Complement Activated by Chimeric Anti–Folate Receptor Antibodies Is an Efficient Effector System to Control Ovarian Carcinoma

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Abstract

Two chimeric monoclonal antibodies (mAb), cMOV18 and cMOV19, recognizing distinct epitopes of folate receptor highly expressed on epithelial ovarian cancer (EOC) cells were analyzed for their ability to activate complement (C) as a means to enhance their antitumor activity. The individual cMOVs failed to activate C on six EOC cell lines as documented by the marginal deposition of C components and the negligible C-dependent cytotoxicity (CDC). Conversely, the mixture of cMOVs was more effective, although the percentage of cell killing did not exceed 25%. Fluorescence-activated cell sorting analysis of EOC cells for surface expression of the membrane C regulatory proteins (mCRP) revealed high levels of CD46, variable expression of CD59, and absence of CD55. This finding was confirmed in tumor tissue specimens obtained from advanced-stage EOC patients and analyzed for the expression of mCRPs mRNA using a cDNA microarray and for the presence of proteins by immunohistochemistry. Incubation of EOC cells with neutralizing mAbs to CD46 and CD59 led to a significant increase in the CDC from 10% - 20% to 45% - 50%. The relative contribution of antibody-dependent cell cytotoxicity (ADCC) and C-dependent killing of two EOC cell lines induced by the mixture of cMOV18 and cMOV19 was about 15% and 25% - 35%, respectively, bringing the total killing to about 40% - 50%. This value increased to 60% - 70% after neutralization of CD46 and CD59 without an appreciable change of CDC. These results suggest that C is the major contributor to the killing of EOC cells induced by the mixture of cMOV18 and cMOV19.

Introduction

An increasing number of chimeric or humanized monoclonal antibodies (mAb) are currently used as therapeutic drugs to control tumor growth, and several others are being tested in preclinical and clinical trials (1). These mAbs have the advantage over more conventional anticancer therapy to be specific for the target antigens and to have less severe side effects. Although the antibodies may cause direct antitumor activity through their F(ab)2 portion (2), they more often mediate damage of cancer cells by binding of these cells to the C receptors CR1 and CR3 expressed on tumor cells and subsequently converted into iC3b promotes binding of these cells to the C receptors CR1 and CR3 expressed on human leukocytes. The cell-cell adhesion may result in C-dependent cell cytotoxicity (CDC) provided that a second signal is delivered to tumor cells by antitumor antibodies that mediate ADCC (3).

The role of ADCC was provided by Clines et al. (4), who showed that treatment of human lymphoma and breast carcinoma with the specific mAb rituximab and trastuzumab in xenograft models was less effective in nude mice deficient in the common γ chain of FcγRI and FcγRIII than in the parental nude mice.

The contribution of C to the in vivo control of tumor growth is less clear. Using murine lymphoma cell line expressing human CD20 molecule, Di Gaetano et al. observed that the killing of lymphoma cells mediated by rituximab was strictly dependent on C because C1q knockout mice, unlike C-sufficient mice, were not protected from tumor growth (5). Similarly, C depletion induced in mice by the i.v. injection of cobra venom factor was found to markedly reduce the therapeutic efficacy of rituximab in a lymphoma xenograft model (2). However, despite the ability of several mAbs to activate C in vitro (3, 6), C is not always involved in the in vivo killing mediated by these mAbs, as suggested by the finding that they are similarly effective in C-sufficient mice as well as in C3- and C4-deficient mice (6).

The low efficiency of C-dependent killing by cell-bound antibodies may depend on the low density of antigenic epitopes that reduces the chance for the IgG to form dimers required for C activation. This was shown by Spiridon et al. (7), who examined the antitumor activity of several murine mAbs to HER-2 overexpressed on tumor cells and found that these mAbs were more effective in causing CDC as a mixture rather than as individual mAbs. The expression of membrane C regulatory proteins (mCRP) on the surface of tumor cells may also account for their resistance to C attack. The mCRPs include CD55, which controls C activation at the level of the C3 convertase; CD46, which interferes with the deposition of functionally active C3; and CD59, which prevents the polymerization of C9 within the terminal complex blocking its
cytolytic activity. These mCRPs are variably expressed on different tumors, and their neutralization by specific antibodies enhances the susceptibility of tumor cells to CDC (8). Overcoming these limitations of C efficiency would be of great help to increase the therapeutic effect of the mAbs.

