with increasing amounts of OV-TL3, OV-TL16 and control antibodies. After incubation, Eu-labelled OV-TL3 was used to detect bound anti-idiotypic anti-OV-TL3 antibodies in a TR-IFMA assay. Only OV-TL3 antibody was able to bind to anti-idiotypic anti-OV-TL3 antibodies, whereas OV-TL16 did not bind. ○, Counts OV-TL16; □, counts OV-TL3; △, counts control.

TR-IFMA assay. At a concentration of 1·8 μg/ml the purified antibodies achieved 50% inhibition of the binding of 0·8 μg/ml Eu-OV-TL3 to OVCAR-3 cells. This clearly indicates that the purified serum does contain anti-idiotypic antibodies to OV-TL3. Figure 3 shows that these immunoaffinity-purified and anti-isotype/allotype-depleted antibodies only bound to OV-TL3-coated wells, whereas no signal could be detected in a heterologous assay using OV-TL16 or OV-TL30 as 'capture' antibody. Similar results were obtained using anti-idiotype MoAb JL1. This antibody reacted exclusively with OV-TL3 and OV-TL3-F(ab′)$_2$, but not with OV-TL16 or its F(ab′)$_2$ fragment. JL1 inhibited the binding of OV-TL3 to OVCAR-3 cells (data not shown). Other, anti-isotype antibodies, derived from the same fusion as JL1, did not inhibit binding of OV-TL3 to OVCAR-3 cells. This suggests that OV-TL3 and OV-TL16, despite their immunohistochemical similarity and their binding to the same epitope, are different antibodies with a distinct idiotype binding site.

Sequence analysis of the heavy and light chain variable regions of OV-TL3 and OV-TL16 and molecular modelling of their 3-D structure

To investigate the idiotypic difference between OV-TL3 and OV-TL16 at the molecular level, the primary structure of the variable regions of the heavy and light chains of both antibodies was determined. Two sets of degenerate oligonucleotide primers were designed to amplify specifically the regions coding for the variable heavy and light chain domains. The primers were based on the FR1 and FR4 regions of the variable domains, since these proteins were intended to be expressed in a bacterial system. The use of degenerated primers in the PCR may introduce mutations in the coding sequences in this area. DNA sequence analysis of the variable regions of OV-TL3 and OV-TL16 (Fig. 4) revealed that the heavy chains of both antibodies are quite different. Both frame work (FR) and complementarity determining regions (CDR) of the heavy chain variable regions showed considerable differences in the deduced amino acid sequence. The lengths of CDR2 and CDR3 differed by one amino acid. In contrast, the deduced amino acid sequence of the light chain variable regions of both antibodies, however, was almost the same. Only one amino acid substitu-

(a)

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          2                            31  35               50             65
OV-TL3:   VQMQESGAELVKPGASVKLSCKASGYTFTNYYIFWVNQRPGQGLEWIGGINPSNGGSNFSNEKFKS
OV-TL16:  --L----P-----R--A-I-------S--G-FMN--K-SH-KS-----R---Y--DT*-Y-Q---G
                                       CDR1                      CDR2

                                         95     102   108
OV-TL3:   KATLTVDKSSSTAYMQLSSLTSEDSAVYFCTRGGLYTMDYRGQGTM
OV-TL16:  -------------H-E-L-------------G--V*-T-SW----L
                                          CDR3
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(b)

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          1                      24               35               50    56
OV-TL3:   DIVMTQSPLSLPVSLGDQASISCRSSQSLVHSNGNTYLHWYLQKPGQSPKLLIYKVSNRFS
OV-TL16:  ---I--T-----------------------------------------------------K---
                                    CDR1                           CDR2

          57                              89       98         107
OV-TL3:   GVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHVPWTFGGGTKLELK
OV-TL16:  ---------------------------------------------------
                                          CDR3
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Fig. 4. Amino acid sequences of cloned OV-TL3 and OV-TL16 heavy and light chain variable domains. Amino acid sequences of variable heavy (a) and light (b) chains of OV-TL3 and OV-TL16 are given in the universal one-letter code. Identical amino acids are replaced by dashes; asterisks indicate that no amino acid is found in that particular position at the complementarity determining region (CDR). CDR are printed bold and shaded. Numbering is according to Kabat *et al.* [31]

tion in CDR2 (Asp → Lys) and two conservative amino acid substitutions in FR1 (Met → Ile and Ser → Thr) could be detected. It thus appears that these two antibodies capable of binding to identical hexapeptide epitopes use structurally different heavy chain variable regions.

