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Mouse Complement Receptor-Related Gene y/p65-Neutralized Tumor Vaccine Induces Antitumor Activity In Vivo†


Two mouse tumor cell lines, Meth A (BALB/c mouse-derived fibrosarcoma) and MM46 (C3H/He mouse-derived mammary tumor), were shown to express high levels of complement receptor-related gene y/p65 (Crry/p65), a membrane-bound complement-regulatory protein. Inhibiting the complement-regulatory activity of Crry/p65 with mAb 5D5 induced high levels of C3 deposition on in vivo tumor-derived Meth A and MM46 cells. To determine the effect of Crry/p65 blockade and increased C3 deposition on in vivo tumor growth, Meth A and MM46 cells were treated with 5D5 mAb and injected into BALB/c and C3H/He mice, respectively. Pretreating MM46 cells with 5D5 mAb significantly suppressed their tumorigenicity when injected s.c. Pretreatment with 5D5 mAb had a modest effect on Meth A s.c. tumor growth. Because complement is involved in the induction of an immune response, we investigated the effect of Crry/p65 blockade and increased C3 deposition on the immunogenicity of the tumor cells in a vaccination protocol. Vaccination of mice with irradiated Meth A cells pretreated with 5D5 mAb protected mice from subsequent challenge. In contrast, vaccination with irradiated Meth A cells without pretreatment was not protective. Survival was correlated with a high titer IgM response and specific CTL activity. These data demonstrate that the functional inhibition of Crry/p65 on tumor cells affects tumor growth and immunogenicity, and that the complement deposition resulting from this inhibition can act in concert with antitumor effector mechanisms to elicit potent antitumor immunity in vivo. The Journal of Immunology, 2004, 173: 205–213.
In this study, we investigate the effect of blocking the function of mouse complement receptor-related protein gene y (Cry/p65) with a mAb, 5D5 (26), on Meth A and MM46 tumor cells in vitro and in vivo. Cry/p65 is a complement-regulatory protein widely expressed on normal tissues and tumor tissues, and it is a functional analog of human DAF and MCP (27, 28).

We also examine the effect of complement deposition on tumor cell immunogenicity in a vaccination procedure. Complement has been shown to positively modulate the induction of an immune response to infectious agents and certain Ags, but such studies in the context of tumor immunity have not been previously reported.

Materials and Methods

Animals

Six- to 8-wk-old female BALB/c (H-2b) and C3H/He (H-2k) mice were used for the tumor implantation and vaccination experiments, and 8-wk-old female mice were used to obtain serum and plasma samples. All mice were obtained from SLC (Hamamatsu, Japan) and were kept in a specific pathogen-free environment. All protocols of animal experiments were approved by the animal experiment committee of Nagoya City University Graduate School of Medical Sciences.

Tumor and hybridoma cells

The methylcholanthrene-induced Meth A fibrosarcoma syngeneic in BALB/c mice was received from Dr. T. Hamaoka of the Osaka University Biomedical Research Center (Osaka, Japan). This tumor was maintained in vivo by passage in BALB/c mice and in vitro by culture at 37°C, 5% CO2 in complete RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) and 5 M 2-ME.

The MM46 mammary tumor was originally generated by R. Irie of the National Cancer Center (Tokyo, Japan) (29). This tumor was maintained in vivo by passage in C3H/He mice and in vitro by culture in complete RPMI 1640 medium. In vivo tumor growth was assessed by periodically measuring perpendicular diameters with calipers after s.c. tumor inoculation. The body weight of mice was measured as an indicator of tumor growth after i.p. tumor inoculation. The 5D5 hybridoma cell line, which produces the anti-mouse Cry rat IgG1 5D5 mAb (26), was maintained in vitro by culture in serum-free medium-101 (Nissui Pharmaceutical) supplemented with 10 µg/ml insulin, 0.002% monoethanolamine, and 10 µg/ml transferrin.

