EUR. J. ONCOL., vol. 18, n. 2, pp. 63-73, 2013

A human monoclonal antibody detecting a Tumor-associated antigen (Taa) expressed on several different solid tumors and its possible use for intracavitary prophylaxis in Non Invasive Bladder Cancer (NIBC)

Giancarlo Pizza[1], Caterina De Vinci[1], Giuseppe Lo Conte[1], Patrizia Brasa[1], Silvia Zuffa[1], Loredana Melchiorri[2], Maura Ferrari[3]

[1] Immunotherapy Module, S. Orsola-Malpighi University Hospital, Bologna; [2] Operative Unit Medical Genetics S. Anna University Hospital, Ferrara; [3] IZSLER Zoo-Prohylactic Institute of Lombardia and Emilia-Romagna Regions, Brescia, Italy

Summary. *Aim:* Production of a human monoclonal antitumour antibody for intracavitary immunoprophylaxis of non infiltrating bladder cancer. *Materials and methods:*A human anti-tumor-antibody-producing lymphoid cell-line was obtained by infection of peripheral blood lymphocytes (PBL) of a cancer patient with Epstein-Barr virus (EBV). Subsequently, the line has been fused with a murine myeloma and some human-antibodies-producing clones were selected by cluster picking. *Results:* No mouse immunoglobulins were detected. We describe here the studies performed on one of these clones, Brit-26/Hy, that produces human M and G1 class immunoglobulins reacting with a human Tumour-Associated Antigen (TAA) of 55 kDa Molecular Weight (MW). The antigen was found on cells from tumors and cell lines from different histology (Transitional Cell Carcinoma of the Bladder –TCCB-, kidney, colon and breast adenocarcinoma, glioblastoma) and does not seem to be expressed on normal cells of the corresponding tissue. *Conclusions:* In consideration of the high percentage of reacting TCCB cells and absence of reaction with normal transitional cells of the bladder we suggest the use of Brit-26/Hy in passive immunoprophylaxis for relapsing Non Invasive Bladder Cancer (NIBC) after transurethral resection (TUR).

Key words: Taa, NIBC, clone Brit-26/Hy

«UN ANTICORPO MONOCLONALE UMANO VERSO UN ANTIGENE TUMORE ASSOCIATO (TAA) ESPRESSO DA DIVERSI TUMORI SOLIDI E POSSIBILE USO NELLA PROFILASSI INTRACAVITARIA DEL CARCINOMA VESCICALE NON INFILTRANTE (NIBC)»

Riassunto. *Scopo:* Produzione di un anticorpo umano per immunoprofilassi passiva intracavitaria del carcinoma vescicale non infiltrante. *Materiali e metodi:* Mediante infezione con virus di Epstein-Barr (EBV) di linfociti del sangue periferico (PBL) di un paziente affetto da carcinoma è stata allestita una linea linfoide in grado di produrre anticorpi anticancerosi. Successivamente la linea è stata fusa mediante glicole polietilenico con un mieloma murino non secernente e sono stati selezionati cloni produttivi. *Risultati:* Non si sono rilevate immunoglobuline murine. Si descrivono gli studi eseguiti sul clone, Brit-26/Hy, che produce immunoglobuline umane di classe M e G1 che reagiscono con un antigene tumore associato (TAA) di 55 kDa di peso molecolare (MW). L'antigene è stato riscontrato in cellule provenienti da tumori solidi e linee tumora-

Grants: Autonomous budgets of Immunotherapy Module, S. Orsola-Malpighi University Hospital and IZSLER Zoo-Prohylactic Institute of Lombardia and Emilia-Romagna Regions, Brescia, Italy

li di diversa istologia (carcinoma transizionale della vescica –TCCB-, rene, colon e adenocarcinoma mammario, glioblastoma) e non sembra reagire con le cellule normali del corrispondente tessuto. *Conclusioni:* Vista l'alta percentuale di cellule reattive nel TCCB e l'assenza di reattività verso le cellule transizionali vescicali si suggerisce l'uso di Brit-26/Hy nell'immunoprofilassi passiva intracavitaria del carcinoma vescicale non infltrante (NIBC) dopo resezione transuretrale.

Parole chiave: Taa, NIBC, clone Brit-26/Hy

Abbreviations: HAMA, Human Anti Mouse Antibodies; IIF, Indirect Immunofluorescence; NIBC, Non Invasive Bladder cancer; PEG, Polyethilenglycol; TAA, Tumour Associated Antigen; PBL, Peripheral Blood Lymphocytes; RCC, Renal Cell Cancer; TUR, Trans Urethral Resection; TCCB, Transitional Cell Carcinoma of the Bladder

Introduction

Bladder cancer is the most common tumor of the urinary tract and approximately 70% of them occur as superficial lesions most of which may be resected by transurethral resection (TUR). Unfortunately, about 80% of tumors shortly relapse, so prevention attempts are under intensive study using chemo- or immunotherapy for intravesical therapies after TUR (1). The latter is reserved to non invasive bladder cancer stage Ta-T1, according to the EAU guidelines (2). In spite of the various treatments the frequency of relapses remains high so new therapeutic strategies are needed, and research is also focused on immunotherapy using monoclonal antibodies.

Since the original report in 1975 (3), hybridoma technology yielded several murine monoclonal antibodies (MoAbs) with different specificities directed against a wide range of TAAs, greatly improving diagnostic tools (4, 5), and suggesting various immunotherapeutic approaches in human cancer treatment (6-8).

