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Folate Binding Protein Distribution in Normal Tissues and Biological Fluids From Ovarian Carcinoma Patients as Detected by the Monoclonal Antibodies MOv18 and MOv19

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Folate-binding proteins (FBP), which are molecules relevant in folate metabolism, are overexpressed in ovarian carcinomas, as detected by the monoclonal antibodies (MAb) MOv18 and MOv19, which recognise two different epitopes of the gp38/FBP. In this paper, features of the FBP such as the distribution on normal tissues and the release in biological fluids of normal and tumour origin have been investigated. Immunohistochemical analyses on frozen sections of normal tissues showed the presence of the gp38/FBP on some epithelia. The reactivity of both the MAb on Fallopian tubes was intense and comparable to that observed on ovary carcinoma sections. The kidney, bronchial glands, alveolar epithelium of the lung, oesophagus, stomach, pancreas, breast and thyroid showed different levels of staining. By MOv18/MOv19 double-determinant immunoradiometric assay (DDIRMA), the gp38/FBP was found in soluble form in ascitic fluid, serum and urine of nude mice in which the human ovary carcinoma cell line IGROV1 grew as ascitic carcinomatosis. In human biological fluids, the gp38/FBP was detected in ascites of 60% of ovarian carcinoma patients, and in 29% of those with other carcinomas, but not in patients with non-epithelial tumours or with other non-tumoral pathologies. The mean serum arbitrary units (a.u.)/ml values of ovary carcinoma patients were significantly different to those of healthy donors or patients with endometriosis ($P < 0.005$ and $P < 0.01$, respectively), but not when compared to the sera of lung carcinoma patients. In addition, the sensitivity of DDIRMA was poor, since only 24% of the ovary carcinoma patients were positive with this assay. When a restricted number of cases selected for the presence of tumour cells in the ascites was examined, the percentage of DDIRMA-positive sera and ascites rose to 41 and 94%, respectively. In the urine, a strong reactivity was observed in the samples of both normal and tumour origin.
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INTRODUCTION

HIGH-AFFINITY folate binding proteins (FBP) are molecules relevant in folate metabolism. They preferentially bind the folate in comparison to reduced folate compounds, and work in the nanomolar range. Two forms have been described: the membrane form, responsible for binding of folate to the cell, and the soluble form, probably derived from the membrane form but still with no clearly defined functions as yet [1–4].

Membrane FBP from different sources, of normal and tumour origin, have been described as proteins of 40 kDa average molecular weight. Glycosylphosphatidylinositol (GPI) anchorage was demonstrated [5] and the gene sequences obtained for several of them [5–8].

A 38–40 kDa GPI-anchored protein [9], originally identified by two monoclonal antibodies (MOv18 and MOv19), which recognise different epitopes on this molecule [10], was found to be overexpressed in ovarian carcinomas [10–12]. Moreover, a soluble form of the protein was detected in the culture medium of different ovary carcinoma cell lines by using the MOv18/MOv19 double-determinant immunoradiometric assay (DDIRMA) [13]. Recently, we obtained molecular evidence [14, 15] that the antigen recognised by MOv18 is a FBP.

In this paper we analysed the distribution of gp38/FBP by immunohistochemistry, detecting MOv18/MOv19 reactivity on cryostatic sections of normal human tissues, and by MOV18/MOV19 DDIRMA, detecting the presence of released soluble protein in various biological fluids of normal and tumour origin. The value of the released protein as an ovarian carcinoma circulating marker was considered.

MATERIALS AND METHODS

Tumour cell lines in vivo

The IGROV1 cell line (Dr. Bénard, Institute G. Roussy, Villejuif, France) was maintained *in vivo* in CD1 nu/nu mice. Five animals received intraperitoneally (i.p.) 2×10^6 viable tumour cells, and tumour growth was monitored for 20–25 days. When the abdomen became visibly stretched due to ascitic formation, the animals were sacrificed and the ascitic liquid removed. Blood and urine were sampled before injection, and then periodically until the animals were killed. All the biological fluids were centrifuged for 10 min at 300 *g* and stored at −30°C.