The aim of the present investigation was to devise a strategy to improve the antitumor activity of two chimeric mAbs to the α isoform of the folate receptor (FR) detected on the majority of ovarian carcinoma. These chimeric mAbs cMOV18 and cMOV19 are of potential clinical interest because they are directed against a molecule that is highly and stably expressed on epithelial ovarian carcinomas (EOC) and mediate ADCC (9). Unfortunately, maximal killing of tumor cells can only be obtained at high effector/target ratio, which is unlikely to occur in vivo. This is a limitation to the therapeutic use of the chimeric mAbs, which led us to explore the possibility that C activated by the cMOVs may provide an efficient means to control tumor growth. We searched for optimal conditions to promote C activation on tumor cells, including the use of a mixture of antibodies, and the selective neutralization of the C regulators involved in the protection tumor cell from C attack. Evidence will be presented, indicating that C activation requires the combined effect of two mAbs directed against two distinct epitopes of FR, and that neutralization of CD46 and CD59 markedly enhances the susceptibility of tumor cells to CDC.

Materials and Methods

Antibodies and sera. The mAb MOV18 (γ1k; ref. 10), directed against the α isoform of the FR, was used in fluorescence-activated cell sorting (FACS) analysis to test antigen expression on target cells. The two chimeric mAbs cMOV18 and cMOV19 (9), recognizing two different FR epitopes and containing CH2 and CH3 domains of human IgG (γ1), were used in functional assays. The anti-CD20 chimeric mAb Rituximab, kindly provided by J. Golay (Bergamo, Italy), was used as a negative control. The anti-C1q, anti-C3, and anti-C4 goat antibodies were purchased from Quidel (San Diego, CA), whereas mAb aE11 directed against a neo-antigen of polymerized C9 in MAC (11) was kindly provided by T.E. Mollnes (Oslo, Norway).

Rabbit IgG and murine mAb GB24 to CD64 were a kind gift from J.P. Atkinson (St. Louis, MO), and rat mAb YTH53.1 to CD59 was kindly provided by S. Meri (Helsinki, Finland). mAb BRIC216 to CD55 was purchased from IBIGEL (Bristol, United Kingdom). Goat anti-CD53 and anti-CD59 antisera were raised in our laboratory using CD55 and CD59 purified from human red cells as previously reported (12). Goat, rat, and mouse IgG were purified by affinity chromatography on Protein G column (Pharmacia Biotech, Milan, Italy; ref. 13). FITC and alkaline phosphatase–labeled secondary antibodies to human, mouse, goat, and rat immunoglobulin were purchased from Sigma-Aldrich (Milan, Italy).

Normal human serum (NHS) was a pool of sera from group AB Rh+ blood donors kindly provided by the Blood Transfusion Center (Trieste, Italy). The C5-deficient (C5D) serum was collected from a patient with a history of meningococcal disease as previously reported (14).

Cells. The following human serous EOC cell lines were used: IGROV1 (a gift from J. Bénard, Institute Gustave Roussy, Villejuif, France); OVCA3 and SKOV3 (American Type Culture Collection, Manassas, VA); OAW42 (kindly provided by A. Ullrich, Max Planck Institute of Biochemistry, Martinsried, Germany); INTOV1 and INTOV2 generated at the Istituto Nazionale Tumori, Milan, Italy (15).

The cell lines were cultured in RPMI 1640 except for OAW42, which was maintained in MEM supplemented with 10% FCS, 2 mmol/L glucose, and streptomycin (100 μg/mL), in a humidified atmosphere of 5% CO2 at 37°C. All cell lines were routinely tested for Mycoplasma infection using the Mycoplasma PCR ELISA kit (Roche, Basel, Switzerland).