Purification of 5D5 mAb

The 5D5 mAb was purified from tissue culture supernatants by precipitation with 45% (v/v) saturated ammonium sulfate, followed by passage over a MonoQ 16/10 column (Pharmacia Biotech, Uppsala, Sweden) and elution with 0.05 M Tris buffer (pH 8.0) containing 0.3 M NaCl (pH 8.0) by a gradient increase of NaCl.

F(ab')2 of 5D5 mAb was prepared by pepsin digestion under the following conditions: 10 mg/ml 5D5 mAb was dialyzed against 0.1 M Na-formate buffer (pH 2.8) for 16 h at 4°C, as preliminary incubation at low pH has been reported to render IgG more susceptible to enzyme cleavage, presumably due to partial unfolding of the Ab molecule (30). Dialysis against 0.1 M Na-acetate buffer (pH 4.5) was then conducted at 4°C for 24 h. This was followed by incubation of the mAb with pepsin at an enzyme/Ab ratio of 1:100 at 37°C for 4 h. The pepsin digestion was stopped with the addition of 2 M Tris-base. The digested sample was diluted to 1 mg/ml with 0.05 M Tris buffer (pH 8.0), and purification was conducted according to the protocol described above, on a MonoQ 16/10 column. The fractions containing F(ab')2 were collected, and SDS-PAGE was conducted to confirm the presence of the 104-kDa band, representing rat IgG1 F(ab')2.

Production of anti-Meth A hyperimmune serum

Anti-Meth A hyperimmune serum was obtained from 50 BALB/c mice injected with 1 × 107 of irradiated in vitro cultured Meth A cells s.c. seven times at 2-wk intervals. Hyperimmune serum was tested for reactivity by flow cytometry using Meth A cells. After heat inactivation at 56°C for 30 min, a 50% (v/v) ammonium sulfate precipitate of antisera was applied to a Superdex 200pg 16/60 column (Pharmacia Biotech). The IgG or IgM fractions were tested for purity by SDS-PAGE and for reactivity by flow cytometry. Fractions were pooled and dialyzed against PBS, pH 7.4.

Flow cytometric analysis

For characterizations of tumor cells, cells were incubated with rat anti-Meth A F(ab')2 (5D5 (26), hamster anti-mouse DAF (RIKO-3) (31), mouse anti-mouse CDS9 (32) donated by B. P. Morgan (University of Wales College of Medicine, Cardiff, U.K.), mouse anti-mouse H-2Kk (BD PharMingen, San Diego, CA), and mouse anti-mouse H-2Kk (BD PharMingen) for 30 min at 4°C. After washing with cold PBS, the cells were incubated with FITC-conjugated goat anti-rat IgG F(ab')2, (Organon Teknika, Durham, NC), FITC-conjugated goat anti-hamster IgG (Southern Biotechnology Associates, Birmingham, AL), or FITC-conjugated goat anti-mouse IgG (Organon Teknika) for 30 min at 4°C. Finally, cells were washed with PBS and suspended in 0.5 ml of Sheath solution (Fujisawa Pharmaceutical, Osaka, Japan) containing 2 µg/ml propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO). Fluorescence intensity of PI-negative (viable) cells was analyzed by FACSCalibur.

For analysis of sera IgG and IgM levels against tumor cells, in vitro cultured tumor cells (2 × 103) were treated with 20 µl of 10% heat-inactivated mouse immune serum, anti-Meth A IgG polyclonal Ab, or anti-Meth A IgM polyclonal Ab, and incubated at 4°C for 30 min. Cells were washed with cold PBS, and incubated with FITC-conjugated goat anti-mouse IgG, Fcγ-specific (Organon Teknika); or FITC-conjugated goat anti-mouse IgM, µ-chain specific (Immunotech, Marseille, France), for 30 min at 4°C. Following washing, cells were suspended in Sheath solution containing 2 µg/ml PI. Fluorescence intensity of PI-negative (viable) cells was analyzed by FACSCalibur.