Monoclonal antibodies (MAb)

The generation and characterisation of MOv18 and MOv19, of IgG1 and IgG2 isotypes, respectively, are reported elsewhere [10]. Purified MOv18 was provided by Centocor (Malvern, Pennsylvannia, U.S.A.). Purification of MOv19 was carried out by affinity chromatography on a Protein A-Sepharose CL 4B column (Pharmacia, Uppsala, Sweden).

The MOv19 MAb was ^{125}I radiolabelled, as described previously [10].

Tissue samples

Normal and tumoral tissues were obtained from surgical specimens from cancer patients. Small blocks of fresh tissue were rapidly frozen in liquid nitrogen and stored at −80°C. Specimens for immunoperoxidase staining were cut into 5 μm slices, fixed for 10 min in cold acetone and air-dried at room temperature.

Biological specimens

Serum was obtained from 51 ovarian carcinoma patients, 31 lung carcinoma patients, 40 patients with endometriosis and 100 healthy donors. Ascitic fluid was collected from 78 ovarian carcinoma patients, 35 patients with other carcinomas, 17 patients with non-epithelial tumours, and 6 patients with non-tumoral pathologies. Urine samples were derived from 39 ovary carcinoma patients and 39 healthy donors. An additional 20 ovarian carcinoma patients were selected for presence of tumour cells in the ascites, as defined by cytological examination, and ascitic fluid and sera were collected.

All samples were centrifuged for 10 min at 300 *g* and stored at −30°C.

Immunoperoxidase technique

The immunoperoxidase technique was performed with the avidin–biotin complex kit (Vector, Burlinghame, California, U.S.A.). Briefly, the sections were rehydrated with 5% fetal calf serum (FCS) in Hank's balanced solution for 15 min at room temperature, and then incubated for 30 min with 300 μl/slide of purified MAbs at 0.7 μg/ml. After rinsing in phosphate buffered saline (PBS), the slides were further incubated with the same amount of biotinylated horse anti-murine immunoglobulins for 30 min. The endogenous peroxidase activity was blocked with 0.03% H_2O_2 in PBS for 40 min before incubating with the avidin–biotin complex (30 min), and the sections stained with a solution of 0.06% 3-3′-diaminobenzidine (Sigma, St Louis, Missouri, USA) in PBS for 5 min. The slides were counterstained with haematoxylin, and mounted under coverslips with resin. Negative controls were carried out using unrelated IgG_1 MAb at the same concentration.

Sections of a reference ovarian carcinoma specimen served as positive controls in each staining session.

The amount of purified MAb was chosen according to a titration curve as the lowest amount which gave strong and homogeneous staining on the reference ovarian carcinoma specimen.

Double-determinant immunoradiometric assay (DDIRMA)

The assay was carried out essentially as described previously [16], using polystyrene beads coated with purified MOv18 and [^{125}I]MOv19 as the tracer. One hundred microlitres of [^{125}I]-MOv19 (2×10^5 cpm) and 100 μl of the samples were added to MOv18-coated beads, and incubated for 20 h at room temperature. After five washings with PBS, the bound radioactivity was measured by a counter. The binding index (B.I.) was calculated as the mean counts per minute (cpm) of two test replicates divided by the mean cpm of two control replicates incubated only with medium and the tracer. A B.I. higher than 2 was considered positive. Alternatively, the values were expressed as arbitrary units (a.u.) by means of a standard curve obtained by serial dilutions of ascitic fluid from an ovarian carcinoma patient. The titration curve was linear between 1:72 and 1:4.5 dilutions; this last dilution was assumed to contain 100 a.u. Positivity cut-off values of 15 and 40 a.u./ml were used for the sera and the ascitic fluids, respectively.

Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment

Cryostatic sections were air-dried and incubated for 1 h at 37°C with 300 μl of PBS (pH 7.4), and 0.1 mg/ml bovine serum

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albumin (BSA) in the presence of protease inhibitors [1 mmol/l phenylmethylsulfonyl fluoride (PMSF) and 0.24 trypsin inhibitor unit (TIU)/ml aprotinin, Sigma], with or without 0.5 mU PI-PLC from *Bacillus thuringiensis* (Funakoski Pharmaceuticals, Sapporo, Japan). After enzymatic treatment, the supernatants were recovered, centrifuged at 10000 *g* for 10 min and analysed undiluted by DDIRMA; the slides were washed in PBS, fixed for 10 min in cold acetone and stained as described.

