Eur J Cancer, Vol. 26, No. 2, pp. 117–127, 1990.
Printed in Great Britain

0277-5379/90$3.00 + 0.00

Monoclonal Antibodies that Discriminate Between Human Ovarian Carcinomas and Benign Ovarian Tumours

O.C. BOERMAN,* W.K. MAKKINK,* C.M.G. THOMAS,† A.G.J.M. HANSELAAR,‡ C.A. YEDEMA,§ P. KENEMANS§ and L.G. POELS*

*Departments of *Cell Biology and Histology, †Obstetrics and Gynaecology, ‡Pathology, University of Nijmegen and §Department of Obstetrics and Gynaecology of the Free University of Amsterdam, The Netherlands*

Abstract—*Three hybridoma cell lines producing monoclonal antibodies (MAbs) against ovarian carcinomas were obtained after immunizing mice with an undifferentiated human ovarian cystadenocarcinoma extract. The hybridoma cell supernatants were initially screened for a positive immunohistochemical reaction with ovarian carcinomas concomitant with a negative reactivity with a benign ovarian cystadenoma.*

The antibodies OV-TL 15 (IgM), OV-TL 30 (IgG1) and OV-TL 31 (IgM) reacted positively with 84, 84 and 74% of the ovarian carcinoma samples ($n = 76$) respectively. Reactivity with ovarian cysts and cystadenomas ($n = 21$), with non-ovarian carcinomas ($n = 77$) and with normal human tissue samples ($n = 63$) was absent or limited to weak reactivity on incidental samples.

All three antibodies were shown by immunoelectron microscopy to react with surface antigens (OA 15, OA 30 and OA 31) of ovarian carcinoma cells. Cross reactivity between OV-TL 15, OV-TL 30, OV-TL 31 and OC 125 monoclonal antibodies was excluded by competitive binding assays on NIH:OVCAR-3 cells.

Antigen levels (OA 15 and OA 31) in tumour extracts and cyst fluids were quantified by immunoradiometric assays and compared to the CA 125 antigen levels. High levels of CA 125, OA 15 and OA 31 were found in cyst fluids from ovarian cancers. In benign ovarian cyst fluids, however, the CA 125 content was also high while OA 15 antigen was hardly detectable. The OA 31 antigen was present at relatively low levels.

The IRMA data confirmed the immunohistochemical data showing that, in contrast to OC 125, the newly developed MAbs OV-TL 15, OV-TL 30 and OV-TL 31 discriminate between benign ovarian cystadenomas and malignant ovarian cancers.

INTRODUCTION

MONOCLONAL ANTIBODIES (MAbs) against ovarian carcinoma-associated antigens (OCAAs) have been developed in several laboratories [1–6], and have been shown to be useful in the discrimination between ovarian and non-ovarian carcinomas [2, 7, 8]. A few antibodies may be useful for assessing the histologic type (mucinous versus other common epithelial types) of ovarian carcinomas [9, 10].

The biological behaviour of ovarian tumours may range from benign to extremely malignant with a number of steps in between. Antibodies described so far are directed against antigens expressed in benign as well as in malignant ovarian tumours. OC 125 has been reported to stain 45% of the benign serous ovarian tumours positively [11], MOv 18 stained 100% [3] and OV 632 stained 56% [4] of the benign serous ovarian tumours.

MAbs against antigens expressed exclusively in malignant ovarian tumours may facilitate an accurate discrimination between benign, borderline malignant and malignant ovarian tumours. In this study, we report the development of MAbs reacting positively and almost exclusively with malignant ovarian cancer cells.

MATERIALS AND METHODS

Tissues, cyst fluids, cell lines and tumour extracts

Human tissue samples were obtained freshly from surgery, snap-frozen in liquid nitrogen and stored at −80°C. Cyst fluids from ovarian carcinomas ($n = 13$) and from benign ovarian tumours ($n = 32$)

Accepted 5 October 1989.
Correspondence to Dr L.G. Poels, Department of Cell Biology and Histology, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

were obtained by thin needle aspiration and centrifuged at 3500 ***g*** (10 min, 4°C). The supernatants were stored at −80°C.

The NIH:OVCAR-3 ovarian carcinoma cell line [12] obtained from the American Type Culture Collection, was grown in RPMI-1640 medium (Gibco, Paisley, U.K.) supplemented with 10% FCS (foetal calf serum, Flow Labs, Herts, U.K.).

Tumour extracts were prepared by homogenizing 0.1 g tissue/ml PBS containing 5 mM EDTA (ethylenediaminetetraacetate, E. Merck, Darmstadt, F.R.G.), 1 mM PMSF (phenylmethylsulphonylfluoride, Sigma, St. Louis, MO) in a glass homogenizer with a Teflon pestle. After centrifuging the crude homogenate (20,000 ***g*** for 30 min at 4°C) the supernatants were stored in small aliquots at −80°C until use.

Production of anti-ovarian carcinoma monoclonal antibodies

BALB/c mice were immunized by four intraperitoneal injections given at 1 month intervals, with 0.5 ml tumour extract prepared from an undifferentiated ovarian carcinoma (code No. 86-753). Four days after the final injection, 5×10^7 immune spleen cells were fused with 2×10^7 myeloma cells (Sp2/0 Ag-14), using polyethylene glycol 4000 (E. Merck, Darmstadt, F.R.G.) as fusogen, as described [13, 14]. The hybridoma supernatants were initially screened using an indirect immunofluorescence assay (IFA) on frozen sections of the same ovarian carcinoma as used for immunization, as described [2]. The supernatants reacting positively with the carcinoma cells in these sections were further selected for a negative reaction on sections of a serious ovarian cystadenoma. The hybridomas were recloned by repeated limiting dilution.

Three hybridoma cell lines (OV-TL 15, OV-TL 30 and OV-TL 31), were selected for further characterization. The hybridomas were grown in pristane-primed BALB/c mice for production of ascites containing antibodies according to standard procedures.

The MAbs were isotyped in a double diffusion assay [15]. Solubilized tumour proteins were subjected to SDS-gel electrophoresis, Western blotting and immunodetection using MAbs as described [16], with minor modifications.