C3 deposition assay

In vitro cultured tumor cells or in vivo tumor-derived cells (1 × 106) were treated at 4°C for 30 min with 5D5, and then washed with PBS. Cells were then incubated for 30 min at 37°C with 30% syngeneic normal mouse plasma in EGTA-GVB (gelatin veronal buffered saline containing 2 mM MgCl2 and 10 mM EGTA) as a source of alternative pathway complement (33), or with 30% syngeneic normal mouse serum in GVB+ (GVB containing 0.15 mM CaCl2 and 1 mM MgCl2) as a source of classical pathway complement. Cells were washed with GVB containing 10 mM EDTA (EDTA-GVB) and then with PBS, after which they were incubated with the FITC-conjugated goat anti-mouse C3 (Organon Teknika) at 4°C for 30 min. Finally, cells were washed with PBS and suspended in 0.5 ml of Sheath solution containing 2 µg/ml PI. Fluorescence intensity of PI-negative (viable) cells was analyzed by flow cytometry on a FACSCalibur.

For analysis of the additional effect on C3 deposition of immune serum, anti-Meth A polyclonal IgG Ab or anti-Meth A IgM Ab, in vitro cultured Meth A cells, were treated at 4°C for 30 min with 5D5, and then washed with PBS. After 5D5 treatment, cells were incubated with 10% heat-inactivated immune serum or various concentration of polyclonal Abs. After washing the cells, they were mixed with mouse complement sources to determine their C3 deposition, as described above.

Treatment of tumor cells with 5D5 mAb

A total of 10 µg/ml 5D5 IgG or its F(ab')2 was added per 106 in vitro tumor-derived Meth A or MM46 cells in PBS. After a 30-min incubation on ice, cells were washed twice with 20 ml of PBS.

Vaccinations

The Meth A vaccine was prepared by harvesting ascites from Meth A-bearing BALB/c mice by i.p. lavage. Cells were made to 1 × 107/ml in PBS and irradiated at 180 Gy on a Softex M-80WE X-ray monitor (Softex, Tokyo, Japan). Following irradiation, cells were washed with PBS and 5 × 107 cells were treated with one of 5D5 IgG, 5D5 F(ab')2, or PBS for 30 min on ice. Cells were then washed with PBS. BALB/c mice were injected s.c. with 5 × 106 cells or an identical volume of PBS. This procedure was conducted again 1 wk later, and then was challenged 2 wk later s.c. or i.p. with 1 × 106 untreated in vivo tumor-derived Meth A cells.

The MM46 vaccine was prepared by harvesting ascites from MM46-bearing C3H/He mice by i.p. lavage. Cells were made to 1 × 107/ml in PBS and irradiated at 130 Gy. Irradiated cells were washed with PBS, and 5 × 107 cells were treated with one of 5D5 IgG, 5D5 F(ab’)2, or PBS for 30 min on ice. Cells were then washed with PBS. C3H/He mice were injected s.c. with 5 × 106 cells or an identical volume of PBS. This procedure was conducted again 1 wk later. Mice were challenged 2 wk later s.c. or i.p. with 5 × 106 untreated in vivo tumor-derived MM46 cells.

51Cr release assay

Cytotoxic activity of tumor-resistant mouse splenocytes was determined by means of a 4-h 51Cr release assay. In vitro cultured Meth A target cells (5 × 104) were incubated with 100 µCi of Na2 51CrO4 in 0.2 ml of complete
RPIM 1640 containing 5% FCS (5% FCS-RPMI 1640) for 45 min at 37°C, 5% CO₂. Spleens were harvested from mice that had survived vaccination and challenge, and spleen cell suspensions were prepared in 5% FCS-RPMI 1640. A total of 5 ml of lysis buffer (0.15 M NH₄Cl in Tris buffer, pH 7.65) was incubated with spleen cells for 5 min at 37°C, 5% CO₂, to remove RBC. Spleen cells were washed and were distributed to each well of 96-well U-bottom microplates in triplicate. Following washing, the 5¹Cr-labeled target cells (1×10⁵) were incubated with 1×10⁶ effector spleen cells in a total of 200 µl of 5% FCS-RPMI 1640 for 4 h at 37°C, 5% CO₂. Following incubation, plates were centrifuged at 1000 rpm for 5 min, and 100 µl of supernatant was taken from each well and determined for radioactivity. Background cpm of the culture of target cells alone.