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from blood donors by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation; maintained in RPMI 1640 supplemented with 5% FCS, 2 mmol/L glucose, and streptomycin (100 μg/mL); and cultured for 2 to 3 days before the functional assays.

ELISA on cells. OVCAR3 cells were grown to confluence in 96-well tissue culture plates (Corning Life Sciences, Corning, NY). To evaluate FR binding saturation, OVCAR3 cells were incubated with 100 μL of medium containing increasing concentration of antibodies for 70 minutes at 37°C followed by washing and further incubation with 100 μL of alkaline phosphatase–labeled goat anti-human IgG (1:7,500). To measure C activation by cMOV18 and cMOV19, the cells were incubated with 100 μL of culture medium containing either the individual chimeric antibodies (2 μg/mL) or the mixture of the two antibodies (1 μg/mL each) for 10 minutes at 37°C. NHS (25%) was then added without washing to a final volume of 200 μL and incubation was continued for further 60 minutes at 37°C. C deposition were evaluated using 100 μL of anti-C1q (1:4,000), anti-C3 (1:1,000), and anti-C4 (1:4,000) goat IgG and, in the case of MAC, aE11 mAb (10 μg/mL) followed by alkaline phosphatase–labeled secondary antibodies at optimal dilutions. Both primary and secondary antibodies were incubated for 1 hour at 37°C. The enzymatic reactions were evaluated using paranitrophenylphosphate and read at 405 nm as previously reported (16).

Immunofluorescence analysis. EOC cells (5×103) were incubated with the primary antibodies (MOV18, GB24, BRIC216, or YTH53.1) or isotype–matched control antibodies (5 μg/mL) in PBS containing 0.1% bovine serum albumin (PBS–BSA) for 1 hour at 37°C and with the appropriate FITC–conjugated secondary antibodies (Sigma–Aldrich). After washing with PBS–BSA, cells were fixed with 2% paraformaldehyde (2% paraformaldehyde) (Sigma–Aldrich).

Cell fluorescence was evaluated on a FACS Calibur instrument (BD Biosciences, Milan, Italy) using CellQuest software.

Evaluation of mCRPs mRNA in ovarian cancer specimens. mRNA expression levels of CD46, CD55, and CD59 in tumor samples were extracted from the cDNA microarray data as recently reported (17). For this purpose, 37 samples of primary EOC, of serous, endometrioid, and undifferentilated histology, were selected for evaluation of mCRPs. All information regarding cDNA microarray preparation, hybridization with patient’s RNA, and data analyses have been previously reported (17). Briefly, tissue samples were taken at the time of initial surgery, immediately frozen in liquid nitrogen, and stored at −80°C until used. All tumor samples were evaluated by a pathologist, and regions containing a tumor cellularity >70% were sharp dissected and homogenized. Total RNA was extracted using Trizol (Life Technologies, Fredrick, MA) following the manufacturer’s instructions. Ten human cell lines of different origins were used to prepare the reference RNA. The target cDNAs were synthesized from total RNA and labeled both directly with Cy5-dCTP (reference RNA) or Cy5-dCTP (sample RNA; Amersham Biosciences, Amersham, United Kingdom) and indirectly with 3DNA Submicro Expression Array Detection kit (Genisphere, Montvale, NJ). Total RNA was reverse transcribed using 5′ end modified oligo–dT primers containing the specific Cy5 or Cy5 3DNA capture sequences. The mRNA levels are reported as relative ratio to the reference RNA as described (17). The list of annotated genes is available from the web sites of Institute of Molecular Oncology Foundation/Italian Foundation for Cancer Research (http://www.ifom.it/) and National Laboratory of the Interuniversity Consortium for Biotechnology (http://www.linc.it/).