However, computer modelling of the 3-D structures of the variable regions of both antibodies demonstrated that considerable differences in amino acid sequence resulted in similar spatial conformations of five of the six CDR. The V_L of the two antibodies presented a perfect match despite the three amino acid substitutions (Fig. 5). The conformation of V_HCDR1 and V_HCDR2 showed for OV-TL3 and OV-TL16 only a slightly different bending of the carbon backbone (Fig. 5). On the other hand, the V_HCDR3 showed, apart from other differences,

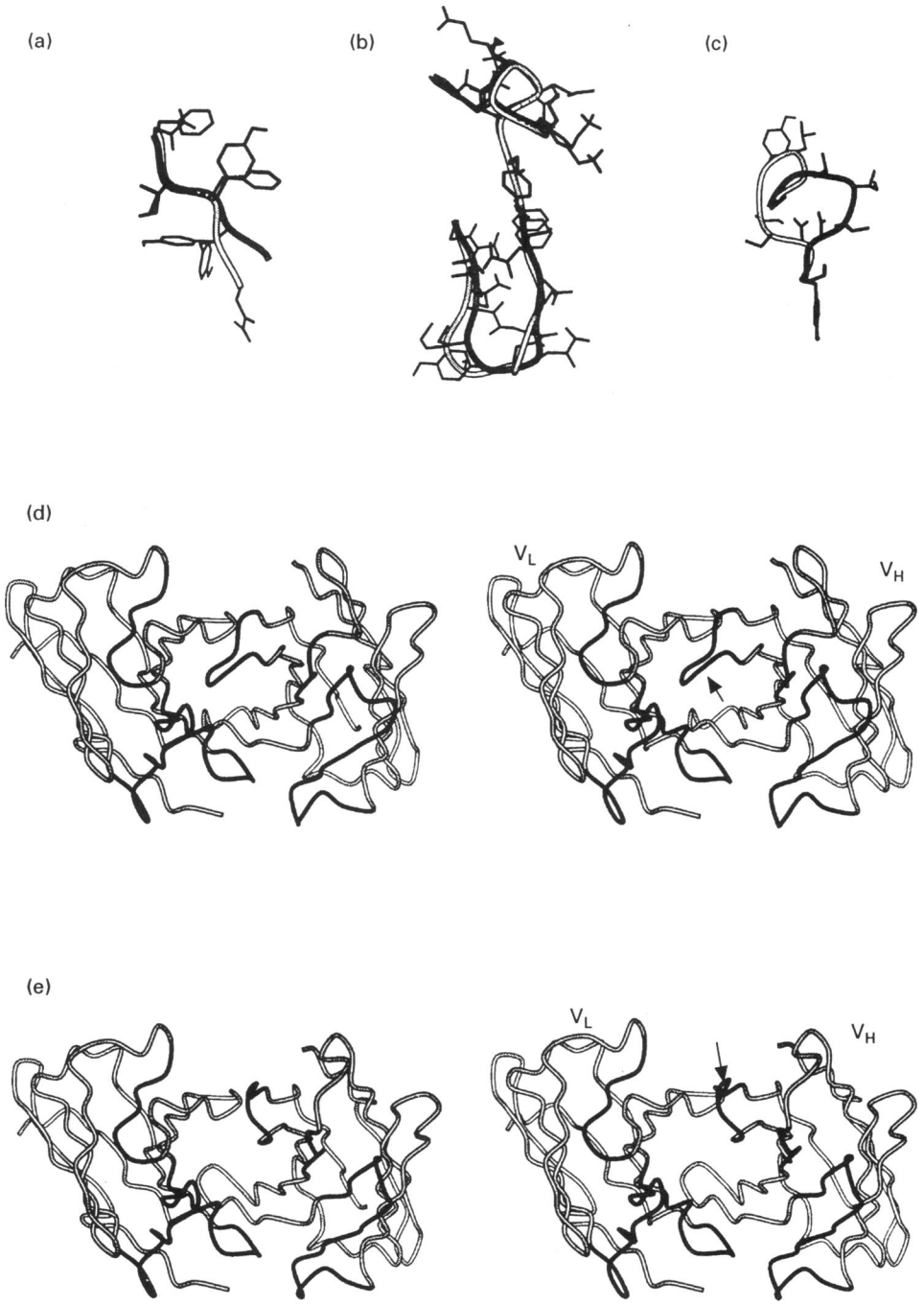

Fig. 5. 3-D structure of OV-TL3 and OV-TL16 variable domains. The spatial conformations of V_HCDR1, CDR2 and CDR3 regions of OV-TL3 (white) and OV-TL16 (black) are depicted (a,b,c). The presented conformation is obtained by energy minimization. Stereo drawings of OV-TL3 (d) and OV-TL16 (e) are composed as described above. The complementarity determining regions (CDR) of the light chains and heavy chains are represented by the black regions. The framework regions are in white. Only the V_HCDR3 (arrow) shows a substantially different orientation.

loops that have an orientation that differed about 90° between the two antibodies (Fig. 5). The loop of CDR3 of OV-TL3 appeared to be directed to the centre of the pocket formed by the CDR, whereas the CDR3 of OV-TL16 had a more outward orientation compared with the other five CDR.