Statistical analysis

The χ^2 test was used to compare the percentage of DDIRMA-positive ovary carcinomas to the percentage of positivity in the other groups; the analyses of the differences between the mean a.u./ml ± S.D. in ovary carcinoma patients and the other groups were performed by the Student's *t*-test.

RESULTS

Detection of the molecule at tissue level

The reactivity of MOv18 and MOv19 MAb on normal tissues was evaluated by immunohistochemistry on frozen sections from surgical specimens and several tissues were found to be positive (Table 1). Confirming the preliminary analysis on surgical specimens from the urogenital tract [17], MOv18 and MOv19 reacted homogeneously on oviduct epithelium and kidney proximal and distal tubules, with a stronger reactivity on the former. With regard to the lungs, the alveolar epithelium showed a weak staining, whereas strong positivity was evident on bronchial glands. The bronchial epithelium was found to be positive only with MOv19 in 1 out of 2 cases. Reactivity of both MAbs was observed on the oesophagus, localised in the glands, but not in the pluristratified epithelium. MOv19 reacted weakly on the glandular stomach. Staining of the pancreas ducts was observed, in addition to some focal reactivity on acinar cells. No reactivity was seen on other tissues of the digestive apparatus, such as the sigmoid colon, rectum and liver, nor on other tissues such as the spleen, muscle and ovary.

Some small follicles of the thyroid were stained by MOv19, whereas mammary glands were stained by both MAbs. The reactivity on acinar cells was homogeneous, whereas on the epithelium of the ducts it was localised in the basal position.

The oviduct epithelium, pancreatic ducts and bronchial glands showed a staining preferentially localised on the apical membranes. In contrast, on kidney tubules the reactivity was diffused over the whole cell and some staining was also observed on the inside of the lumen.

In the kidney and oviduct, the treatment with PI-PLC from *Bacillus thuringensis* was able to partially remove the relevant antigen from the section, as detected by DDIRMA analysis of the supernatants, which gave a B.I. of 4.2 and 9, respectively. The supernatant from PI-PLC-treated ovary carcinoma gave a mean B.I. of 23.5. The immunoperoxidase staining carried out after the enzymatic treatment showed a drastic reduction of MOv18 and MOv19 reactivity, but no changes in the staining of an anti-HLA class I MAb. In Fig. 1 the effect of the PI-PLC treatment on the oviduct epithelium (panels c and d) and on an ovarian carcinoma (panels a and b), used as a positive control is illustrated.

Table 1. MOv18 and MOv19 reactivity on normal tissues by immunoperoxidase staining

Organ	Reactivity MOv18	Reactivity MOv19	No. of positive cases/ total cases
Ovary, germinative epithelium	−	−	0/2
Fallopian tubes	+++	+++	2/2
Kidney			
Glomeruli	−	−	0/4
Proximal tubules	++	++	4/4
Distal tubules	++	++	4/4
Collecting tubules	−	−	0/4
Lung			
Bronchial epithelium	−	+	1/2
Bronchial glands	+	++	4/4
Alveolar epithelium	+	+	4/4
Digestive apparatus			
Oesophagus	+	++	2/2
Stomach	+/−	+	3/3
Sigma	−	−	0/2
Rectum	−	−	0/2
Pancreas	+	++	3/3
Liver	−	−	0/1
Mammary gland	+	++	3/3
Spleen	−	−	0/1
Thyroid	+/−	+	1/2
Muscle	−	−	2/2

+++, intense staining; ++, moderate staining; +, weak staining; +/−, focal staining.

Detection of gp38/FBP soluble form: preclinical analysis

We investigated, in an experimental model, whether the release of the protein occurs *in vivo*. Five nude mice were injected i.p. with the IGROV1 ovary carcinoma cell line and their sera and urine were tested repeatedly.