Immunohistochemistry. Nine surgical frozen specimens from serous ovarian carcinomas were used to evaluate FR and mCRPs expression by immunohistochemistry on cryostat sections. The clinical specimens used for this study were obtained with institutional review board approval from patients who underwent exploratory laparotomy at the Istituto Nazionale Tumori and gave informed consent to use left over biological material for investigative purposes. Cryostat sections of EOC (5 μm) were then incubated for 10 minutes at −20°C. Sections were then rehydrated and processed as previously reported (17). Immunoperoxidase staining was done by incubating the sections with the primary antibodies (MOV18 (10 μg/mL), rabbit anti-CD46 (1:100), goat anti-CD55 (1:500), and goat anti-CD59 (1:200) for 1 hour at room temperature. Immunoperoxidase staining was done by incubating the sections with the primary antibodies mAb MOV18 (10 μg/mL), rabbit anti-CD46 (1:100), goat anti-CD55 (1:500), and goat anti-CD59 (1:200) for 1 hour at room temperature. After washing, the sections were incubated with biotin–conjugated secondary antibodies (Sigma–Aldrich). Immunoperoxidase staining was done by incubating the sections with the primary antibodies (MOV18, GB24, BRIC216, or YTH53.1) or isotype–matched control antibodies (5 μg/mL) in PBS containing 0.1% bovine serum albumin (PBS–BSA) for 1 hour at 37°C and with the appropriate FITC–conjugated secondary antibodies (Sigma–Aldrich). After washing with PBS–BSA, cells were fixed with 2% paraformaldehyde (2% paraformaldehyde) (Sigma–Aldrich).

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The mixture of cMOV18 and cMOV19 activates the C system. Two chimeric mAbs cMOV18 and cMOV19, which recognize different epitopes of human FR, were tested for their ability to activate the classic pathway of the C system. Because C1q only binds to two molecules of IgG deposited close to each other on the cell surface, we searched for the maximal number of antibody molecules that bind to the cell surface at sites sufficiently close to allow fixation of C1q. To this end, OVCAR3 cells overexpressing FR were incubated with increasing amounts of the individual mAbs for 1 hour at 37°C. At the end of the incubation, the supernatants from samples containing NHS were analyzed for the 51Cr release measure cell lysis (CDC), whereas the supernatants from the other samples containing either the cMOV or the cMOV + C5D serum were removed and replaced by the mixture of the cMOV (1 μg/mL each), PBMCs were then added at 50:1 effector to target ratio, and incubation was continued for additional 4 hours at 37°C to evaluate ADCC and CDC. The contribution of CDC, ADCC, and CDCC to the total killing of EOC cells was evaluated by incubating the cells with the mixture of cMOV supplemented with NHS (25%) for 1 hour at 37°C to measure CDC. After removing the supernatant, the cells were incubated with the cMOVs and PBMC for further 4 hours at 37°C to measure both ADCC and CDCC.

Spontaneous and maximum release were evaluated by incubating the labeled cells with medium alone or with 2% Triton X-100, respectively. The radioactivity released in a 60-μL aliquot of supernatant aspirated from each well and transferred to a lumaplate (Packard, Groningen, the Netherlands) was measured using a β-counter (Microbeta, Wallac, Turku, Finland). Percent specific lysis was determined as \([(\text{test release} - \text{spontaneous release}) / (\text{total release} - \text{spontaneous release})] \times 100 \).\n
Results

The mixture of cMOV18 and cMOV19 activates the C system. Two chimeric mAbs cMOV18 and cMOV19, which recognize different epitopes of human FR, were tested for their ability to activate the classic pathway of the C system. Because C1q only binds to two molecules of IgG deposited close to each other on the cell surface, we searched for the maximal number of antibody molecules that bind to the cell surface at sites sufficiently close to allow fixation of C1q. To this end, OVCAR3 cells overexpressing FR were incubated with increasing amounts of the individual mAbs for 1 hour at 37°C and analyzed for the extent of mAbs binding by ELISA. The concentration of each antibody required to fully coat the cell surface was 2 μg/mL (data not shown). This concentration was then used for all the experiments of C fixation and was reduced to half this value when the two mAbs were combined and tested as a mixture. Having established the optimal conditions for antibody binding to tumor cells, we next analyzed the two mAbs for their ability to activate the C system by ELISA as detailed in Materials and
Methods. The results presented in Fig. 1 indicate that cMOV18 and cMOV19 tested individually failed to trigger the C system. By contrast, the mixture of the two chimeric antibodies proved to be very efficient in inducing deposition of C1q as well as C4, C3, and the terminal C complex C5b-9 on the cell surface.