Radioimmunotherapy, for instance, which involves the conjugation of cytotoxic nuclides to tumor-specific antibodies to deliver a lethal dose of radiation specifically to tumor cells, is an attractive concept if intravesically administered. Pfost et al (9), using an orthotopic human bladder carcinoma mouse model found significant increase of the survival of mice treated with radioimmunotherapy using ^{213}Bi-Anti-EGFR-mAb in comparison with mice treated with Mitomicin C. They concluded that this option could be promising in humans. Previously, Murray et al (10) reported the production and initial studies using a ^{188}Re-antibody complex directed against high-molecular-weight glycoprotein MUC1 mucin, usually overexpressed on bladder tumors, and representing a useful target for intravesical radioimmunotherapy of superficial bladder cancer. Nonetheless, no additional reports followed the initial one.

The antibody response of the human recipient against mouse immunoglobulins (HAMA), when intravenously delivered, affects their half-life and diminishes their ability to react with TAAs (11). In addition, the HAMA causes, in some instances, important adverse side effects, with life threatening risk, reducing the wide use of mouse monoclonals for therapeutic purposes. Although , to our knowledge, no studies have been published about HAMA following intravesical administration of mouse immunoglobulin, the use of human MoAbs (h-MoAbs) could avoid the aforementioned problems as the absence of the specie's barrier renders them much less immunogenic and their prolonged half-life could permit a better recognition of the antigenic epitopes; so, h-MoAbs appear a very attractive specific anticancer molecule.

However, until now, the production of h-MoAbs has been hampered by several factors due to different technology drawbacks and their therapeutic efficacy has been proven only in a minority of clinical studies. h-MoAbs, produced by established EBV+ lymphoid B cell-lines, obtained from immunized cancer patients, have been used both *in vitro* to identify several TAAs as diagnostic tools (12,13) and *in vivo* for the prophylaxis of relapses of the superficial transitional-cell carcinoma of the bladder (TCCB) (14-17). Encouraged from the clinical results observed,

and to avoid the risk of the interruption of production of h-MoAbs from the EBV+ cell lines (it is well known that these cells may interrupt the antibody production after days or months), we decided to attempt the stabilization of their ability to produce antibodies by fusion with a mouse-human lymphoid hybrid cell-line. In the present study, we report the preparation and characterization of a h-MoAb, named Brit-26/Hy, obtained by polyethilenglycol (PEG) fusion of an EBV producing cell-line with the non-antibody-producing mouse-human murine myeloma SP2/0-Ag14. Because of *in vitro* observations, we suggest it could be used in immuno-passive prophylaxis of relapsing NIBC after TUR as parental antibody produced by the EBV+ human lymphoblastoid cell lines (14-17).

Brit-26/Hy recognizes a common cytoplasmic antigen of 55 kDa expressed in bladder, kidney, breast, colon, liver and brain tumors, and in some *in vitro* established tumor cell lines. It is worth mentioning here that the antibody does not react with cells from normal tissue nor with peripheral blood cells. Furthermore, preliminary data suggest that Brit-26/Hy MoAb presents an *in vitro* inhibitory growth effect on tumor cell lines when complement is added in the culture.

Materials and Methods

Peripheral Blood Lymphocytes (PBL) source

Patients with transitional TCCB or renal cell cancer (RCC) were used as the source of PBL for infection with EBV, after detection in their serum of antibodies against TCCB or RCC cells by indirect immunofluorescence test (IIF) (12).

EBV source

As EBV source, we used a marmoset lymphoid cell line spontaneously producing virus i.e. the B95-8 line (18). Fresh virus was produced every two months, and stored at +4°C. Titration of the virus was performed using standard techniques, and concentration was adjusted to 100 transforming units per ml of supernatant fluid by addition of medium (Iscove's modified Dulbecco MEM (minimum essential medium) medium +10% fetal calf serum (FCS) (Hyclone Lab. Logan, Utah, USA), the same medium in which the line was grown).

Fusion partner

The murine myeloma non-producing-immunoglobulins SP2/0-AG14, HAT-sensitive and ouabain resistant, is well reported in the literature (19).

Tumor cells

Human tumors were obtained during surgery and immediately processed. Briefly, the necrotic areas are discarded and the remaining tissue passed through a metal mesh. After two gentle washings in Hank's Balanced Salt Solution, the cells were fixed with a formalin buffer at room temperature overnight (20). After two washings in saline, the cells were suspended in saline solution at $2x10^6$ per ml and stored at +4°C until use. Samples of the fresh tumor were immediately frozen after surgery using a liquid nitrogen container, and stored at −70°C. Cryostatic sections were also obtained for IIF and immunoperoxidase testing on fresh tissues according to standard techniques.

Tumor Cell lines

T24 human bladder carcinoma, MCF7 human breast adenocarcinoma, U373-MG human glioblastoma, HEP-G2 human liver cancer and CACO-2 human colon adenocarcinoma lines were obtained by ATCC American Type Culture Collection (Rockville, Maryland, USA). LG14 is an EBV transformed cell line from a healthy donor. PBL were isolated by Lympholite-H gradient (Cederlane Hornby, Ontario, Canada) from blood samples of healthy subjects. In the experiments performed for the identification of the antigenic determinant recognized by Brit-26/Hy, PBL were stimulated 48hs with 1 μg/ml of phytohaemoagglutinin-P (PHA) (Murex, Darford, England). Cell lines and PBL were grown in Iscove's modified Dulbecco MEM medium,

supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine and 10 μ/ml of penicillin. Human AB serum was used as source of complement.