As shown in Fig. 2, the gp38/FBP was clearly detectable in the animal sera starting from day 16, when the abdomen became stretched. In the urine, a consistent increase in the antigen level was evident by day 11 and always exceeded the serum content. When the animals were sacrificed on day 23, the tumour appeared as a disseminated carcinomatosis associated with abundant ascites which presented high antigen levels (60 a.u./ml).

No reactivity was recorded in the urine and sera from animals injected with a control cell line (data not shown).

Clinical evaluation of the gp38/FBP release

The presence of released antigen was evaluated in human biological fluids of different origin (Table 2). Forty-seven out of 78 (60%), and 10 out of 35 (29%) ascitic fluids from ovarian and non-ovarian carcinoma patients, respectively, were found to be positive. No reactivity was observed in the ascitic fluid from patients with non-epithelial tumours and from non-tumour pathologies. The distribution of the levels of reactivity is reported in Fig. 3. The percentages of positive cases in patients with ovarian carcinomas was found to be significantly higher as compared to the other three groups, but the mean a.u./ml value of ascitic fluid from patients with ovarian carcinoma was not statistically different from that of the other pathologies.

Fifty-one sera samples from patients with ovarian carcinoma were tested for the presence of circulating antigen. The majority of the patients (88%) were stage III–IV. 12 (24%) were found to be positive, compared with only 1/31 with lung carcinoma, 0/40 with endometriosis, and 1/100 healthy donors (Fig. 4). The percentage of positive sera from patients with ovarian carcinomas