DISCUSSION

In this study we show that OV-TL3 and OV-TL16, two MoAbs that appear to bind the same epitope of the ovarian carcinoma membrane antigen OA3, show considerable heavy chain variability, and therefore constitute a different idiotope. The OV-TL3 antibody was obtained after immunization with a tumour extract prepared from a patient with endometrioid ovarian carcinoma [2], while OV-TL16 was produced independently, using a completely different procedure by immunization with cyst fluid from a poorly differentiated ovarian carcinoma [9]. Both antibodies are of the IgG1 subtype, and have been shown to have similar binding characteristics [10]. The affinity constants of both antibodies are virtually identical (approximately $1{\cdot}1-1{\cdot}4 \times 10^9$ M^{-1}). Also, the number of antigenic determinants per cell is the same for both reagents ($100-120 \times 10^4$). The kinetics of association and dissociation, immunohistochemical reactivity patterns and immunotargeting capacity were found to be very similar, and internalization studies with OV-TL3 and OV-TL16 bound to OVCAR-3 cells also showed identical kinetics [7]. So far, the only difference observed is a loss of reactivity of OV-TL16 after Eu- or SASD-labelling, while OV-TL3 reactivity is not affected by these labelling procedures. Taken together, these results indicate that OV-TL3 and OV-TL16 may react with an identical antigenic determinant.