EBV infection

The lymphoid antibody-producing cell-lines have been established as previously described (12). Briefly, 20 ml of peripheral blood (PBL) were drawn from patients with elevated serum titers of IgG-IgM class antibodies against TCCB or RCC cells. The mononucleated cells were obtained by the Ficoll-Hypaque technique. Plastic sterile tubes were incubated 1 hour in a CO_2 incubator at 37°C with 1 ml of EBV containing supernatant, and 10^7 PBL. After two gentle washings with Iscove medium containing 10% FCS, $5x10^5$ cells in 1 ml of medium were dispensed in 2-ml-well plastic plate (Costar 3548, Cambridge MA, USA), and incubated in a CO_2 incubator at 37°C. Clusters of cells appeared 15-30 days later and were collected using a fine pasteur pipette and placed individually in new wells with 1 ml of medium and allowed to grow. The presence of antibodies in the supernatant fluid was evaluated using the IIF test against formalin-fixed tumor cells (12). The antibody producing line, named Brit-26, was selected for the fusion with the murine myeloma line SP2/0-AG14 aiming at the definite stabilization of antibody production.

Cell-lines Fusion Protocol

The antibody-producing EBV transformed lymphoid cells ($2x10^6$) were washed three times in Iscove serum free medium, and admixed with the SP2/0-AG14 cells (10^7) at 5:1 ratio in 50% PEG 1500 for 1 min at 37°C. Afterwards, the cells were gently washed two times and plated in Iscove 10% FCS in Costar 3548 plates at $5x10^5$/well; 24 hours later HAT ($1x10^{-4}$M hypoxanthine, $4x10^{-7}$M aminopterin and 1.6 $x10^{-5}$ M thymidine) and ouabain 10^{-6}M were added to culture medium for new hybrids selection. Supernatants obtained from growing cultures were screened for presence of antibodies against patients cancer cells by IIF, as previously described , and positive wells were sub-cloned by limiting dilution (0.8 cell per well) two times. The clone BRIT-26/Hy-44.1, characterized by high immunoglobulin secretion was further sub-cloned three additional times, obtaining the GF 449.4F1.3.12 cell line (referred as GF 449) secreting a human IgM antibody continuously for the last 24 months. The latter clone has been re-named Brit-26/Hy.

Analysis of immunoglobulin products

The analysis of the antibodies secreted both by the EBV cell line and Brit-26/Hy was performed by ELISA. Briefly, affinity purified anti-human IgG and IgM (200 μl, 1mg/ml DAKO, Denmark), diluted in borate saline buffer (BSB) with 0.1% Tween, was added to each well of a 96-well plate (Falcon 3915, B-D Lincoln Park N.J. USA) and incubated overnight at 4°C. The plates were then washed 3 times with BSB and the excess binding sites were blocked by incubation for 1h at room temperature (RT) with BSB-Tween containing 3% bovine serum albumin (BSA, NEN Life Sciences, Boston MA, USA). After 3 washings with BSB-Tween, 0.1 ml of culture supernatant was added to triplicate wells of the plates that, after an additional 1 h incubation at RT, were subsequently washed 3 times with BSB-Tween. Tissue culture medium alone was used as "blank" control. Peroxidase labeled anti-human IgG or anti-human IgM (DAKO, Denmark) was then added to each well and incubated 1 h at room temperature. After 3 washings with BSB-Tween the plates were added of 200 μl of ABTS (ROCHE, Italy) to each well and evaluated by photometry at 405 nm using an ELISA Microplate Reader 400 (Packard, Meridien, CT, USA)

Immunofluorescence indirect test (IIF)

Two slides with 5 drops of 5μl each of tumor cells were prepared, air dried and incubated 30 minutes (min) in a humidified incubator at 37°C with 5 μl of supernatant fluid of cultured lymphoid clusters or PBS diluted (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128) patients' serum. Five μl of the same suspension was incubated with control medium. After gentle washings with 20 ml of phosphate buffer saline (PBS), the slides were air dried and incubated again for 30 min

with 5µl of rabbit fluorescein-conjugated immunoglobulins (FITC-Ig) diluted 1:20 with PBS against human Ig (Dako, Denmark and Sigma-Aldrich, USA). Afterwards the slides were washed, prepared with a mounting liquid, and observed with a UV light microscope. For characterization of the positive supernatant fluids against specific tumor targets, we used the rabbit rhodamine-Ig or FITC-Ig against the different human Ig classes (IgG, IgM) and kappa (*k*) and lambda (λ) light chain. When cryostatic sections were used, they were processed as for tumor cells suspension.

Immunohistochemistry

We used human tumor cell lines grown on coverslips for 4-6 days and fixed in 10% formalin at RT for 10 minutes; five micrometer thick sections from formalin fixed and paraffin embedded tumors were prepared. Endogenous peroxide activity was blocked using 5% hydrogen peroxide in methanol for 30 min at 4°C, then the slides were immersed in citrate buffer and treated with microwaves 20 min for the antigen unmasking. The cell lines and the histology sections were then incubated 1 h at 4°C with a rabbit anti-human IgG , and subsequently incubated 2 hs at RT with Brit-26/Hy culture supernatant. The antibody binding was visualized with the PAP (Peroxidase anti-peroxidase) complex and the label developed with 3-3' DAB (diaminobenzidine). All the slides of the cell lines and the tumor sections were counter-stained with Mayer's haematoxylin.

Affinity purification of IgG Brit-26/Hy antibody

MoAb affinity purification of IgG from supernatants was performed using MabTrap GII (Pharmacia Biotech AB, Uppsala, Sweden) using the procedures suggested by the manufacturer.