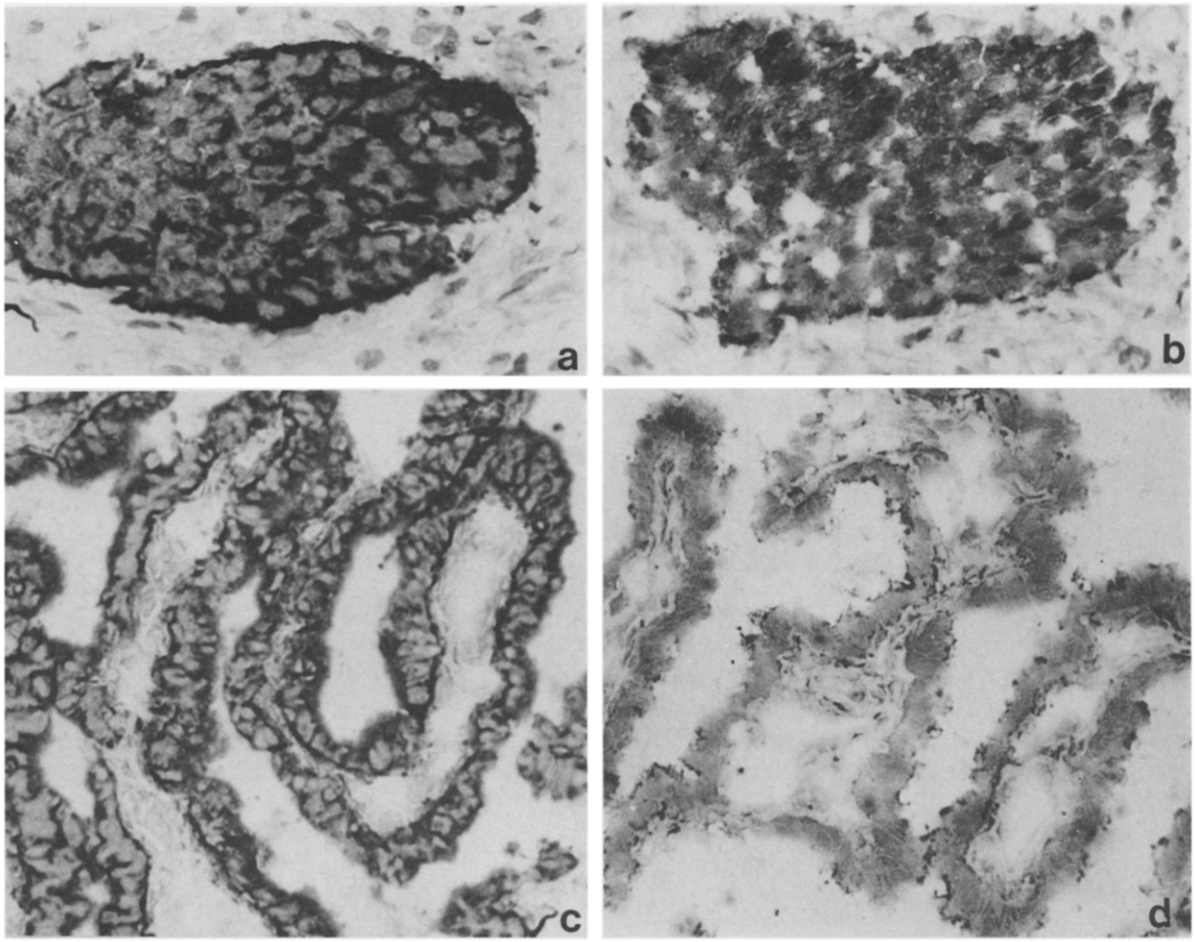

Fig. 1. Immunoperoxidase on frozen sections of an ovary carcinoma specimen (panels a and b) and of a Fallopian tube (panels c and d) as tested by the MOv18 MAb. Before the immunostaining the sections were incubated for 1 h at 37°C in the absence (panels a and c) or in the presence (panels b and d) of PI-PLC.

was significantly higher than those in the other groups, whereas the difference between the mean a.u./ml value was statistically significant only between sera from patients with ovarian carcinoma and those with endometriosis, or from healthy donors. The correlation between the presence of tumour cells in the peritoneal cavity and the release of gp38/FBP in the respective ascitic fluid and serum was simultaneously tested in 20 patients (Table 3). Seventeen ascites, examined cytologically, contained tumour cells (85%), and of these 16 (94%) were positive when analysed by DDIRMA. The number of positive sera was limited to 7 (41%). The 3 cases which were cytologically negative were also negative when tested by DDIRMA.

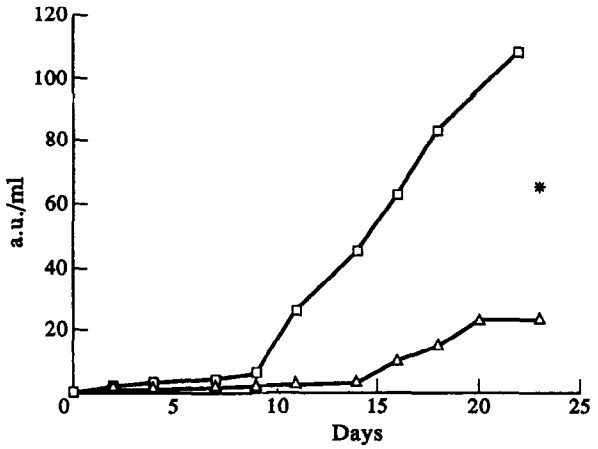

Fig. 2. MOv18/MOv19 DDIRMA in biological fluids of a representative nude mouse injected i.p. with IGROV1 cells. Ascitic fluid *; serum —△—; urine —□—. A similar pattern was observed with samples from the other animals analysed.

When urine samples, either from patients with ovarian carcinoma or healthy donors, were tested, all revealed the presence of high levels of antigen (Table 2). 35/39, and 30/39, respectively, exceeded 100 a.u./ml, ranging from 20 to 10000 a.u./ml in tumour patients, and from 28 to 2900 a.u./ml in healthy donors (Fig. 5). No statistically significant differences were found between the mean values of the two groups.

DISCUSSION

Results obtained from different clinical investigations [18–20] indicate that the MOv18 MAb is a suitable antibody for *in vivo* ovarian tumour diagnosis and therapy. Its relevant antigen, i.e. the gp38/FBP, with its overexpression on this kind of tumour, can be considered a tissue marker of ovary carcinoma.