Results
Characterization of Meth A and MM46 cells
Meth A and MM46 cells were assessed for expression of complement-regulatory proteins and MHC class I by flow cytometry. Both cell lines expressed Crry/p65 (Fig. 1, a and b), but did not express detectable DAF (Fig. 1, c and d) or CD59 (Fig. 1, e and f). Meth A, but not MM46, expressed MHC class I (Fig. 1, g and h).

C3 deposition on tumor cells
Complement activation by tumor cells following the inhibition of Crry/p65 was determined by measuring homologous C3 deposition. Classical pathway and alternative pathway activation were determined by incubation of tumor cells in either serum or EGTA serum, respectively (see Materials and Methods). In vitro cultured Meth A and MM46 cells did not activate the alternative pathway of complement whether Crry/p65 function was blocked or not (data not shown). In contrast, in vivo tumor-derived MM46 cells activated the alternative pathway of complement, but only following treatment with Crry/p65-neutralizing mAb (Fig. 2c). Very little or no C3 deposition occurred on in vivo tumor-derived Meth A cells via the alternative pathway (Fig. 2a). Following incubation of in vivo tumor-derived cells with normal mouse serum in which mainly classical pathway is functional, a high level of C3 deposition occurred on both tumor cells (Fig. 2, b and d).

In vivo Meth A and MM46 tumor growth
To determine the effect of neutralizing Crry/p65 on Meth A and MM46 on tumor growth in vivo, cells were pretreated with 5D5 IgG or 5D5 F(ab’)₂ before injection into mice of the appropriate strain either i.p. or s.c.

In the Meth A model, groups of five BALB/c mice were injected with 1×10⁵ in vivo tumor-derived cells with or without pretreatment with 5D5 mAb. Mice injected i.p. with 5D5 mAb-treated Meth A cells showed no significant differences in tumor growth rates or survival periods from mice injected with untreated Meth A cells (data not shown; p > 0.05). Mice injected s.c. with 5D5 IgG-treated Meth A cells showed a somewhat delayed tumor growth pattern and longer survival times, with one permanent survivor (the mice were monitored for 80 days; Fig. 3, a and b). Mice injected s.c. with 5D5 F(ab’)₂-treated Meth A cells showed similar tumor growth rates as those injected with untreated Meth A cells, although one mouse did display delayed tumor growth (Fig. 3, a and b).

To monitor the effects of Crry/p65 neutralization on MM46 tumor growth rates and mouse survival, groups of five C3H/He mice were injected i.p. or s.c. with 1×10⁶ 5D5 mAb-treated or untreated in vivo tumor-derived cells. There was a significant difference in tumor growth rates or survival times for mice injected i.p. with either 5D5 mAb-treated or untreated MM46 cells (data not shown; p > 0.05). In contrast, there was a significant difference in both the tumor growth rate and survival time between mice injected s.c. with either 5D5 mAb-treated or untreated MM46 cells (Fig. 3, c and d). In the MM46 s.c. model, 100% of mice inoculated with untreated tumor cells die by day 20. However, pretreatment of MM46 cells with 5D5 IgG or F(ab’)₂ resulted in long-term survival of 80% and 40% of injected mice, respectively (the mice were monitored for 60 days; Fig. 3c).