Immunoprecipitation of the antigenic determinant recognised by Brit-26/Hy

Cell lines and PHA activated PBL were labeled overnight with 50 µCi/ml of ^{35}S methionine (NEN Life Sciences, Boston MA, USA) in RPMImet$^-$ medium supplemented with 10% FCS. Pellets were suspended in 1 ml of a solution containing 150 mM NaCl, 1% NP40, 0.5% DOC (deoxicorticosterone), 0.1% SDS (sodium dodecyl sulfate), 50 mM TRIS pH 7.5, 1mM PMSF (phenyl-methyl-sulfonyl-fluoride) (RIPA buffer). The lysates were precipitated with Brit-26/Hy IgG and protein A/G agarose (Oncogene Scientific Inc. Cambridge, MA, USA). Immunocomplexes were washed twice with solution I : 1% sodium dodecylsulphate, 0.5% sodium deoxicholate, 1% BSA in TNEN (20 mM Tris-HCl pH 7.6, 0.5% Nonidet P40, 10 mM EDTA, 0.1 M NaCl) and once with solution II: 0.5% NaCl in 1/10 TNEN. Pellets were dissolved in 50 µl of 2x Laemmli buffer (20% glycerol, 3% SDS, 3% B-mercaptoethanol, 10 mM EDTA and 0.5% bromophenol blue). Samples were separated by SDS-PAGE on 10% acrylamide gel using the Laemmli discontinuous buffer under reducing conditions.

Results

Brit-26

Brit-26 line, the EBV cell-line selected for fusion, showed a doubling cell number time of 4 days; 48% of the cells show surface Ig of class M and 1% of class G; no CD3+ve cell were scored. The line produces 4 mg /liter for 10^6cells in 24 hs of IgM and 0,02 mg/liter of IgG1 class complement fixing antibodies with *k* light chain (Table 1). When assessed by IIF, Brit-26 reacted with surface antigens of formalin-

Table 1. Antibody production and doubling time of the cells in culture.

Cells/antibody	Brit-26	Brit-26/Hy
Doubling time	96 hs	18 hs
Ig produced*	4 mg/l	14 mg/ml
Ig Class (percentage)	M (99%) G1 (1%)	M (99%) G (1%)
TAA MW	55kD	55kD
Light chain	*k*	*k*

*= production per 10^6cell/24hs
kD = kilo daltons
MW = molecular weight

fixed tumors cells with a typical membrane fluorescence pattern in 82 over 106 (77%) TCCB and 32 over 80 (40%) RCC patients studied. The percentage of positive cells varied between 10 to 100% with an intensity of reactivity between 1 and 4 for TCCB, and 1 to 3 for RCC, being 1 the minimum and 4 the maximum brightness (Table 2).

In the immunoprecipitation studies performed with the label of T24 and MCF7 cells with ^{35}S-methionine and subsequent reaction with Brit-26 IgG we noticed a common antigenic product of about 55 kD. No reaction was observed when EBV lymphoblastoid cells and PHA activated PBL treated were used in the same manner.

Brit-26/Hy

The IgM production of the eteromyeloma Brit-26/Hy is 14 mg/1x10^{6}cells/24 hs and the doubling cell number time of the line is 18 hs. In spite of the seven sub-cloning procedures (0,8 cell per well) performed for the Brit-26/Hy a slightly presence of IgG1 class antibodies (0.2 mg/1x10^{6}cells/24h) have been detected in culture supernatant (Table 1). When evaluated by IIF on tumor cell suspension Brit-26/Hy reacted with 10-100% of the cells of 38 over 49 (79%) of TCCB and with 8 over 20 (40%) of the RCC studied (Table 2). The IIF pattern appeared as a typical membrane fluorescence with, in some cells, cytoplasmic staining. The results obtained using the immunoperoxidase technique on 22 tumors are reported on Table 3. All cases tested (6 TCCB, 6 colon and 6 breast adenocarcinomas, and 4 glioblastomas) appeared reactive, although the percentage of positive cells was higher for the first 2 tumor types (35-36%) than for the remaining two (27-28%).

Table 2. Results of IIF testing

Tumor type	Brit-26	Brit-26/Hy
TCCB	82/106 (77%)	38/49 (79%)
RCC	32/80 (40%)	8/20 (40%)

Table 3. Brit-26/Hy: results of immunoperoxidase testing

Tumor tested	n. Pos./n. tested	n. Pos.cells ± sd
TCCB	6/6	36±4
Colon	6/6	35±3
Breast	6/6	27±2
Glioblastoma	4/4	28±2

The immunohistochemistry patterns observed with Brit-26/Hy supernatants are reported in Fig. 1A: the comparative analysis showed that IgM and IgG1 antibodies recognize the same antigenic epitope. The antibodies reacted with the tumor cells of each tissue sections tested (TCCB, colon, breast adenocarcinoma, glioblastoma) and with cell lines of the corresponding immortalized phenotype including liver cancer (HEP-G2 cell line). No reactivity was observed in tissue section against the normal cells and against both resting and mitogen activated peripheral blood lymphocytes or EBV transformed cell lines from healthy subjects. In tissue sections, the antibodies stain homogeneously about 20-30% of tumor cells, apparently reacting with a cytoplasmic structure. In tumor cell lines, Brit-26/Hy also reacts with a cytoplasmic structure according to a scattered pattern in MCF-7 and to a "Golgi like" structure pattern in U-373-MG and Caco-2. In Fig. 1B are reported immunofluorescence studies showing reaction with cells of TCCB tumour and negative reaction against normal mucosa of the same patient. In addition membrane and cytoplasmic reaction is better observed in cells when tumour cells are tested in suspension.

In the immunoprecipitation studies performed labeling T24, MCF7, U-373-MG, HEP-G2 and CACO-2 cells lines with ^{35}S-methionine and the subsequent reaction with Brit-26/Hy IgG, we noticed the presence of a common antigenic product of about 55 kD. On the contrary, by using the same procedure on LG14 EBV+ lymphoid cells and PHA activated PBL we failed to notice any immunoprecipitated product (Fig. 2).