Immunohistochemistry

Reactivity of MAbs on unfixed cryostat sections of human tissue samples was tested both in immunofluorescence (IFA) [2] and in immunoperoxidase assays (IPO) [17], using undiluted hybridoma supernatant or optimally diluted hybridoma-induced mouse ascitic fluid. A broad spectrum anti-keratin MAb, OV-TL 12-5 [18, 19], staining all epithelial cells in a section was used as a positive control. The IFA and IPO staining were scored arbitrarily: (+) for clearly positive, (±) for weakly positive and (−) for negative staining.

Immunoelectron microscopy

A freshly obtained sample from a serous ovarian carcinoma was fixed in periodate–lysine–paraformaldehyde–glutaraldehyde solution (PLPG), for 1½ h [20] and further processed for electron microscopy as described previously [21].

Radioiodination of the antibodies

The IgM-isotype MAbs OV-TL 15 and OV-TL 31 were purified by 45% ammoniumsulphate precipitation from hybridoma-induced ascitic fluid [17], followed by gel filtration on a G-200 column (Pharmacia, Uppsala, Sweden) [22]. The IgG1 isotype MAb OV-TL 30 was purified by affinity chromatography on a protein A–Sepharose column (Pharmacia, Uppsala, Sweden) [23].

Purified MAbs were radioiodinated by the chloramine-T procedure [24] followed by chromatography on Sephadex G-25 (Pharmacia, Uppsala, Sweden) resulting in a specific radioactivity of 10–15 Ci/g.

Competitive binding assay

NIH:OVCAR-3 cells were grown in eight-well strip plates (Costar, Cambridge, MA) until 90% confluency. After the cells were fixed in 0.25% glutaraldehyde (E. Merck, Darmstadt, F.R.G.) (10 min, 20°C) the wells were washed and saturated (1 h, 20°C) with 0.5% BSA (bovine serum albumin, Sigma, St Louis, MO) in PBS. After washing, the cells were incubated with 50 µl of a fixed amount of ^{125}I-labelled MAb (50 ng/ml) in the presence of various dilutions of the unlabelled MAbs OV-TL 15, OV-TL 30, OV-TL 31, as well as OC 125 [25], for 4 h at 20°C. From the displacement of the labelled antibodies by the corresponding unlabelled antibody, the affinity constants of the antibodies were calculated as the reciprocal of the molar concentration of unlabelled MAb required for 50% displacement [26].

Immunoradiometric assays

Two-step IRMAs were developed essentially as described previously for an OC 125-based immunofluorometric assay [27]. The wells of 12-well EIA/RIA strips (Dynatech, Billingshurst, U.K.) were coated by overnight incubation with 200 µl purified, unlabelled MAb (1–25 µg/ml in PBS). Wells were saturated with 250 µl 0.5% BSA (Sigma, St. Louis, MO) in PBS for 2 h and subsequently incubated for 4 h with 100 µl tumour extract or cyst fluid and 100 µl assay buffer (50 mM Tris–HCl, pH 7.75, 150 mM NaCl, 0.5% BSA). ^{125}I-labelled MAb in 200 µl assay buffer (50 ng/ml) was incubated in each well for 2 h. All incubations were carried out at room temperature and after each incubation the wells were washed four times with PBS containing 0.05% Tween-20 (E. Merck, Darmstadt, F.R.G.).

Finally, the bound portion of the tracer was determined in a gamma counter. Serial dilutions of an ovarian carcinoma extract (code No. 86-753) were used as standard preparations in each assay, and equivalent antigen (OA 15 and OA 31) concentrations for experimental samples were determined by interpolation of the standard curve run on each assay plate using the RIA-Calc software package (Pharmacia-Wallac Oy, Turku, Finland) on an IBM-XT compatible personal computer. The OA 15 and OA 31 contents of the 86-753 ovarian carcinoma extract were calibrated as 100 arbitrary units per ml (100 arb. U/ml). As a rule the standard error of triplicates (intra-assay precision) remained within 10% of the mean, while the inter-assay precision for the same samples on five different occasions remained within 15% of the mean. Wells retaining a radioactivity less than the 99% upper confidence limit of the control (assay buffer) were considered below the limit of the assay sensitivity.

To investigate whether the epitopes defined by OV-TL 15, OV-TL 30, OV-TL 31 and OC 125 are located on the same molecule we performed double determinant IRMAs. In these assays the antigen preparation (ovarian carcinoma extract or cyst fluid) was incubated in wells coated with one antibody and a second, different antibody was used as a tracer.

CA 125 determinations

Levels of CA 125 in cyst fluids were assayed in an OC 125-based IRMA as described by Klug *et al.* [28]. To avoid the high-dose 'hook effect' [29], a two-step incubation protocol was applied. For all samples multiple dilutions up to 1/10,000 were assayed. Dilutions were made using a standard serum diluent. The reagents were obtained from Centocor Inc. (Malvern, PA).

Statistical methods

As antigen levels in cyst fluids were not normally distributed, non-parametric statistical methods were used. A Mann–Whitney *U*-test was used for comparison of antigen levels in benign and malignant ovarian tumour cyst fluids.

RESULTS

Characterization of three anti-ovarian carcinoma monoclonal antibodies.

Based on the strategy of immunization with an extract of an undifferentiated ovarian carcinoma, followed by a differential screening for a positive reaction on the same carcinoma concomitant with a negative reaction on sections of an ovarian cystadenoma, three hybridoma cell lines were selected. Radial immunodiffusion analysis indicated that the hybridomas OV-TL 15 and OV-TL 31 produced IgM antibodies, while OV-TL 30 produced IgG1 antibodies.

The immunoreactivity of the tumour antigens (OA 15, OA 30 and OA 31) defined by the three monoclonal antibodies OV-TL 15, OV-TL 30 and OV-TL 31 respectively, was not preserved after SDS-gel electrophoresis followed by immunoblotting. The immune reactivity of these antibodies was lost on paraffin processed tissue blocks, but resisted cold methanol as well as PLPG fixation.