The 5D5-treated tumor vaccine
The binding or deposition of C3 to an Ag or a pathogen surface can enhance the induction of specific immunity. Therefore, we investigated whether pretreatment of tumor cells with 5D5 mAb, which results in increased levels of C3 deposition in vivo, influenced tumor cell immunogenicity in a vaccination protocol.

In vivo tumor-derived cells were x-irradiated before treatment with 5D5 IgG, 5D5 F(ab’)₂, or PBS. Groups of five mice were vaccinated with pretreated cells, or with an equal volume of PBS s.c. Vaccinations were repeated 1 wk later, and all mice were challenged s.c. and i.p. with viable in vivo tumor-derived cells 1 wk after the second immunization. There was no significant difference in protection from s.c. tumor challenge for mice vaccinated with either 5D5 mAb-treated or untreated irradiated cells. Irrespective of 5D5 mAb treatment of tumor vaccine, all mice vaccinated with irradiated tumor cells survived following s.c. challenge (data not shown; p > 0.05).
Vaccination of mice with irradiated untreated Meth A cells protected 20% of mice following i.p. challenge with viable Meth A cells. In contrast, vaccination of mice with irradiated Meth A cells that had been pretreated with 5D5 IgG or F(ab')2 resulted in 80 and 100% protection from subsequent tumor challenge, respectively (Fig. 4a).

In the MM46 model, 60% of mice vaccinated with untreated irradiated MM46 cells survived subsequent tumor challenge. However, in contrast to the data using the Meth A model, vaccination with irradiated MM46 cells pretreated with 5D5 mAb or F(ab')2 did not improve survival. In fact, pretreating MM46 cells with 5D5 mAb or F(ab')2 appeared to compromise the efficacy of the vaccination (Fig. 4c).

**Humoral and cellular immune response in Meth A-vaccinated BALB/c survivors**

BALB/c mice that survived vaccination and Meth A tumor challenge underwent an i.p. implantation of $1 \times 10^5$ viable in vivo tumor-derived Meth A cells on day 110 to investigate the immunological memory against Meth A. One week after implantation, mice were bled by cardiac puncture to examine humoral responses, and spleen cells were harvested to investigate cellular responses.

Spleen cells isolated from the lone survivor of the group that had been vaccinated with PBS-treated irradiated Meth A cells showed relatively high cytotoxic activity (Fig. 5a; B1 of group B). Surviving mice from the group that had received the 5D5 IgG-treated Meth A vaccine showed lower spleen cell cytotoxicity (Fig. 5a; C1, C2, C3 of group C), whereas spleen cells from mice vaccinated with 5D5 F(ab')2-treated Meth A cells showed high cytotoxicity in two of three samples (Fig. 5a; D2 and D4 of group D).

The levels of anti-Meth A IgG and IgM against Meth A cells in the sera of surviving mice and the effect of these sera on C3 deposition on Meth A in vitro were analyzed by flow cytometry (Fig. 5b). IgM levels correlated with C3 deposition levels on Meth A cells treated with the serum from surviving mice ($p = 0.011, R^2 = 0.968$), but IgG levels did not ($p = 0.284, R^2 = 0.002$).

**C3 deposition on Meth A cells treated with purified anti-Meth A polyclonal Abs**

To verify the effects of anti-Meth A IgM on C3 deposition on Meth A cells in a Meth A-BALB/c vaccination model, we produced anti-Meth A polyclonal IgG and IgM Abs, as described in Materials and Methods. As shown in Fig. 6, purified polyclonal IgM Ab from anti-Meth A hyperimmune serum promoted C3 deposition on Meth A cells treated with 5D5 mAb dose dependently (Fig. 6b). In contrast, purified polyclonal IgG Ab did not elicit such C3 deposition (Fig. 6b).