Discussion

To our knowledge, this is the first time that a non-immunoglobulin-producing murine myeloma, after the fusion with a human anti-tumor immunoglobulin-producing EBV+ cell line, produces the anti-tumor antibodies of the parental human line,

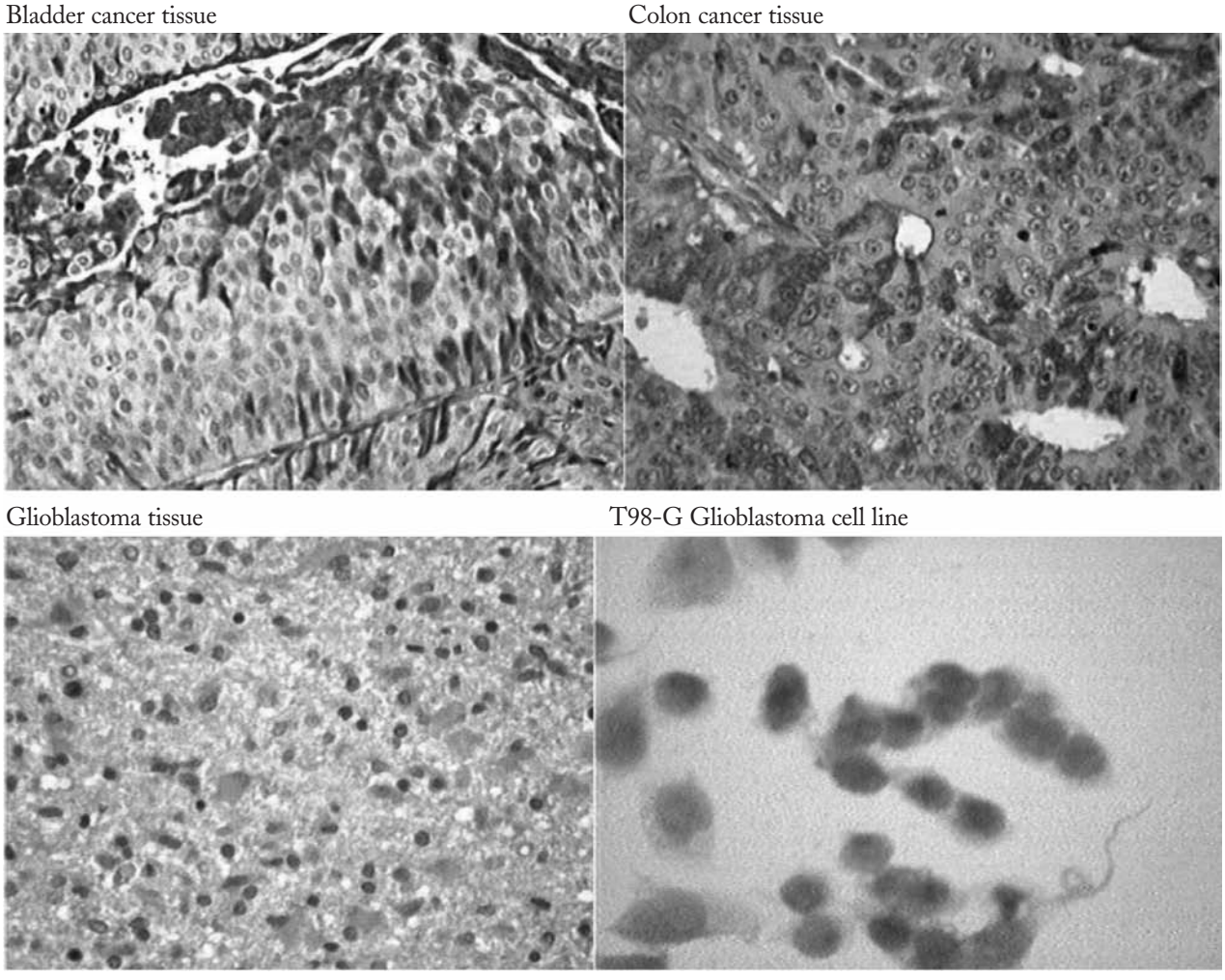

Figure 1A. Immunohistochemistry. Immunoperoxidase label of the cytoplasmic epitope recognized by Brit-26/Hy in tissue sections and cell lines of bladder carcinoma, colon adenocarcinoma, glioblastoma, and T-98G glioblastoma cell line with typical cytoplasmic reaction

carrying the same specificity and reacting with the same antigenic structure. In spite of seven sub-cloning procedures, Brit-26/Hy continues to produce, not only human IgM, but also small amounts of human IgG antibodies, both reacting with the same antigenic epitope. We have no explanations for this phenomenon partially contrasting with the hypothesis of "one cell" equal to "one antibody class". We can only speculate that few IgM producing cells switch towards the IgG production, this phenomenon being observed seven times consecutively.

The possibility of using MoAbs as "magic bullets" in cancer therapy was immediately conceived after the discovery of the hybridoma murine technology by Kohler and Milstein when anti-tumor specific antibodies were produced in unlimited amounts (3). However, twenty five years later, although thousands of murine MoAbs have been generated against a variety of targets, and in spite of their relative easy production, only a few limited therapeutic applications in humans are noticeable (21). The promise for the generation of therapeutic molecules in cancer therapy is not yet achieved. In fact, although mouse MoAbs are greatly effective in protecting mice against infectious diseases and tumors, being foreign proteins, when used in humans