In this study, we used the MOv18 and MOv19 MAbs to define some more features of the gp38/FBP, such as its distribution in normal tissues and its release in biological fluids of normal and tumour origin. This last investigation should be the basis on which to determine whether the protein can be detected with

Table 2. Reactivity in biological fluids by MOv18/MOv19 DDIRMA

Biological specimen	Origin	No. of positive cases*/total cases	%	P†
Ascitic fluid				
	Ovarian carcinoma	47/78	60	
	Non-ovarian carcinoma	10/35	29	< 0.005
	Breast	3/9	33	
	Lung	1/3	33	
	Gastrointestinal	1/9	11	
	Uterine	5/6	83	
	Others	0/8	0	
	Non-epithelial tumour	0/17	0	< 0.001
	Non-tumour pathology	0/6	0	< 0.02
Sera				
	Ovarian carcinoma	12/51	24	
	Lung carcinoma	1/31	3	< 0.05
	Endometriosis	0/40	0	< 0.05
	Healthy donors	1/100	1	< 0.001
Urine				
	Ovarian carcinoma	35/39	90	
	Healthy donors	30/39	77	

*Cut-off = 40 a.u./ml ascitic fluid, 15 a.u./ml sera and > 100 a.u./ml urine. **P was evaluated by χ^2 test. The percentage of positive ovarian carcinoma cases was compared to the percentage of positive cases with other pathologies.

sufficient specificity and sensitivity by MOv18/MOv19 DDIRMA to be further investigated as a possible new serological marker for ovarian carcinomas.

The presence of the high affinity membrane FBP has been detected on the plasma membrane of various cells and tissues by using biochemical methods and/or antisera produced against the purified protein [2, 3]. The MOv18 and MOv19 Mab were the first monospecific reagents able to identify the gp38/FBP [10].

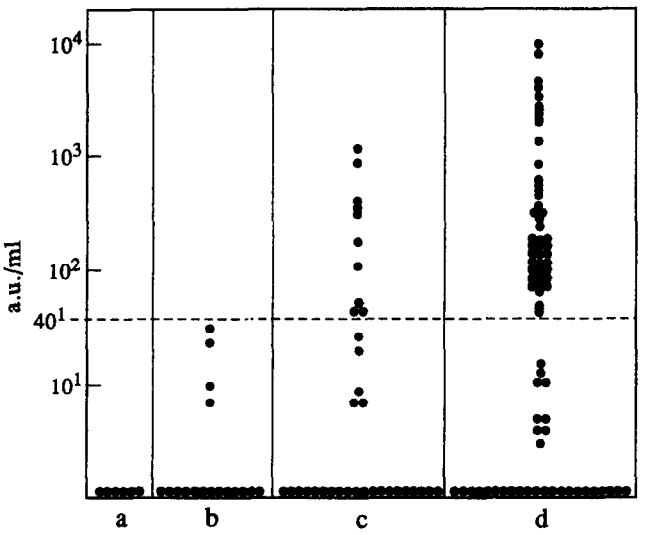

Fig. 3. Evaluation of the gp38/FBP in human ascitic fluid by MOv18/MOv19 DDIRMA. (a) non-tumour pathologies (n = 6); (b) non-epithelial tumours (n = 17); (c) non-ovarian carcinomas (n = 35); (d) ovarian carcinomas (n = 78). P was evaluated by a one-sided t-test: d versus a, b and c gave values > 0.01 (non-significant).

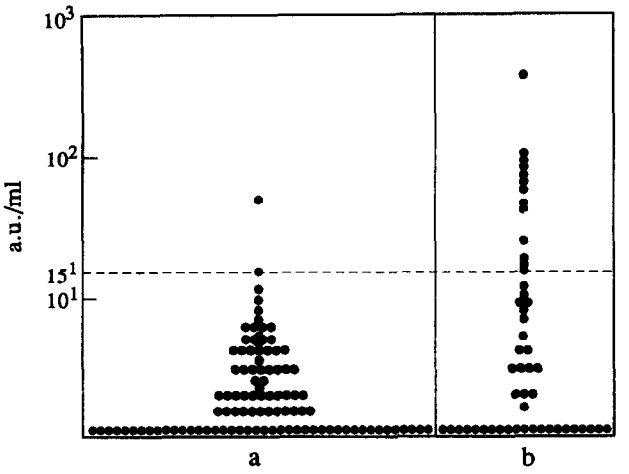

Fig. 4. Evaluation of the gp38/FBP in human sera by MOv18/MOv19 DDIRMA. (a) healthy donors (n = 100); (b) ovarian carcinomas (n = 51). P was evaluated by a one-sided t-test; b versus a, $P < 0.005$. A statistically significant difference in the distribution was also observed when ovarian carcinomas were compared to endometriosis ($P < 0.01$).