Evaluation of C-dependent killing of EOC cells by cMOV18 and cMOV19. The finding that the mixture of cMOV18 and cMOV19 caused deposition of MAC on OVCAR3 cells prompted us to investigate the ability of the two mAbs to induce C-dependent killing of several EOC cell lines. To this purpose, the cells were exposed to the individual mAbs or the mixture of the two mAbs and NHS, as a source of C, and the number of cells killed by MAC was measured after 1 hour of incubation at 37°C. As expected from the results of C fixation, cMOV18 and cMOV19 had a negligible cytotoxic activity as single agents, causing a percentage of lysis of about 5%. The number of cells killed by C activated by the antibody mixture was significantly higher, although the value did not exceed 25% (Fig. 2), raising the possibility that the C-dependent lysis of these cells may be restricted by C regulators and/or a relatively low expression of FR.

Expression of FR and C regulators on EOC cells and ovarian carcinoma. The observation that the mixture of cMOV18 and cMOV19 antibodies, unlike the individual cMOV, were able to trigger the C system, although with a low lytic efficiency, led us to find out if the levels of FR and mCRPs expressed on the various EOC cell lines may account for their low susceptibility to C-dependent lysis. To address this issue, the cells were incubated with MOV18, which reacts with FR, or with GB24, BRIC216, and YTH53.1, which recognize CD46, CD55, and CD59, respectively, and analyzed by FACS using appropriate FITC-labeled secondary antibodies. The results, presented in Fig. 3, show that FR is differently expressed on the cell lines, as evaluated by the mean fluorescence intensity, with the highest value on OVCAR3 and the lowest value on SKOV3. Interestingly,
OVCAR3 cells were relatively more susceptible to C lysis than SKOV3 cells, suggesting a possible correlation between the degree of FR expression and the extent of C-mediated killing, although the low percentage of cell lysis induced by C precluded an accurate statistical analysis. Figure 3 also shows that CD46 was the C regulator mostly expressed on the cell surface in amounts essentially similar on all the cell lines, whereas CD55 was almost totally absent, and the expression of CD59 differed widely on the EOC cell lines examined.

We have also evaluated the differential expression levels of the three mCRPs on specimens from advanced-stage EOC patients, extracting the mRNA expression data from a wide cDNA microarray analysis (Fig. 4A). CD46 and CD59 were expressed in the majority of cases at levels above the references mRNA, whereas CD55 was very slightly expressed because only three samples showed levels above reference mRNA. These data were supported by the immunohistochemical analysis of cryostat sections of EOC from nine patients among the 37 analyzed by cDNA technology (Fig. 4B). FR was detected in all the samples examined as were CD46 and CD59, whereas none of the samples expressed CD55. It is important to emphasize that the expression of FR was restricted to tumor cells, unlike the mCRPs that were expressed both on stromal and cancer cells, although at different levels.

Neutralization of mCRPs increases CDC induced by cMOV18 and cMOV19. To evaluate the relative contribution of CD46 and CD59 to the protection of EOC cells from C attack, we repeated the experiments of CDC induced by the mixture of cMOV18 and cMOV19 in the presence of neutralizing antibodies to CD46 and CD59. As shown in Fig. 5, treatment of the target cells with GB24 or YTH53.1, which neutralize the inhibitory activity of CD46 and CD59, respectively, resulted in a nearly 2-fold increase in the number of cells lysed by C activated by cMOV18 and cMOV19. No apparent correlation was seen between the amount of CD59, which was differently expressed on the various cell lines, and their susceptibility to C-dependent lysis. The two antibodies GB24 and YTH53.1, tested as a mixture, had an additive effect causing a 3-fold increase in cell lysis. The failure of these antibodies to lyse the tumor cells in the presence of NHS ruled out the possibility that they contributed to enhance cell killing as a result of a direct C activation. To further prove that mAbs GB24 and YTH53.1 did not act synergistically with anti-FR antibodies to activate C, we measured the levels of C components deposited on tumor cells treated with anti-FR antibodies and neutralizing mAbs. The values did not differ from those obtained solely with anti-FR antibodies. Thus, the additive effect observed with GB24 and YTH53.1 is compatible with the fact that CD46 and CD59 control the C sequence at different levels, and they both contribute to protect the tumor cells from C attack.