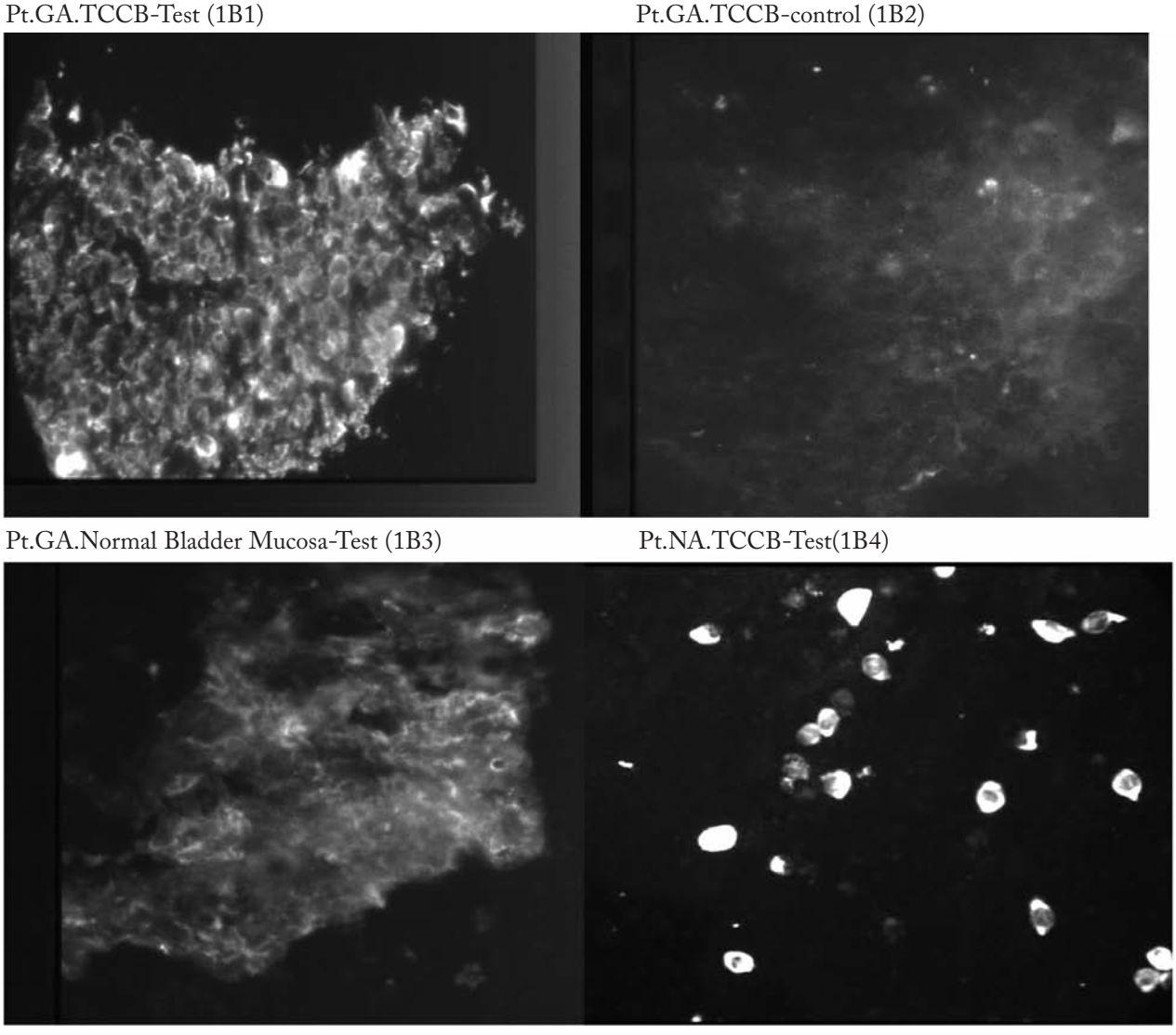

Figure 1B. Immunofluorescence Testing. **1B1** cryostatic section of TCCB of the patient GA in presence of Brit-26/Hy and fluoresceinated antibodies anti human IGg – positive membrane reaction; **1B2** cryostatic section of TCCB of the patient GA in presence of fluoresceinated antibodies anti-human IgG only negative reaction; **1B3** cryostatic section of normal bladder mucosa of the patient GA in presence of Brit-26/Hy and anti-human fluoresceinated antibodies anti-human IgG – negative reaction; **1B4** formalin-fixed suspension of TCCB cells of the patient NA in presence of Brit-26/Hy and anti-human fluoresceinated antibodies anti-human IgG – positive membrane and cytoplasmic reactions

they induce an immune response. Consequently, the monoclonal antibody activity is blocked by the human antibody response with formation of immune complexes with adverse side effects, including, in some instances, anaphylactic shocks. This problem can be avoided by using human MoAbs: the latter have a much longer half-life in the human body, and are more effective in the interaction with human effector cells. For these reasons, many efforts have been made, in the past years, in an attempt to produce human MoAbs by using the transformation by Epstein Barr virus of B lymphocytes and fusion of human B cells with murine myelomas. Biochemical and molecular techniques for the production of antibodies containing only small part of the original murine molecules (chimerical antibody, humanized antibody)

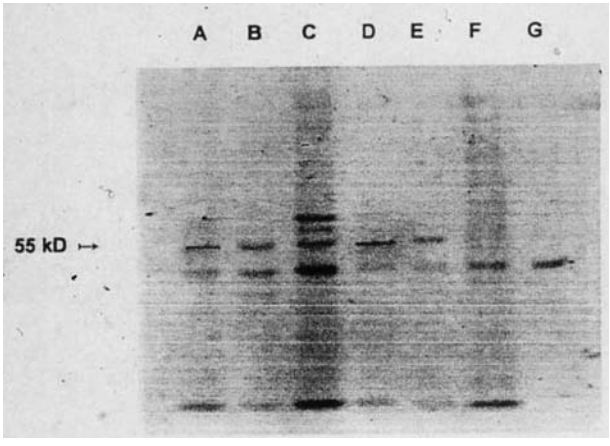

Figure 2. Detection of a common antigenic structure of about 55 CKD identified in different cell lines by Brit-26/Hy. Cell lines and PBL were labeled overnight with ^{35}S methionine lysate then left to precipitate with Brit-26/Hy and resolve in SDS–PAGE.
A: CACO-2 human colon adenocarcinoma; **B**: MCF7 human breast adenocarcinoma; **C**: T24 human bladder carcinoma; **D**: U373-MG human glioblastoma; **E**: HEP-G2 human liver cancer; **F:** peripheral blood lymphocyte of an healthy donor ; **G**:LG14 EBV transformed cell line from a healthy donor