Immunohistochemical reactivity of ovarian tumours

The immunohistochemical reactivity of the three monoclonal antibodies on ovarian tumours was studied in combination with the reference MAb OC 125. A broad spectrum anti-keratin monoclonal antibody, OV-TL 12-5, was applied in order to stain all epithelial cells in the tissue sections. The results as summarized in Table 1 show that OV-TL 15, OV-TL 30 and OV-TL 31 hardly reacted

Table 1. Immunohistochemical reactivity of anti-ovarian carcinoma monoclonal antibodies OV-TL 15, OV-TL 30, OV-TL 31 and OC 125 with ovarian tumours

Tumours	OV-TL 15	OV-TL 30	OV-TL 31	OC 125
Cystoma simplex	0/8*	0/8	0/8	6/8
Cystadenomas	3/14†	3/14†	3/14†	10/14
Borderline tumours	2/4†	3/4†	2/4†	3/4
Serous carcinomas	37/42 (88%)	39/42 (93%)	35/42 (83%)	41/42 (98%)
Mucinous carcinomas	3/5	3/5	2/5	2/5
Endometrioid carcinomas	6/8	6/8	5/8	5/8
Clear cell carcinomas	5/6	4/6	4/6	5/6
Non-classified adenocarcinomas	13/15	12/15	10/15	15/15
Total carcinomas	64/76 (84%)	64/76 (84%)	56/76 (74%)	68/76 (89%)
Granulosa cell tumours	0/2	0/2	0/2	0/2
Fibrothecomas	0/4	0/4	0/4	0/4

*The number of positively reacting samples per total number of samples.
†Weak immunoreactivity was found on the indicated number of samples.
All specimens were assayed by both immunofluorescence and the immunoperoxidase assays.

with benign ovarian tumours. Figure 1A shows an example of OV-TL 15 staining negatively a serous ovarian cystadenoma. Weak and heterogeneous staining was seen with these antibodies in only three cystadenoma samples, while OC 125 (Fig. 1B) stained the majority of benign ovarian tumours clearly positively.

A few of the samples of the borderline tumours were stained focally (10–25% of the epithelial cells in a section) and heterogeneously. However, in these samples both the obviously proliferating areas as well as the areas showing no atypical features displayed immune reactivity.

OV-TL 15 reacted with 88% of the 42 serous ovarian cancers and with 84% of the 76 ovarian carcinoma samples tested. On the same samples OV-TL 30 reacted with respectively 93 and 84% and OV-TL 31 with 83 and 74%, while OC 125 stained 98% of the serous ovarian cancers and 89% of all ovarian carcinomas examined. In most samples OV-TL 15 showed a homogeneous, diffuse positive staining pattern (Fig. 2D), OV-TL 30 usually displayed a heterogeneous pattern (Fig. 2E), while OV-TL 31 reactivity was confined to the apical side of the tumour cells (Fig. 2F).

Immunoelectron microscopy showed that the three antibodies reacted with the tumour cell membrane (Fig. 3). OV-TL 15, however, also showed some submembranous cytoplasmic staining (Fig. 3B), indicating the OA 15 antigen is not exclusively localized in the cell membrane.

Immunohistochemical reactivity of non-ovarian carcinomas

With regard to the gynaecologic carcinomas (Table 2), all three antibodies positively stained both carcinomas of the Fallopian tube examined, while OV-TL 15 and OV-TL 30 positively stained three of the 13 endometrial carcinomas tested. OV-TL 31 did not react with either endometrial carcinomas or cervical carcinomas.

The OV-TL 15 and OV-TL 31 antibodies did not show any reactivity with any of the non-gynaecologic tumours ($n = 48$), including breast ($n = 10$) and gastro-intestinal carcinomas ($n = 21$) (Table 2). OV-TL 30 reacted with some renal cell carcinomas (five out of nine specimens) and breast carcinomas (three out of 10 specimens).

Immunoreactivity with normal human tissues

Staining sections of 64 samples of different types of normal human tissues with the three antibodies indicated that the MAb-defined antigens were hardly expressed in normal human tissues (Table 3). Sections of ovarian tissue clearly showed the antibodies to be non-reactive with the germinal epithelium. Mesothelial linings in sections of three non-ovarian samples also stained negatively. OV-TL 15 displayed weak positive reactivity with the apical side of oviductal epithelium and traces of reactivity were observed on some renal tubules. OV-TL 30 stained faintly positive the glomerular mesangial cells (two out of six samples), while tubules remained negatively. OV-TL 31 stained positively with less than 10% of the renal tubules, probably the Henle loops.

Competitive binding assay

We investigated whether the MAbs compete for the binding of identical epitopes on the ovarian carcinoma cell line NIH:OVCAR-3. The binding of ^{125}I-labelled MAbs was measured in the presence of varying concentrations of unlabelled antibodies.

Table 2. Immunohistochemical reactivity of anti-ovarian carcinoma monoclonal antibodies OV-TL 15, OV-TL 30 and OV-TL 31 with non-ovarian tumours

Tumours	OV-TL 15	OV-TL 30	OV-TL 31	OC 125
Gynaecologic carcinomas				
Tubal carcinomas	2/2*	2/2	2/2	2/2
Endometrial carcinomas	3/13	3/13	0/13	5/13
Cervical carcinomas				
Adenocarcinomas	2/4	1/4	0/4	2/4
Squamous cell carcinomas	0/4	2/4	0/4	0/4
Non-gynaecologic carcinomas				
Breast carcinomas	0/10	3/10	0/10	2/10
Renal cell carcinomas	0/9	5/9	0/9	0/9
Colon cancers	0/12	0/12	0/12	1/12
Other carcinomas (stomach, ileum, pancreas, bladder, rectum, a.o.)	0/17	0/17	0/17	1/17†
Non-epithelial tumours (seminomas, melanomas, lymphomas)	0/8	0/8	0/8	0/8

*The number of positively reacting samples per total number of samples.

†1/2 positively reacting pancreas carcinoma.

All specimens were assayed using both an immunofluorescence and an immunoperoxidase assay.