In the original histochemical characterisation, no reactivity was found on frozen sections of normal tissues when tested by immunofluorescence [10], but further studies with more sensitive techniques, like immunoperoxidase, showed staining of the Fallopian tubes and kidney proximal and distal tubules [17].

In the present study, histochemical reactivity at different degrees of intensity, depending on the MAb used (MOv19 staining was stronger than that of MOv18), and the cellular localisation of the protein, was also observed in bronchial glands, alveolar epithelium of the lung, oesophagus, stomach, pancreas, thyroid and breast. The above results agree substantially with those reported by Stein [21], who, in addition, found reactivity of the MOv19 MAb on the trachea, salivary glands, prostate, uterus and endocervix, and by Weitman [22], who by northern and western blotting, demonstrated the expression of FBP on the thyroid, kidney, lung and pancreas, but not on the intestine, liver, spleen and muscle.

On some of the positive tissues, we found that the reactivity was apically distributed, whereas in the breast, the ductal epithelium was stained in basal areas. These particular cell localisations suggest different functional roles which the protein may assume in the folate metabolism within different tissues.

In keeping with our previous demonstration of the GPI anchorage of the gp38 on ovarian carcinoma cells [9, 13], even the FBP expressed *in vivo* on the Fallopian tubes and the kidney

Table 3. Relationship between the presence of tumour cells in ascites and presence of released gp38/FBP in ascitic fluid or serum, as detected by MOv18/19 DDIRMA

DDIRMA result	Cases with tumour cells in ascites* Ascites	Sera
Positive	16 (94%)	7 (41%)
Negative	1	10

*20 cases were subjected to cytological examination and 17 of them (85%) contained tumour cells. All cases with negative cytology (n = 3) showed no reactivity for gp38/FBP in ascites or serum.

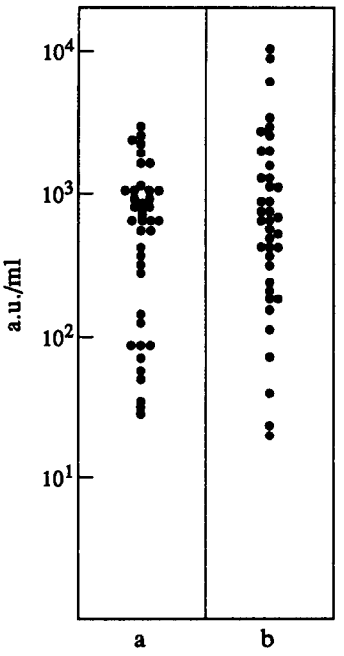

Fig. 5. Evaluation of the gp38/FBP in human urine by MOv18/MOv19 DDIRMA. (a) healthy donors (n = 39); (b) ovarian carcinomas (n = 39). P was calculated by a one-sided t-test. The differences in the distribution of the two groups were found to be statistically non-significant.

was found to be sensitive to PI-PLC treatment and, therefore, can be considered a GPI protein.

The reactivity of the anti-FBP MAbs on normal tissues did not appear to be relevant when MOv18 was injected in patients for clinical purposes. Either after intraperitoneal or intravenous administration of [^{131}I]MOv18, no false positive staining was observed [18]. The apical distribution of the protein on some epithelia, resulting in direct exposition to the external environment, is probably relevant in avoiding contact with the MAb present in the blood stream.

No direct correlation between the reactivity found on normal tissues and the respective carcinomas has been observed. From the MOv18-negative surface epithelium of the ovary develop tumours which express the FBP in 80–90% of cases. In ascites from ovary carcinoma patients, the antigen released by the cells was found in 60% of the examined cases. The percentage of positive cases rose to 94% when evaluated in a limited number of cytologically positive cases.