C is the major contributor to the cytotoxic damage of EOC cells induced by cMOV18 and cMOV19. ADCC is believed to be an important mechanism of cell killing mediated by mAb used in...
cancer therapy (2). We sought to evaluate the relative contribution of ADCC and C-mediated destruction of EOC cells induced by cMOV18 and cMOV19 under the experimental conditions detailed in Materials and Methods. The results presented in Fig. 6 show that the mixture of cMOV18 and cMOV19 induced killing of about 15% of OVCAR3 and IGROV1 cells through ADCC, whereas CDC was responsible for the lysis of 30% OVCAR3 and 20% IGROV1 cells. C contributed to cellular cytotoxicity (CDC) with a value of about 5%. The total killing, which results from the contribution of ADCC, CDC, and CDC, was about 50% for OVCAR3 and 40% for IGROV1. We also analyzed the effect of the neutralizing antibodies to CD46 and CD59 under these experimental conditions (Fig. 6) and found that the addition of these mAbs enhanced CDC to values of 40% - 50% and the total killing to 60% - 70% of tumor cells.

**Discussion**

EOC is the most lethal gynecologic malignancies in the industrialized countries with an overall 5-year survival rate of <30% (19). Although chemotherapy has played an increasingly important role in the treatment of ovarian carcinoma, the EOC survival rate has remained largely unchanged for many years mainly due to the difficulties of an early diagnosis and the frequent resistance of these tumors to chemotherapy. A major problem in the clinical management of EOC patients is that the response to the first-line treatment is largely unpredictable. In addition, relapse occurs after complete response to initial treatment and is associated with broad cross-resistance to even structurally dissimilar drugs (20). There is therefore a need for alternative therapies, and our data suggest that C activating chimeric mAbs to FR expressed on EOC cells may provide an effective means to substantially reduce the tumor mass, allowing the eradication of residual tumor cells by the host immune system.

The two mAbs cMOV18 and cMOV19 used in this study recognize the α isoform of the FR, which is homogeneously overexpressed in the majority of ovarian carcinomas and is associated with signaling molecules in specific membrane sub-regions (21). These antibodies are directed against two distinct epitopes of a 38- to 40-kDa glycosylphosphatidylinositol-anchored molecule (22) subsequently identified as a family member of homologous proteins that bind folic acid with high affinity (23). Immunohistochemical studies revealed that the FR is a tissue-specific fetal antigen expressed at high levels on malignant ovarian tumor cells (10).

The failure of the individual chimeric antibodies to activate C on EOC cells can not be attributed to the isotypes of cMOV18 and cMOV19, because they, as many other chimeric or humanized antibodies with antitumor specificity, were constructed with the constant region of human γ1 heavy chain with the purpose to promote Fc-mediated effector functions (24). The fact that both cMOV18 and cMOV19 induced ADCC, when used as single antibodies, and were as effective as the mixture of both antibodies (9) indicates that this aim was, at least in part, achieved. Furthermore, the efficient ADCC of EOC cells mediated by the two cMOVs under the same experimental conditions that failed to induce CDC rules out the possibility that C activation and completion of the C sequence on the cell surface is prevented by the instability of the antibodies bound to the antigenic sites.