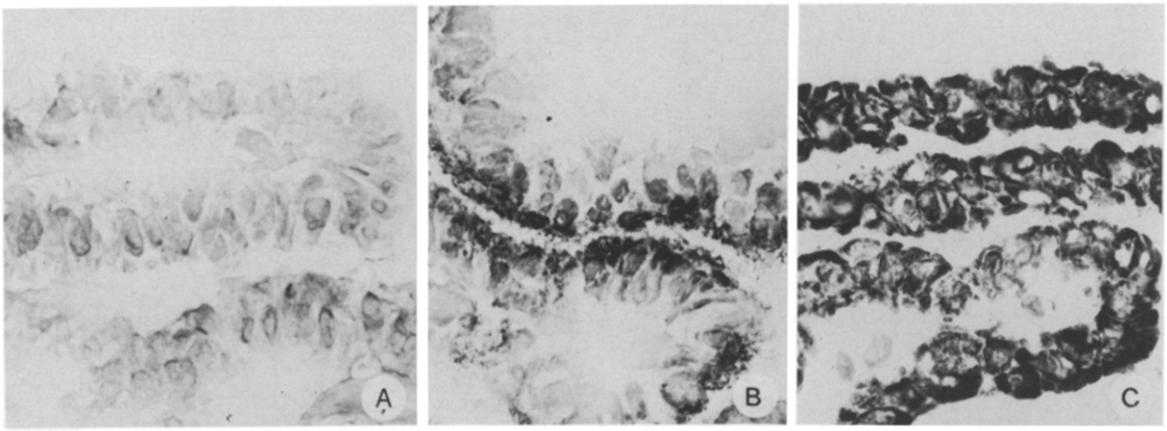

Fig. 1. *Immunohistochemical staining pattern (IPO) of a serous ovarian cystadenoma* (188×) *stained with OV-TL* 15 (*A*) *showing no immune reactivity, OC* 125 (*B*) *and OV-TL* 12-5 (*C*).

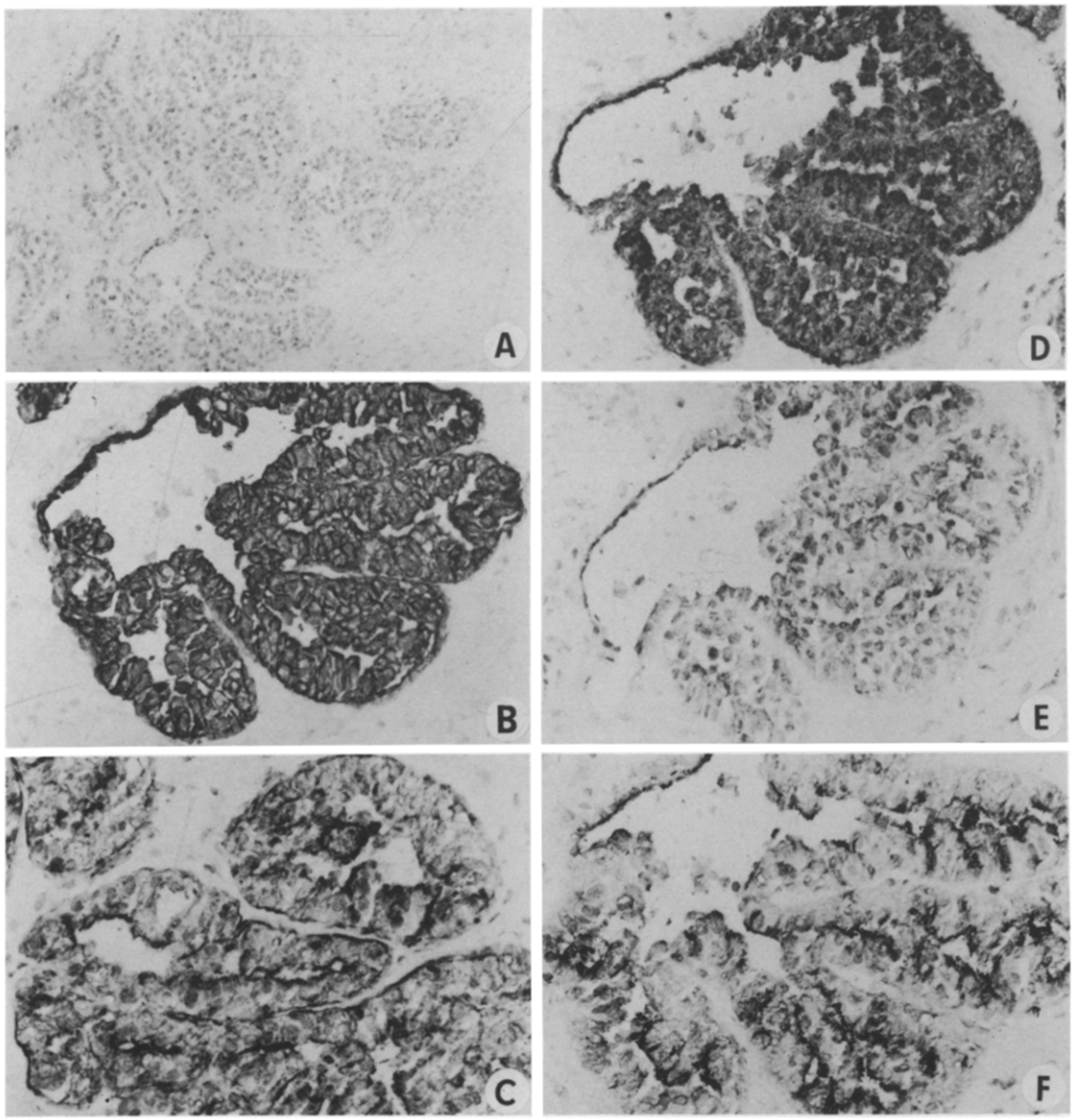

Fig. 2. *Immunohistochemical staining (IPO) of serial sections of a serous ovarian carcinoma* (188×), *stained with OV-TL* 12-5 (*B*), *OC* 125 (*C*), *OV-TL* 15 (*D*), *OV-TL* 30 (*E*) *and OV-TL* 31 (*F*). *Sections were counterstained with haematoxylin. A negative control section* (75×) *is shown in Fig.* 2*A*.