**Discussion**

In humans, complement-regulatory proteins are overexpressed on many kinds of tumor cells and tumor cell lines, and their expression prevents homologous complement attack in vitro (11–16). However, the ability of complement-regulatory proteins to protect tumor cells from complement, and the ability of anti-complement regulator neutralizing mAbs to sensitize tumor cells to complement have been extensively studied in vitro (17, 34–38). However, the in vivo consequences of blocking the function of complement regulators remain largely unexplored. In one previous study, it was shown that rats injected with KDH-8 rat hepatoma cells expressing low levels of Crry survived longer than rats injected with KDH-8 expressing high levels of Crry (18), and that pretreatment of the tumor cells with the Crry-neutralizing mAb 5I2 or its F(ab')2 significantly increased the survival time of recipient rats (18). In this study, we investigated the role of complement activation on tumor growth and immunity in vitro and in vivo.

In initial experiments, we determined whether the culture conditions of Meth A and MM46 cells play a role in their susceptibility to C3 deposition following neutralization of Crry/p65. When cultured in vitro, both tumor cell lines are resistant to C3 deposition via the alternative pathway even in the presence of 5D5 mAb. Conversely, in vivo tumor-derived cells are susceptible to C3 deposition following treatment with 5D5 mAb (Fig. 2). The difference between in vitro and in vivo tumor-derived cells in C3 deposition may be due to several factors.

One might be the disparity of expression levels of complement-regulatory proteins between in vitro and in vivo tumor-derived cells. Therefore, we determined the expression levels of complement-regulatory protein on cells cultured in vitro and grown in vivo. We found no significant difference in Crry/p65 and DAF expression levels between in vitro and in vivo tumor-derived MM46 and Meth A cells (data not shown). It is possible that these
cells express different levels of MCP, although MCP expression in mouse is restricted to the testis (39–41).

Another may be the disparity of culture conditions. We thus investigated whether ascites fluid from MM46 tumor-bearing mice affected C3 deposition on in vitro cultured MM46 cells. When Crry/p65 function was blocked with 5D5 mAb, we found a significant difference between alternative pathway-dependent C3 deposition on in vitro cultured MM46 in 10% FCS and in vitro cultured MM46 in 10% ascites (percentages of cells with deposited C3 were 4.6 and 20.6%, respectively; data not shown). This finding indicated the presence of a factor in ascites from tumor-bearing mice that induces C3 deposition on tumor cells. One such factor may be Abs that bind to the tumor cells. Indeed, we found low levels of IgG and IgM deposited on in vivo tumor-derived cells (data not shown), but high level of C3 was only detected on in vivo tumor-derived cells when they were subsequently incubated with a complement source in the presence of 5D5 mAb (Fig. 2).

Previous data demonstrated that pretreatment of KDH-8 rat hepatoma cells expressing rat Crry with neutralizing 5I2 mAb substantially increased survival time of recipient rats (18), and that expression of rat Crry on a human tumor cell line enhanced tumorigenicity in nude rats (42). In addition, it has been shown that expression levels of human DAF can change following in vivo growth of tissue-cultured cells (19). To determine the effects of Crry/p65 neutralization on tumor growth, MM46 and Meth A cells were pretreated with 5D5 IgG or F(ab')2 before inoculation in s.c. and peritoneal tumor models. Mice injected i.p. with Meth A or MM46 died between 15 and 20 days, and pretreatment of cells

FIGURE 3. Tumor growth and survival following s.c. challenge in naive mice. Three groups of mice (n = 5) were injected s.c. with 1 × 10⁵ 5D5 IgG-treated, 5D5 F(ab')2-treated, or untreated tumor cells. a, Shows Kaplan-Meier survival analysis of BALB/c mice challenged with Meth A. Differences among three groups were not statistically significant (p > 0.05). b, Shows tumor growth rate of Meth A cells in individual mice. c, Shows Kaplan-Meier survival analysis of C3H/He mice challenged with MM46. The difference between groups A and B was significant (p = 0.012, log rank test). The difference between groups A and C was not significant (p > 0.05). d, Shows tumor growth rate of MM46 cells in individual mice. Tumor size was assessed by measuring perpendicular diameters with caliper.
with 5D5 mAb did not affect survival (data not shown). A similar result was obtained with Meth A cells injected s.c. In contrast, however, mice inoculated s.c. with 5D5 IgG- or F(ab\(^{-}\))\(_{2}\)-treated irradiated MM46 cells survived longer than mice inoculated with untreated cells, although the difference was statistically significant only for 5D5 IgG-treated MM46 cells. It is not clear why there is a difference between the i.p. and s.c. models, but it may be due to nutritional conditions in the different environments. Perhaps a faster growth rate of tumor cells in the peritoneum may outpace the induction of an immune response.