Since neither antibody reacts with denatured antigens in Western blotting, it was suggested that both recognize conformational epitopes. Photoaffinity labelling revealed the molecular weight of the OV-TL3 antigen to be 30–33 kD. An additional, much weaker band at 16 kD was also observed, the significance of which is not clear (data not shown). Recently, the antigen, designated OA3, has been cloned and characterized as a 323 amino acid membrane spanning protein [4]. Transfected COS cells expressing OA3 reacted strongly with both OV-TL3 and OV-TL16 (Dr I. Campbell, personal communication), thus elegantly proving that the antigens of OV-TL3 and OV-TL16 are identical. Interestingly, 3′-shortened RNA transcripts resulting in C-terminally deleted OV-TL3 antigen products have been identified, probably originating from differential splicing events. All of these transcripts contain an open reading frame coding for C-terminally deleted proteins of 295 and 312 (instead of 323) amino acids, and all were reactive with OV-TL3 as well as OV-TL16. The occurrence of alternative splicing products in OVCAR-3 cells may account for the reactivity of the antibodies with a doublet of 30–33 kD. The OA3 protein seems to be expressed ubiquitously in humans as determined by Northern blotting, but is in normal tissue not recognized by both OV-TL3 and OV-TL16 [4]. This corroborates the fact that both OV-TL3 and OV-TL16 recognize a conformational epitope, not occurring in normal cells. From competition assays it has become apparent that OV-TL3 and OV-TL16 interfere with each other in binding to the antigen on OVCAR-3 cells. This may be caused either by binding to identical or overlapping regions on the antigen, or by steric hindrance of the antibodies binding to epitopes located close to each other. To identify a (possibly common) epitopic region of OV-TL3 and OV-TL16 we used a filamentous phage epitope library, displaying random hexapeptide sequences at the surface of the phages. The construction and use of filamentous phage epitope libraries for epitope mapping of MoAbs have been described for several such libraries [21–26]. We found that seven out of 15 (=46%) sequences selected from the filamentous phage epitope library are shared by both antibodies. In view of the number of independent phage clones present in the library ($6{\cdot}4 \times 10^7$), this percentage is significant, and indicates efficient binding of these phages to both antibodies. The obtained sequences are not found in the primary protein sequence of the cloned antigen. This may be explained by the absence of the epitope in the phage library, which contains only 70% of all possible hexapeptide motifs [19]. Also, the epitope to be recognized may comprise more than six amino acids. However, the most likely reason is that the phage epitope system works efficiently for linear epitopes only. Since both antibodies do not react in Western blotting, but do react in ELISA and immunoprecipitation assays, it is likely that the epitope recognized by OV-TL3 and OV-TL16 is not linear. Therefore, the hexapeptide sequences of the selected phages will merely mimic the structure of the genuine (conformational) epitope, or represent better binding sequences than the genuine epitope. These results and the finding that the antibodies are able to compete for the epitope, make it plausible that the two antibodies recognize the same epitope.

The idiotopes of both antibodies were found to be different, since affinity-purified polyclonal anti-idiotypic antibodies to OV-TL3, which were depleted from anti-isotypic/allotypic antibodies, as well as anti-idiotypic MoAb JL1, did not react with the idiotope of OV-TL16. This was corroborated by molecular cloning of the variable regions of the antibodies. The sequences of the V_H regions of the antibodies differ extensively, and may originate from different germ-line genes, but the V_L regions are homologous with only one amino acid substitution in CDR2 and two conservative amino acid substitutions in FR1. The latter may be the result of the use of degenerate primers for PCR amplification, since this region of the V_L domain is encoded by the VL1 oligonucleotide. The considerable difference between the V_HCDR seems to indicate that the structures of the antigen-binding regions of OV-TL3 and OV-TL16 are quite different. Nevertheless, the two antibodies with different heavy chain variable regions appear to bind to identical epitopes. Previously other antibodies have been described to bind to identical or very closely related epitopes, despite differences in their primary structure. For example, antibodies against $\alpha(1 \rightarrow 6)$ dextrans use different V_H and V_L genes to form combining sites specific for the internal linear sequence [27]. Diverse V_H and V_L genes can be used to produce antibodies reactive with a defined protein epitope [28]. Also, two structurally different antibodies have been characterized that are specific for an epitope of the influenza virus haemagglutinin protein different for only one amino acid [29]. Although the epitope in this case was very similar, the antibodies were shown to be structurally very different.

As can be deduced from published data, differences in amino acid sequence of the variable domains of antibodies do not necessarily exclude recognition of the same epitope.