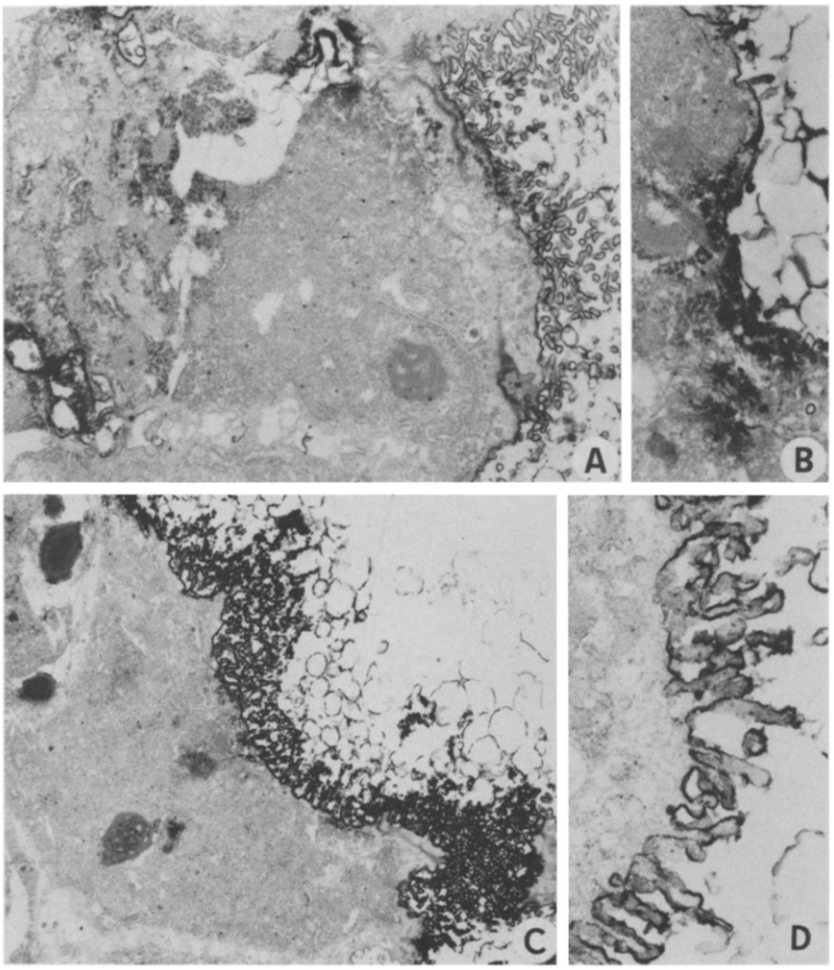

Fig. 3. Immunoelectron micrograph showing immunoperoxidase reaction on a sample of a serous ovarian carcinoma stained with OV-TL 15 (A,B) and OV-TL 31 (C,D) at the tumour cell surface. The microvillous cell membranes are stained clearly positive. Some submembranous cytoplasmic immune reactivity is observed with the OV-TL 15 antibody (Fig. 3B). Magnification A,C (6000×) and B,D (12,000×).

Table 3. Immunohistochemical reactivity of anti-ovarian carcinoma monoclonal antibodies OV-TL 15, OV-TL 30 and OV-TL 31 with normal human tissue

Tissues (number of samples examined)	OV-TL 15	OV-TL 30	OV-TL 31
Mesothelium (3)	—	—	—
Ovary, uterus, cervix, vulva, vagina (18)	—	—	—
Oviductal epithelium (8)	5/8*	—	—
Digestive tract epithelium (9)	—	—	—
Liver, gall bladder, pancreas (4)	—	—	—
Respiratory tract epithelium (3)	—	—	—
Kidney (6),			
glomeruli	—	2/6*	—
tubules	3/6*	—	1/6*
Testis, prostate (3)	—	—	—
Adrenal gland (2)	—	—	—
Spleen, tonsil (3)	—	—	—
Skin (5)	—	—	—

*Weak positive reaction was found on the indicated number of samples per total number of samples. All specimens were assayed using both an immunofluorescence and an immunoperoxidase assay.

*Table 4. Competitive inhibition of binding of radiolabelled MAbs on ovarian carcinoma cells**

^{125}I-MAbs	Unlabelled MAb	Percentage inhibition at 1 μM unlabelled antibody	Affinity constant K_a (M^{-1})
OV-TL 15	OV-TL 15	97	6.8×10^8
	OV-TL 30	0	
	OV-TL 31	0	
	OC 125	0	
OV-TL 30	OV-TL 15	0	
	OV-TL 30	96	16.0×10^8
	OV-TL 31	0	
	OC 125	0	
OV-TL 31	OV-TL 15	0	
	OV-TL 30	0	
	OV-TL 31	92	1.4×10^8
	OC 125	0	

*The binding studies were carried out on NIH:OVCAR-3 cells as described in 'Materials and Methods'. All numbers represent the mean of three different experiments (1 μM IgG = 0.15 g/l; 1 μM IgM = 0.8 g/l).

As shown in Table 4 the binding of labelled antibody could only be inhibited by using the identical unlabelled antibody, indicating that each antibody recognized a unique epitope on the cell surface.

From these displacement studies the affinity constants of the three MAbs were calculated as the reciprocal of the molar concentration of unlabelled MAb required for 50% displacement of the homologous ^{125}I-labelled antibody. The affinity constants of the three antibodies were in the same range, being highest for OV-TL 30 (16×10^8 M^{-1}), and lowest for OV-TL 31 (1.4×10^8 M^{-1}).

Immunoradiometric assays

To investigate whether the MAb-defined antigenic determinants could be quantified in tumour extracts and ovarian cyst fluids, three 'sandwich'-type IRMAs based on the three MAbs (OV-TL 15, OV-TL 30 and OV-TL 31) were developed.