These data indicate that Crry/p65 is providing MM46 with protection from complement in vivo, and that complement-associated mechanisms are controlling tumor growth in this model. The 5D5 IgG pretreatment of MM46 was more effective than 5D5 F(ab\(^{-}\))\(_{2}\) pretreatment in terms of increasing survival of recipient mice. This may be due to a contribution by Fc receptor-mediated Ab-dependent cell-mediated cytotoxicity. The reason that Crry/p65 neutralization has a more profound effect on MM46 tumor growth and mouse survival compared with Meth A may be related to their relative antigenicities or to the expression of MHC class I molecules. The presence of the

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**FIGURE 4.** Tumor growth and survival following i.p. challenge in vaccinated mice. Four groups of mice (n = 5) were vaccinated with either PBS, untreated irradiated tumor cells, 5D5 IgG-treated irradiated tumor, or 5D5 F(ab\(^{-}\))\(_{2}\)-treated irradiated tumor cells. All mice were subsequently challenged i.p. with untreated viable tumor cells on day 14, and survival and body weight were monitored. a, Shows Kaplan-Meier survival analysis of BALB/c mice challenged with Meth A. The difference between groups A and B was significant (p = 0.004, log rank test). The difference between groups A and C was significant (p = 0.004). The difference between groups A and D was significant (p = 0.001). The difference between groups B and D was significant (p = 0.015). b, Shows Meth A tumor growth as estimated by measuring body weight in individual mice. Results are expressed as a percentage of body weight on day 0. c, Shows Kaplan-Meier survival analysis of C3H/He mice-MM46 model. The difference between groups A and B was significant (p = 0.043). The difference between groups B and D was significant (p = 0.031). d, Shows MM46 tumor growth as estimated by measuring body weight in individual mice. Results are expressed as a percentage of body weight on day 0. Similar results were obtained in two independent experiments.
MM Ag on MM46 cells confers high immunogenicity, and it has been shown that some mAbs against MM Ags can suppress the growth and the spread of tumor cells in vivo (43). High levels of complement were deposited on MM46 cells via both the classical and alternative pathways, while no complement was deposited on Meth A cells via the alternative pathway (Fig. 2). Furthermore, MM46 do not express detectable levels of MHC class I, while Meth A cells are MHC class I positive (Fig. 1, g and h). It is conceivable that the lack of MHC I expression on MM46 may have contributed to increased susceptibility of these cells to NK cells and Ab-dependent cell-mediated cytotoxicity. These differences may account, at least in part, for the differences observed between the two tumor models.