Computer modelling of the 3-D structure offers an alternative approach to determine the structures involved in epitope recognition. The program applied in this study uses a database of 3-D structured data of a number of antibodies. These analyses revealed that the spatial conformation of the framework regions of the molecules is well conserved, and modelling can concentrate on the CDR. The two antibodies under investigation have remarkable similarities in their 3-D structures. Not only are five of the six CDR identical (V_L) or nearly identical (V_H), but their position in the structural conformation is basically the same. The loops formed for the V_HCDR3 of the two antibodies have different orientations. This would suggest that the CDR3s are not involved in antibody–antigen interaction. This seems to be remarkable, since the antigen binding specificity is supposed to be largely determined by the CDR3 of the heavy chain variable region, which generally is the most variable region of the antibody. However, recently conformational changes have been reported that occur during antibody–antigen interaction [30]. Such a change, a flip-over, of the OV-TL3 CDR3 would bring this CDR3 in a position similar to the OV-TL16 CDR3. The modelling analysis indicates that the monoclonal JL1 is directed against the V_HCDR3, since it is specific for OV-TL3. If this is the case, then the inhibited binding of OV-TL3 to OVCAR-3 cells after interaction with JL1 suggests not only an involvement of this CDR3 in the binding of the epitope, but also a conformational change of the CDR during interaction with the antigen.

Thus, the conformational analysis of the variable regions of OV-TL3 and OV-TL16 provides arguments that indicate similarities of the antigen-binding regions. The 'mimitopes' represented by the peptide epitopes of the selected phages appear to favour conformational similarity, as do the results of the computer modelling study. On the other hand, amino acid sequences differ considerably, and anti-idiotypic antibodies to OV-TL3 do not react with OV-TL16.

For ovarian cancer management the use of two structurally different antibodies recognizing identical epitopic regions on the same antigen may be of use. After administration of (fragments of) mouse immunoglobulins to cancer patients for immunoscintigraphical or -therapeutical reasons often a human anti-mouse antibody (HAMA) and an anti-idiotypic antibody response are observed [16]. This has a great influence on the tumour binding efficiency, since after subsequent injection this MoAb will rapidly be removed from circulation. The use of two structurally different antibodies with identical binding characteristics may therefore circumvent this problem and greatly improve the applicability of these MoAbs for immunoscintigraphy and immunotherapy.

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REFERENCES

1 Campbell IG, Freemont PS, Foulkes W, Trowsdale J. An ovarian tumor marker with homology to vaccinia virus contains an IgV-like region and multiple transmembrane domains. Cancer Res 1992; **52**:5416–20.

2 Boerman OC, Massuger L, Makkink K, Kenemans P, Poels L. Comparative *in vitro* binding characteristics and biodistribution in tumor-bearing athymic mice of anti-ovarian carcinoma antibodies. Anticancer Res 1991; **10**:1289–96.

3 Poels L, Peters D, van Megen Y *et al.* Monoclonal antibodies against ovarian tumor-associated antigens. J Natl Cancer Inst 1986; **76**:781–91.

4 Van Niekerk C, Ramaekers F, Hanselaar A, Aldeweireldt J, Poels L. Changes in the expression of differentiation markers between normal ovarian cells and derived tumors. Am J Pathol 1993; **142**:157–77.

5 Massuger L, Kenemans P, Claessens R *et al.* Immunoscintigraphy of ovarian cancer with indium-111-labelled OV-TL3 F(ab')2 monoclonal antibody. J Nucl Med 1990; **31**:1802–10.

6 Massuger L, Claessens R, Kenemans P *et al.* Kinetics and biodistribution in relation to tumor detection with Indium-111-labelled OV-TL3 (F(ab')2 in patients with ovarian cancer. Nucl Med Comm 1991; **12**:593–609.

7 Poels LG, Boerman OC, Massuger L *et al.* Biodistribution and internalization of monoclonal antibodies to human ovarian carcinoma cells. In: Crommelin J, Schellekens H, eds. Clone to clinic. Dordrecht: Kluwer Academic Publishers, 1990:73–81.

8 Nassander UK, Steerenberg PA, Poppe H, Storm G, Poels LG, De Jong WH, Crommelin DJA. *In vivo* targeting of OV-TL3 immunoliposomes to ascitic ovarian carcinoma cells (OVCAR-3) in athymic nude mice. Cancer Res 1992; **52**:646–53.

9 Boerman OC, Makkink K, Massuger L, Thomas C, Kenemans P, Hanselaar T, Poels L. Monoclonal antibodies against ovarian carcinoma associated antigens, raised by immunization with cyst fluids. Anticancer Res 1991; **9**:551–8.