The optimized standard curve of the OA 15 IRMA, using serial dilutions of the 86-753 ovarian carcinoma extract as standards, is shown in Fig. 4A. The curve of a serially diluted cyst fluid was similar to the standard curve, indicating OA 15 is immunochemically similar in both cyst fluid and tumour

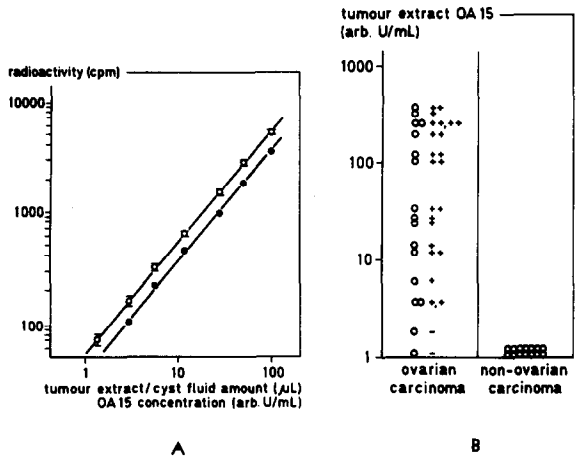

Fig. 4. A: Standard curve of the OA 15 IRMA (●) and the curve of a serially diluted ovarian carcinoma cyst fluid (○). Serial dilutions of an ovarian carcinoma extract were used as standards (error bars represent $\sqrt{X_1 + X_2/2}$*). B: Relative OA 15 levels in tissue extracts of 17 ovarian carcinomas and extracts of 14 non-ovarian carcinomas (four colon, three breast, three renal, two endometrial and two cervical carcinomas). IFA scores of the corresponding samples are indicated: ++ = strong positive IFA reactivity on cryostat sections, + = positive IFA reaction, ± = weak IFA reaction, − = negative IFA reaction.*

extracts. Similar data were obtained with the OV-TL 31-based IRMA (not shown). With the OV-TL 30-based IRMA, wells did not retain significant amounts of radioactivity, either after incubation with ovarian carcinoma extracts or with ovarian carcinoma cyst fluids, suggesting that the OA 30 antigenic determinant was not preserved during the procedure or does not appear as a repetitive epitope on the antigen.

Figure 4B shows that using the OV-TL 15-based IRMA, no significant OA 15 levels could be detected in extracts of a selection of 14 non-ovarian cancers exhibiting no OA 15 expression as determined immunohistochemically, while significantly elevated antigen levels were found in the extracts of ovarian cancers (n = 17). The immunohistochemical reactivity of the MAb OV-TL 15 in sections of the ovarian carcinoma samples was arbitrarily scored on a range from negative (−) to strongly positive (++) and corresponded well to the IRMA data (Fig. 4B). Ovarian carcinomas of different differentiation grade (well, moderately and poorly differentiated), were used to prepare the tumour extracts measured in the IRMA, but no relationship between OA 15 content and differentiation grade as assessed histologically, was observed (data not shown). Analogous results were found for the level of OA 31 antigen in extracts of ovarian carcinoma extracts.

The ovarian carcinoma-associated antigen OA 15 was also detectable in 12 out of 13 cyst fluids from ovarian cystadenocarcinomas, significantly lower levels were found in the fluids from 32 benign ovarian tumours ($P < 0.001$, Mann–Whitney U-test). In fact, only two benign ovarian tumour cyst fluids showed detectable OA 15 levels as shown in Fig. 5A. These findings, again, are in line with the immunohistochemical assays on tissue sections of the corresponding tumour specimens.

Although the OA 31 antigen was not detected immunohistochemically in sections of benign ovarian tumours, relatively low OA 31 levels were found in some cyst fluids from these tumours (n = 27) as determined in the IRMA (Fig. 5B). Significant higher levels, however, were found in the fluids of cystadenocarcinomas (n = 11) ($P < 0.001$, Mann–Whitney U-test). Cyst fluids from only two ovarian carcinomas contained OA 31 concentrations that were in the range of the benign tumour cyst fluids (0–10 arb. U/ml). In these two ovarian carcinoma samples no OA 31 expression could be demonstrated with immunohistochemical staining techniques.

The CA 125 levels in cyst fluids from ovarian carcinomas were not significantly higher than CA 125 levels in benign ovarian tumour cyst fluids, as

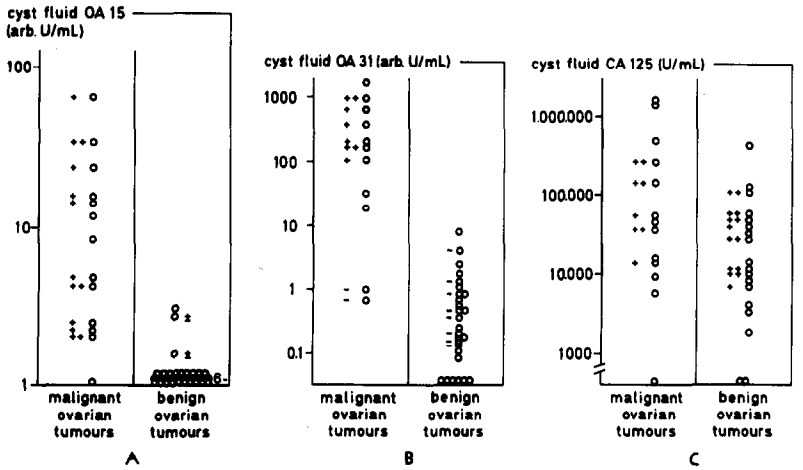

Fig. 5. Relative levels of the ovarian carcinoma-associated antigens OA 15 (A), OA 31 (B) and CA 125 (C) in cyst fluids of malignant and benign ovarian tumours. If a frozen tissue sample of the corresponding tumour was available, the IFA score is indicated: ++ = strongly positive, + = positive, ± = weakly positive, − = negative staining.

determined in Mann–Whitney *U*-test ($P = 0.0911$) (Fig. 5C).

In contrast to the homologous IRMAs, no significant antigen levels could be detected with double determinant IRMAs in tumour extracts in the presence of 10 mM CHAPS (3-[3-cholamideopropyl)-dimethylammonia]-1,1-propanesulfonate, Boehringer, Mannheim, F.R.G.). This indicates that the MAb-defined epitopes (OA 15, OA 30, OA 31 and CA 125) are located on different molecules.

DISCUSSION

In this paper we describe the generation and characterization of three monoclonal antibodies against ovarian carcinoma-associated antigens. The MAbs OV-TL 15, OV-TL 30 and OV-TL 31 were obtained after immunization with an undifferentiated ovarian carcinoma extract, followed by screening of the immunoreactive hybridoma culture supernatants for a negative reactivity on the epithelium of a serous ovarian cystadenoma.