Binding of multiple globular heads of C1q to closely spaced IgG on the cell surface is an absolute requirement for an effective antibody-mediated CDC. This effect depends on the deposition of a high number of antibody molecules on tumor cells, which, in turn, is directly related to the expression of the antigenic epitopes on the cell surface. FR is highly expressed on EOC cells, and its level has been estimated to be around 1 × 10^6 molecules per cell on IGROV1 (9). This figure is higher than the 40,000 - 70,000 CD20 molecules per cell detected on B cells from patients with chronic lymphocytic and prolymphocytic leukemias, which were found by Golay et al. (25) to be highly susceptible to CDC mediated by rituximab. This indicates that the density of the antigenic sites favors but is insufficient to justify the mAb-mediated CDC. Additional factors may be required, besides the high number of antigenic targets, as suggested by the finding that CDC of B cells correlates with the segregation of this molecule into the lipid raft (26).

Difference in the total number of cell-bound antibodies may provide a plausible explanation for the ability of the mixture of the two cMOVs to induce CDC and for the failure of the individual mAb to have a similar effect. However, this possibility is unlikely because each cMOV was present in the mixture at half the concentration at which the same mAb were used as single agents. C activation observed under these conditions is more compatible with the close deposition of two IgG molecules on the cell membrane as a result of binding of cMOV18 and cMOV19 to two

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**Figure 5.** Neutralizing antibodies to mCRPs enhance CDC of EOC cell lines induced by chimeric anti-FR mAbs. C-dependent killing was done, incubating EOC cells with NHS and the mixture of cMOV18 and cMOV19 in the presence or absence of neutralizing mAbs to CD46 and CD59 used alone or in combination. Columns, mean of six different experiments; bars, SD. *, P < 0.01 versus “cMOV18 + cMOV19” group. **, P < 0.001 versus “cMOV18 + cMOV19” group. †, P < 0.01 versus “cMOV18 + cMOV19 + GB24” or “cMOV18 + cMOV19 + YTH53.1” group.

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Cancer Res 2006; 66: (7). April 1, 2006

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distinct epitopes of the FR molecule. Similar results were obtained by Spiridon et al. (7) using multiple mAb to different epitopes on the extracellular domain of HER-2 expressed on human breast cancer and various other carcinomas. Further evidence that interaction of antibodies with multiple epitopes on target cells improves their biological activities was more recently provided by Meng et al., who showed that a chimeric tetravalent mAb against human CD22 had an enhanced antitumor activity and an increased ability to bind C1q and to induce ADCC compared with divalent mAb (27).

Despite the binding of the early C components and of the terminal complex C5b-9 to tumor cells, which suggests activation of the whole C sequence on the cell surface, cMOVs-mediated CDC of EOC cells does not seem to be an efficient process. As observed with many other tumors, these cells seemed to be scarcely susceptible to C-dependent killing, and their resistance was related to the surface expression of mCRPs. However, not all three mCRPs contributed to EOC protection because CD55 was not expressed on the cell lines and on the tumor tissues examined both at protein and RNA levels. By contrasts, CD46 and CD59 were both detected on tumor cells with a slight prevalence in the expression of CD46. These data are in agreement with similar observations made by Thorsteisson et al. (28) in human breast and colorectal carcinomas using immunohistochemical and immunoblot analysis and, more recently, by Rushmere et al. (29) in human breast cancer at RNA level. Although CD46 was expressed more strongly than CD59 in tumor tissue, both these mCRPs were found to protect EOC cells from C attack with some difference among the various cells. This conclusion is supported by our finding that each neutralizing antibodies increased CDC, and cell lysis was further enhanced by the mixture of the two neutralizing antibodies.

The antibody-mediated C-dependent killing of nearly 50% EOC cells observed in this study clearly indicates that C provides an important information to develop a more specific model to evaluate the therapeutic potentiality of chimeric or humanized antibodies.

**Acknowledgments**

Received 9/23/2005; revised 1/17/2006; accepted 1/20/2006.

Grant support: Italian Association for Cancer Research, Fondo Italiano per la Ricerca di Base grant RBAU01C13CJ and RBNE01B4C, European Noci "EMBIC" within FP6 grant LSHN-CT-2004-512040, and COFIN 2003 grant 062203_004 provided by Ministero dell'Istruzione, Università e Ricerca (MIUR).

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We thank the Immunohematology Unit of the Istituto Nazionale Tumori for providing buffy coats and the Blood Transfusion Center Trieste for providing NHS.
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