10 Boerman OC, Makkink WK, Thomas CMG, Hanselaar AGJM, Ydema CA, Kenemans P, Poels LG. Monoclonal antibodies that discriminate between human ovarian carcinomas and benign ovarian tumors. Eur J Cancer 1990; **2**:117–27.

11 Hamilton TC, Young RC, McKoy WM *et al.* Characterization of a human ovarian carcinoma cell line (NIH:OVCAR3) with androgen and estrogen receptors. Cancer Res 1983; **43**:5379–89.

12 Miotti S, Canevari S, Menard S *et al.* Characterization of human ovarian carcinoma-associated antigens defined by novel monoclonal antibodies with tumor-restricted specificity. Int J Cancer 1987; **39**:297–303.

13 Lowry OH, Rosebrough NJ, Farr Al, Randall RJ. Protein measurement with the Folin reagent. J Biol Chem 1951; **193**:265–75.

14 Laemmli EK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; **227**:680–5.

15 Hemmila I, Dahabu S, Muhhala VH, Siitari H, Lovgren T. Europium as a label in time-resolved immunofluorometric assays. Anal Biochem 1983; **137**:335–43.

16 Massuger L, Thomas C, Segers M, Corstens F, Verheijen R, Kenemans P, Poels L. Specific and non-specific immunoassays to detect HAMA after administration of Indium-111-labeled OV-TL3 F(ab')2 monoclonal antibody to patients with ovarian cancer. J Nucl Med 1992; **33**:1958–63.

17 Soini H, Kojola H. Time-resolved fluorometer for lanthanide

chelates—a new generation of non-isotopic immunoassays. Clin Chem 1983; **29**:65–68.

18 Bruccolerie RE, Harber E, Novotney J. Structure of antibody hypervariable loops reproduced by a conformational search algorithm. Nature 1988; **335**:564–8.

19 Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. In: Nolan J, ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989.

20 Kraulis PJ. Molscript; a program to produce both detailed and schematic plots of protein structures. J Appl Crystalog 1991; **24**:946–50.

21 Scott JK, Smith GP. Searching for peptide ligands with an epitope library. Science 1990; **249**:386–90.

22 Parmley SF, Smith GP. Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. Gene 1988; **73**:305–18.

23 Devlin JJ, Panganiban LC, Devlin PE. Random peptide libraries: a source of specific protein binding molecules. Science 1990; **249**:404–6.

24 Christian RB, Zuckerman RN, Kerr JM, Wang L, Malcolm BA. Simplified methods for construction, assessment and rapid screening of peptide libraries in bacteriophage. J Mol Biol 1992; **227**:711–8.

25 Stephen CW, Lane DP. Mutant conformation of p53. Precise epitope mapping using a filamentous phage epitope library. J Mol Biol 1992; **225**:577–83.

26 Bottger V, Lane EB. A monoclonal antibody epitope on keratin 8 identified using a phage peptide library. J Mol Biol 1994; **235**:61–67.

27 Akolkar NP, Sikder SK, Bhattacharya SB, Liao J, Gruezo F, Morrison SL, Kabat EA. Different V_H and V_L germ line genes are used to produce similar combining sites with specificity for α(1-6)dextrans. J Immunol 1987; **138**:4472–9.

28 Andria ML, Levy S, Benjamini E. Diverse V_H and V_L genes are used to produce antibodies against a defined protein epitope. J Immunol 1990; **144**:2614–9.

29 Stark S, Caton AJ. Antibodies that are specific for a single amino acid interchange in a protein epitope use structurally distinct variable regions. J Exp Med 1991; **174**:613–24.

30 Rini JM, Schulze-Gahmen U, Wilson IA. Structural evidence for induced fit as a mechanism for antibody–antigen recognition. Science 1992; **255**:959–65.

31 Kabat EA, Wu TT, Reid-Miller M, Perry HM, Gottesman KS. Sequences of proteins of immunological interest. 4th edn. Bethesda: US Dept. of Health and Human Services, NIH, 1987.