Based on competitive binding assays on NIH: OVCAR-3 cells, the three monoclonal antibodies are directed against different epitopes. No cross reactivity was found with CA 125 as defined by the OC 125 antibody. Experiments using double determinant assays indicated that the epitopes defined by the MAbs used are located on different molecules. The antibodies exhibited differing affinity constants, being highest for OV-TL 30 (16×10^8 M^{-1}). This might be related to the subclass of this antibody (IgG1); the other antibodies belonged to the IgM class.

Monoclonal antibodies against ovarian carcinoma-associated antigens described until now, such as: OC 125 [7, 25], MOv1, MOv2 [1], MT179, MW162, MW207, MX35 [5], MOv 18, MOv 19 [3] and OV 632 [4, 7] have all been reported to react with most benign ovarian tumours as well. The ovarian carcinoma-associated antigens defined by these antibodies are expressed in endometrial and endocervical epithelium and are thought to be antigens expressed in the Müllerian duct, the embryologic origin of the epithelia of the ovaries, the Fallopian tubes and the endocervix. In contrast, the MAbs described here are directed against antigens apparently not expressed on endometrial and cervical epithelium, and thus may be non-Müllerian duct-related antigens. This may explain the observation that these anti-ovarian carcinoma antibodies (OV-TL 15, OV-TL 30 and OV-TL 31) are non-reactive with benign ovarian tumours.

It has been suggested that ovarian carcinogenesis starts with inclusion cyst formation from the germinal epithelium of the ovary. Ovarian cystadenomas arising from these cysts are thought to give rise to ovarian carcinomas [30]. According to this concept, OA 15, OA 30 and OA 31 expression is triggered during malignant transformation.

The negative or low immunohistochemical reactivity of the MAbs OV-TL 15, OV-TL 31 wih benign ovarian tumours was supported by IRMA determinations in cyst fluids from a larger series of ovarian tumours: high concentrations of OA 15 and OA 31 were found in ovarian carcinoma cyst fluids, but the antigens were absent or found at low levels in cyst fluids from benign ovarian lesions. Although no OA 31 antigen was found by immunohistochemical methods in the tissue sections of the benign ovarian tumours, the antigen was detectable in the cyst fluid of the corresponding samples. This might be due to accumulation of shed antigen in cyst fluids. CA 125 determinations in cyst fluids of benign and malignant ovarian tumours revealed comparable CA 125 levels, being in line with the data reported by Fleuren *et al.* [31]. They proposed the basal membrane in benign ovarian tumours to be an effective barrier for CA 125, explaining the observation that, in spite of the high CA 125 contents of cyst fluids from benign tumours, elevated CA 125 serum levels are observed only occasionally in patients with benign ovarian lesions [32].

The OV-TL 15 and OV-TL 31-based IRMA, facilitated quantitative determination of the OA 15 and OA 31 contents of ovarian carcinoma specimens. High levels were found in ovarian carcinomas, while the antigens were hardly expressed in benign ovarian tumours, thus suggesting that the expression levels of the MAb-defined antigens are related to the biological behaviour of the tumour. However, a clear relationship between the differentiation grade of ovarian carcinomas as determined histologically, and their OA 15 or OA 31 content as determined immunoradiometrically, was not observed. Further studies are required to determine whether these antibodies can be used to assess the malignant potential of ovarian tumours.

The antibodies OV-TL 15 and OV-TL 31 showed no reactivity with breast and colon carcinomas together representing the majority of adenocarcinomas metastatic to the ovary. Therefore, these antibodies are considered to be useful as additional tools for differential diagnosis between ovarian cancers and other cancers.

By immunoelectron microscopy it was shown that the antigens OA 15, OA 30 and OA 31 were asssociated with the membrane of the tumour cells. In addition, the antigenic determinants OA 15 and OA 30 could not be traced in serum from ovarian carcinoma patients (data not shown). It is thought that tumour-associated antigens located in the cell membrane and not detectable in serum are suitable targets for in vivo application of antibodies. Preliminary biodistribution studies of OV-TL 30 in tumour-bearing athymic mice support this suggestion.

Preliminary studies indicate that the OA 31 epitope is detectable in serum of ovarian cancer patients.

In summary, we have generated three new monoclonal antibodies against ovarian carcinoma-associated antigens which may be useful adjuncts in differential diagnosis of ovarian tumours.

Acknowledgements—We are indebted to Mr M.F.G. Segers for his cooperation in the IRMA assays; to Dr W.B.K.N.V. de Goey, Dr P.M. Arkenbout (Canisius Wilhelmina Hospital, Nijmegen) and Dr F.J.J.M. van de Molengraft (Medical Centre Alkmaar, The Netherlands) for their generous cooperation. We are grateful to Dr V.R. Zurawski, Jr (Harvard Medical School, Boston, MA) for providing the OC 125 antibodies. We thank Dr P.H.K. Jap and Dr D. Iles for critically reading the manuscript. This work was supported by a grant from the Dutch Praeventiefonds (no. 28-1248).