(22) or the direct use of phage libraries technology that avoid "in vivo" or "in vitro" immunization have also been attempted (23). However, in spite of the availability of these cellular and molecular approaches, few human MoAbs are currently used in diagnosis or therapy in solid tumors, and any new antibody that recognizes specific antigenic structures expressed by cancer cells may be of interest.

The human MoAb reported in this paper recognizes a common antigenic structure expressed in tumor cells in both fresh tissue or immortalized cell lines of different solid tumor tissues, while no reactivity was observed against any no tumor cell. Brit-26/Hy hybridoma stable produces from more than 24 months an antibody that reacts against 20-30% of tumor cells in the different phenotypes, the strength of reactivity do not appear related to the differentiation stage of the cells suggesting that the antigenic epitope recognized is expressed since early stages of tumor differentiation. The antibody secreted by Brit-26/Hy belongs to the IgM isotype and shows *in vitro* an inhibitory ability on tumor cell growth, suggesting its possible utilization *in vivo* in a passive immunotherapy approach and/or for an enhancement of biological functions against tumor cells in antibody-dependent cellular and/or complement-dependent cytoxicity. Our studies in the treatment of TCCB patients, using the antibodies secreted by the EBV+ cell line used for obtaining Brit-26/Hy hybridoma, appeared encouraging (14-17).

We already showed (14) that the intravesical administration of BRIT-26 antibodies of the parental EBV-cell lines and human complement in patients with TCCB in three hs were able to activate cell death as evaluated by trypan blue dye inclusion in over 90% of cells obtained by bladder washings. In addition bladder biopsies performed after treatment showed wide areas of the mucosa without epithelium accompanied by degenerative phenomena in the remaining epithelium (14). In addition, the superficial cells, although strongly linked each other, appeared detached from deep strata and the cells of the middle strata had many inflammatory cell around them, i.e., granulocytes, lymphocytes and monocytes. Some of the latter appeared to migrate from the lamina propria which showed intense oedema and infiltration through small ruptures of the basement membrane. These effects of BRIT-26 antibodies on TCCB cells encouraged us in the use of the antibody in the treatment, the rationale being the evidence of the complement-mediated lysis of TCCB cells presenting in their surface the reacting antigen as the antibodies appeared complement fixing as showed by indirect IIF anti fraction C3 (12).

The extension of these studies was impeded by the difficulties of producing large amounts of antibodies with the EBV lines; this limitation may be now considered overcome by the BRIT-26/Hy generation that may virtually assure an unlimited source of specific antibody. It must also be taken into consideration that, in preliminary experiments, Brit-26/Hy shows an *in vitro* inhibitory effect on the growth of tumor cells lines (data not shown).

In the immunotherapy of cancer, much attention has been paid to the role of CD8+ T lymphocytes recognizing tumor antigens restricted to the MHC class I, because of their ability to kill tumor cells, and much have been learned, in the last years, about the development of specific vaccination procedures. Vacci-

nation should direct the immune response on specific antigenic epitopes, and different approaches, using either autologous tumor lysates or natural/synthetic peptides derived by the tumor associated antigens, have been proposed. For instance, dendritic cells, pulsed with autologous tumor lysates, have been showed to be able to induce significant clinical responses in melanoma and renal cancer patients (24-26). In this approach, the purity of antigens is essential for the specific recruitment of cytotoxic T lymphocytes. Brit-26/Hy could permit, in several types cancers, the purification from autologous tumor tissues of the recognized TAAs and their use in vaccination approaches. Furthermore, the knowledge of the aminoacid sequence of the antigenic structure recognized by this antibody could allow a more specific identification of more effective peptides in the induction of specific cellular immune response.

In summary we have produced a human MoAb that recognizes a TAA common to several solid tumors. This antibody could provide opportunities both for a "direct" clinical utilization in anticancer immunotherapy with or without interleukins as well as for an "indirect" use by selection of the pertinent antigens for vaccine approaches and/or for the induction of specific anti-tumor immune responses, i.e. by the pulse of dendritic cells.

As regards the clinical setting, because Brit-26/Hy recognizes a common cytoplasmic antigenic structure of 55 kDa expressed, among others, in bladder tumours without reacting with the corresponding normal tissue cells, we suggest its use in an attempt to prevent NIBC relapses after TUR. Its intravesical administration by catheter being a reasonable possibility of replicating the positive clinical data observed using antibodies produced by the parental EBV+ cell lines (17).