From breast and lung epithelial cells, originate a low percentage of MOv18 and MOv19 reactive tumours [10–12]. However, some ascitic fluid derived from breast and lung carcinomas was found to be DDIRMA-positive (3/9 and 1/3, respectively). The uterine carcinomas at the immunohistochemical level [12, 17], as well as the majority of the fluids derived from them (5/6), were positive, whereas the corresponding normal tissue was described as MOv18-positive [21] or -negative [17], depending on the authors. No fluids from patients with benign diseases or non-epithelial tumours were found to be positive.

The preclinical model of a human ovarian xenograft, which first indicated that the release of the protein specifically took place *in vivo*, also gave indications on the behaviour of the protein in humans. In fact, although in the ascitic fluid of mice, where the tumour cells were growing, gp38/FBP was found at high levels, low levels could be detected in sera. The higher concentration observed in the urine, in addition to the earlier detectability than in the serum, suggest a fast kidney clearance of the protein.

However, in contrast, even normal urine samples were found to be strongly positive in humans. This FBP could derive from both the local release by the kidney, and the ultrafiltration of the serum protein produced and released by other normal tissues [23]. As far as the presence of antigen in the serum is concerned, low levels of gp38/FBP were found in ovary carcinoma patients in comparison to the ascites and urine and statistically significant differences of the mean serum a.u./ml values were observed when compared to healthy donors or to the sera from endometriosis patients. The assay, however, is not sensitive enough to allow any diagnostic or monitoring application, since the percentage of positive ovary carcinoma cases was low, and only rose when the cases, selected for the presence of tumour cells in the ascites, were considered.

By using non-immunological methods, based on the displacement of the endogenous folate from the serum protein, low levels of FBP have been detected in normal serum [24, 25], and increased levels, in comparison to healthy donors, have been reported in the sera of breast cancer patients [26].

These analyses were carried out when the biological and biochemical knowledge of the FBP was still limited, and no monoclonal antibodies were available to detect the protein. It should now be interesting to apply the non-immunological assay to the sera of patients with tumours which overexpress the gp38/FBP, such as ovarian carcinomas, to investigate whether it could be more sensitive than the MOv18/MOv19 DDIRMA.

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C-*raf*-1 Proto-oncogene Expression Relates to Radiosensitivity Rather Than Radioresistance

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The transfection of several oncogenes, particularly c-*raf*-1, into mammalian *in vitro* cell lines has been reported to be associated with increased radioresistance. We have thus investigated (by scanning photodensitometry of western blots) the phenotypic expression of the c-*raf*-1, c-*myc* and c-*ras* protein products in 19 human *in vitro* cell lines, whose intrinsic cellular sensitivity to 4 MeV photon irradiation has also been determined. High levels of c-*raf*-1 proto-oncogene product expression did not correlate with increased cellular radioresistance, but rather showed a significant correlation with intrinsic cellular radiosensitivity to photon irradiation for α ($r = 0.664$, $P = 0.002$), and SF_2 ($r = -0.655$, $P = 0.002$). There was no significant correlation for the *ras* family, c-*myc* or actin. These results conflict with those of previous studies in which transfection of the activated forms of the c-*raf*-1 oncogene were associated with increased radioresistance, and suggest the possibility that the full length proto-oncogene may influence cellular radiosensitivity in a different manner from that of the activated oncogene.
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INTRODUCTION

SEVERAL STUDIES have indicated that cellular and viral oncogenes, in particular c-*myc* [1, 2], c-Ha-*ras* [3, 4], N-*ras* [5] and c-*raf*-1 [6–8], known to be involved in the malignant transformation of mammalian cells, may also increase the resistance of these cells to ionising radiation. Some reports have found no general correlation between cellular radiosensitivity and oncogene product levels [9–11], and others have described increased radioresistance in cells which have only been transfected with a neomycin-resistance marker [12]. The previous studies demonstrating a positive relationship between oncogene expression and relative radioresistance were mainly conducted on NIH3T3 or embryonic cells transfected with viral or activated cellular oncogenes. It has been noted that the use of NIH3T3 cells for the study of radioresistance may be problematic [4], since they are highly aneuploid, have a very high rate of spontaneous transformation, and there is known to be a wide variation among laboratories in their properties which makes comparisons difficult [13,14]. In addition, transfected murine fibroblastic or embryonic cell lines are not representative of the