REFERENCES

1. Taglibue E, Ménard S, Della Torre G *et al.* Generation of monoclonal antibodies reacting with human epithelial ovarian cancer. *Cancer Res* 1985, **45**, 379–385.
2. Poels LG, Peters D, van Megen Y *et al.* Monoclonal antibodies against human ovarian tumor-associated antigens. *J Natl Cancer Inst* 1986, **76**, 781–791.
3. Miotti S, Canevari S, Ménard 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.
4. Fleuren GJ, Coerkamp EG, Nap M, van de Broek LJCM, Warnaar SO. Immunohistological characterization of a monoclonal antibody (OV 632) against epithelial ovarian carcinomas. *Virchows Arch A* 1987, **410**, 481–486.
5. Mattes MJ, Look K, Furukawa K *et al.* Mouse monoclonal antibodies to human epithelial differentiation antigens expressed on the surface of ovarian carcinoma ascites cells. *Cancer Res* 1987, **47**, 6741–6750.
6. Boerman O, Makkink K, Massuger L *et al.* Monoclonal antibodies against ovarian carcinoma-associated antigens, raised by immunization with cyst fluids. *Anticancer Res* 1989, **9**, 551–558.
7. Koelma IA, Nap M, van Steenis GJ, Fleuren GJ. Tumor markers for ovarian cancer: a comparative immunohistochemical and immunocytochemical study of two commercial monoclonal antibodies (OV 632 and OC 125). *Am J Clin Pathol* 1988, **90**, 391–396.
8. Nouwen EJ, Hendrix PG, Dauwe S, Eerdekens MW, De Broe ME. Tumor markers in the human ovary and its neoplasms: a comparative immunohistochemical study. *Am J Pathol* 1987, **126**, 230–242.
9. Tsuji Y, Suzaki T, Mishiura H, Takemura T, Isojima S. Identification of two different surface epitopes of human ovarian epithelial carcinomas by monoclonal antibodies. *Cancer Res* 1985, **45**, 2358–2362.
10. Sakakibara K, Ueda R, Ohta M, Nakashima N, Tomoda Y, Takahashi T. Three novel mouse monoclonal antibodies, OM-A, OM-B and OM-C, reactive with mucinous type ovarian tumors. *Cancer Res* 1988, **48**, 4639–4645.
11. Kabawat S, Bast RC, Welch WR, Knapp RC, Colvin RB. Immunopathologic characterization of a monoclonal antibody that recognizes common surface antigens of human ovarian tumors of serous, endometrioid, and clear cell types. *Am J Clin Pathol* 1983, **79**, 98–104.
12. Hamilton TC, Young RC, McKoy WM *et al.* Characterization of a human ovarian carcinoma cell line (NIH:OVCAR-3) with androgen and estrogen receptors. *Cancer Res* 1983, **43**, 5379–5389.
13. Fazekas St. Groth S, Scheidegger D. Production of monoclonal antibodies: strategy and tactics. *J Immunol Meth* 1980, **35**, 1–21.
14. Lane RD. A short-duration polyethylene glycol fusion technique for increasing production of monoclonal antibody-secreting hybridomas. *J Immunol Meth* 1985, **81**, 223–228.
15. Hudson L, Hay FC. *Practical Immunology*. Oxford, Blackwell Scientific, 1980, 1–7.
16. Towbin H, Stahelin I, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Nat Acad Sci USA* 1979, **76**, 4350–4354.
17. Vandesande F. A critical review of immunocytochemical methods for light microscopy. *J Neurosci Meth* 1979, **1**, 3.
18. Poels LG, Jap PHK, Ramaekers FCS *et al.* Characterization of a hormone-producing ovarian carcinoma cell line. *Gynecol Oncol* 1989, **32**, 203–214.
19. Van de Molengraft F, Poels LG, Van Niekerk CC, Mungyer G, Steerenberg P, Jap PHK. Changing tumour antigen expression in metastatic hepatocellular carcinoma cells of the guinea pig. *Virchows Archiv Cell Pathol* 1989, **56**, 211–219.
20. Gendelman HE, Moench TR, Narayen O, Griffin DE. A fixative for identifying T cell subsets, B cells and macrophages in paraffin embedded mouse spleen. *J Immunol Meth* 1983, **65**, 137–145.
21. Van Niekerk CC, Poels LG, Jap PHK *et al.* Characterization of a human ovarian carcinoma cell line OTN 14 derived from a mucinous cystadenocarcinoma. *Int J Cancer* 1988, **42**, 104–108.
22. Parham P, Androlewicz MJ, Brodsky FM, Holmes NJ, Ways JP. Monoclonal antibodies: purification, fragmentation and application to structural and functional studies of class I MHC antigens. *J Immunol Meth* 1982, **53**, 133–173.

23. Ey PL, Prowse SJ, Jenkins CR. Isolation of pure IgG_1, IgG_{2a} and IgG_{2b} immunoglobulins from mouse serum using protein A-sepharose. *Immunochemistry* 1978, **15**, 429–436.
24. Greenwood FC, Hunter WH, Glover JS. The preparation of ^{125}I-labelled human growth hormone of high specific radioactivity. *Biochem J* 1963, **89**, 119–123.
25. Bast RC, Feency M, Lazarus H, Nadler LK, Colvin RB, Knapp RC. Reactivity of a monoclonal antibody with human ovarian carcinoma. *J Clin Invest* 1981, **68**, 1331–1337.
26. Giacomini P, Natali P, Ferrone S. Analysis of the interaction between a high molecular weight melanoma-associated antigen and the monoclonal antibodies to three distinct antigenic determinants. *J Immunol* 1985, **135**, 696–702.
27. Boerman OC, Thomas CMG, Segers MFG *et al.* Time-resolved immunofluorometric assay for the ovarian carcinoma-associated antigenic determinant CA 125 in serum. *Clin Chem* 1987, **33**, 2191–2194.
28. Klug TL, Bast RC, Niloff JM, Knapp RC, Zurawski VR. Monoclonal antibody immunoradiometric assay for an antigenic determinant (CA 125) associated with human epithelial ovarian carcinoma. *Cancer Res* 1984, **44**, 1048–1053.
29. Miles LEM, Bilber CP, Eng LF, Lipschitz DA. Properties of two-site immunoradiometric (labeled antibody) assay systems. In: *Radioimmunoassays and Related Procedures in Medicine.* Vienna, International Atomic Energy Agency, 1974, Vol. 1, 149–163.
30. Cramer DW, Welch WR. Determinants of ovarian cancer risk. II. Inferences regarding pathogenesis. *J Natl Cancer Inst* 1983, **71**, 711–721.
31. Fleuren GJ, Nap M, Aalders JG, Trimbos JB, de Bruijn HW. Explanation of the limited correlation between tumor CA 125 content and serum CA 125 antigen levels in patients with ovarian tumors. *Cancer* 1987, **60**, 2437–2442.
32. Einhorn N, Bast RC, Knapp RC, Tjernberg B, Zurawski VR. Preoperative evaluation of serum CA 125 levels in patients with primary epithelial ovarian cancer. *Obstet Gynecol* 1986, **67**, 414–416.