References

1. Logan C, Brown M, Hayne D. Intravesical therapies for bladder cancer - indications and limitations. BJU Int 2012; 110 Suppl 4: 12-21.
2. Babiuk M, Oostelink W, Sylvester R, *et al*. EAU guidelines on non-muscle invasive urothelial carcinoma of the bladder, the 2011 update. Eur Urol 2011; 59: 997-1008.
3. Kohler G, Milstein C. Continuos cultures of fused cells producing antibodies to predefined specificity. Nature 1975; 256: 495-7.
4. Pollock RR, Aguila HL, Bargellesi A, *et al.* Advances in monoclonal antibody technology. In Pinchera A, Doria G, Dammacco F, Bargellesi F. (eds). Monoclonal Antibodies '84: biological and clinical applications. Milan: Kurtis, 1985: 15-38.
5. Baricordi OR, Sensi A, De Vinci C, *et al.* A monoclonal antibody to human transitional cell carcinoma of the bladder cross-reacting with a differentiation antigen of neutrophilic lineage. Int J Cancer 1985; 35: 781-6.
6. Houghton AN, Chapman PB, Bajorin DR. Antibodies in cancer therapy: clinical applications - melanoma. In De Vita VT Jr, Hellman S, Rosenberg SS (eds). Biologic therapy of cancer. Philadelphia: Lippincot, 1991: 533-49.
7. Welt S, Scott AM, Divgi CR, *et al*. Phase I/II study of iodine-125-labeled monoclonal antibody A33 in patients with advanced colon cancer. J Clin Oncol 1996; 14 (6): 1787-97.
8. Riethmuller G, Schneider-Gadicke E , Schlimok G, *et al*. Randomised trial of monoclonal antibody for adjuvant therapy of resected Dukes'C colorectal carcinoma. Lancet 1994; 343: 1177-83.
9. Pfost B, Seidl C, Autenrieth M, *et al*. Intravesical alfa-radioimmunotherapy with ^{213}Bi-Anti-EGFR-mAb defeats human bladder carcinoma in xenografted nude mice. J Nucl Med 2009; 50: 1700-8.
10. Murray A, Simms MS, Scholfield DP, *et al*. Production and characterization of ^{188}Re-C595 antibody for radioimmunotherapy of transitional cell bladder cancer. J Nucl Med 2001; 42: 726-32.
11. Courtney-Luck NS, Epenetos AA, Moore R, *et al.* Development of primary and secondary immune responses to mouse monoclonal antibodies used in the diagnosis and the therapy of malignant neoplasm. Cancer Res 1986; 46: 6489-94.
12. Pizza G, De Vinci C. In vitro production of human antibodies against bladder cancer: characterization and diagnostic implications. J Exp Pathol 1987; 3 (4): 335-46.
13. Corrado F, Pizza G, De Vinci C, *et al*. Use of human antibodies for in vitro detection of bladder cancer in urine. Acta Urol Ital 1989; 3: 185-9.
14. Corrado F, Pizza G, Casanova S, *et al*. In vitro produced human antibodies in the treatment of superficial bladder cancer. J Exp Pathol 1987; 3 (4): 347-58.
15. Pizza G, De Vinci C, Corrado G, *et al*. Anticorpi umani nell'immunoprofilassi dei tumori superficiali vescicali (TCCB). In Corrado F, Maltoni C, Corrado G, *et al.* (eds). I Tumori urologici: recenti progressi e nuovi obiettivi della ricerca. Bologna: Monduzzi Editore, 1992: 639-43.
16. De Vinci C, Pizza G, Corrado G, *et al*. Basi razionali e risultati clinici dell'uso adiuvante dell'interferone nella profilassi dei tumori vescicali superficiali dopo resezione transuretrale. In Corrado F, Maltoni C, Corrado G, *et al.* (eds). I Tumori urologici: recenti progressi e nuovi obiet-

tivi della ricerca. Bologna: Monduzzi Editore, 1992: 607-11.
17. Pizza G, De Vinci C, Maver P, *et al*. Immunoprofilassi delle recidive neoplastiche superficiali del carcinoma a cellule di transizione della vescica (TCCV) con anticorpi umani prodotti in vitro. In Martinelli G (ed). Bladder Sparing: Terapia Conservativa del carcinoma della vescica. Atti del III Convegno AUER, Il Ponte Vecchio Editore, 1997: 50-5.
18. Miller G, Lipman M. Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. PNAS 1983; 70: 190-2.
19. Schulman M, Wilde CD, Kohler G. Introduction and derivation of the Sp2/0-Ag14 cell line for fusion. Nature 1978; 276: 269-72.
20. Ross CE, Cochrane AJ, Hoyle DE, *et al*. Formalin-fixed tumour cells in the leukocyte-migration test. Lancet 1983; 2: 1087.
21. Fleckenstein G, Osmers R, Puchta J. Monoclonal antibodies in solid tumours: approaches to therapy with emphasis to gynecological cancer. Med Oncol 1998; 15: 212-21.
22. Reichmann L, Clark M, Waldmann H, *et al*. Reshaping human antibodies for therapy. Nature 1988; 332: 323-7.
23. Marks C, Marks JD. Phage libraries- a new route to clinically useful antibodies. N Engl J Med 1996; 36: 730-3.
24. Nestle' FO, Alijagic S, Gilliet M, *et al*. Vaccination of melanoma patients with peptide-or tumour lysate-pulsed dendritic cells. Nat Med 1998; 4 (3): 328-32.
25. Holtl L, Rieser C, Papesh C, *et al*. CD38+ blood dendritic cells as a vaccine for immunotherapy of metastatic renal-cell cancer. Lancet 1998; 352: 1358.
26. Holtl L, Rieser C, Papesh C, *et al*. Cellular and humoral immune responses in patients with metastatic renal cell carcinoma after vaccination with antigen pulsed dendritic cells. J Urol 1999; 161: 777-82.

Received: 25.1.2013
Accepted: 12.4.2013
Address: Giancarlo Pizza, MD,
Head Immunotherapy Module,
S.Orsola-Malpighi University Hospital,
Via P. Palagi, 9 - 40138 Bologna, Italy
Tel. +39-0516362478
Fax +39-0516362476
E-mail: giancarlo.pizza@aosp.bo.it