Complement activation and deposition on tumor cells result in opsonization and can lead to direct cell lysis by membrane attack complexes, an important effector function of complement. Complement activation also generates proinflammatory factors such as C3a and C5a that attract and activate immune effector cells. Moreover, the complement cascade links the innate with the specific immune response (44–46). In this study, we determined whether blocking the function of a complement regulator and enhancing C3 deposition on tumor cells would affect the efficacy of a tumor cell-based vaccine. Indeed, we found that vaccination with 5D5 F(ab')2-treated Meth A cells was significantly more effective than vaccination with untreated Meth A cells at conferring protection from subsequent tumor challenge (Fig. 4a; p = 0.015). As shown in Fig. 2, the tumor cells only become targets for complement deposition when Crry/p65 activity is neutralized by 5D5-IgG or 5D5-F(ab')2, and it is likely that the effect of 5D5 mAb on Meth A tumor vaccine is complement dependent. We cannot completely rule out the possibility that 5D5 induces a biological response and alters the immunogenicity of the cells via a signaling mechanism, although we consider this unlikely because the cells used for vaccination were irradiated. Transduction of viable mouse T cells by Crry/p65 has been demonstrated, although the signal from Crry/p65 alone does not induce cell proliferation (47). Our data support the hypothesis that blocking complement-regulatory proteins on tumor cells enhances the induction, as well as the effector phase (see above), of an antitumor immune response. Analysis of the cellular and humoral response following Meth A vaccination revealed the development of a memory T cell response. Spleen cells harvested from surviving mice that had received an induction implantation of Meth A cells demonstrated high specific lysis of Meth A target cells (Fig. 5a). This was particularly true for the surviving mice that had been vaccinated with 5D5 F(ab')2-treated Meth A cells, indicating that a CTL response was the primary effector cell response induced in this case. The low cytotoxicity exhibited by spleen cells of mice that had been vaccinated with 5D5 IgG-treated Meth A cells could be related to the presence of the Fc portion of the IgG in the vaccine. The presence of the Fc portion may have caused preferential activation of macrophages and/or NK cells, because these cells express FcγRs. It is also conceivable that a negative signal may have been transmitted to the immune response through the FcγRs (48). It is noteworthy that all surviving mice had high levels of specific IgM that promoted C3 deposition on Meth A cells. The levels of IgM correlated with C3 deposition levels on Meth A cells in vitro (Fig. 5b). The dependence of C3 deposition on a specific IgM response is further supported by the fact that anti-Meth A polyclonal IgM promoted C3 deposition on Meth A cells, but anti-Meth A polyclonal IgG did not (Fig. 6). These results suggest an important role for IgM in complement activation and tumor regression in this model. In contrast, 5D5 F(ab')2-treated MM46 cells were a less effective vaccine than untreated MM46 cells with regard to mouse survival following tumor challenge (Fig. 4c; p = 0.031). This result may be due to the high susceptibility of MM46 to complement deposition, and in particular, to the high levels of alternative complement activation in addition to classical complement activation. Due to the high susceptibility of MM46 to complement deposition, the 5D5 mAb-treated cells inoculated as a vaccine may have been rapidly eliminated by scavenger phagocytes before it could confer that function. A long-lasting stimulation with a tumor vaccine may be required for the desired effect, because vaccination of mice with overirradiated MM46 cells showed that no protective immunity

**FIGURE 5.** Cellular and humoral response of Meth A-vaccinated BALB/c survivors. Spleens and serum were harvested from mice who had survived the vaccination procedure, and who had received an induction implantation of 1 × 10⁶ Meth A cells. a, Specific lysis was examined by incubating 1 × 10⁶ ⁵¹Cr-labeled Meth A cells with 1 × 10⁶ spleen cells. Cytotoxic activities were determined after incubation for 4 h, and values represent means plus SD of triplicate samples. B1, C1, C2, C3, D1, D2, and D3 represent each surviving mouse. Naive mouse is an age-matched control mouse. b, The y-axis represents the reactivity of serum IgG and IgM levels against Meth A, as measured by the mean fluorescence intensity, and the x-axis represents the amount of C3 deposition. ○ and □. Show the IgG and IgM level of an age-matched naive mouse serum, respectively.

**FIGURE 6.** C3 deposition on Meth A cells treated with anti-Meth A polyclonal Abs. The x-axis represents the reactivity of anti-Meth A IgG Ab (a) and anti-Meth A IgM Ab (b) against Meth A, as measured by the mean fluorescence intensity, and the y-axis represents the amount of C3 deposition, as measured by the mean fluorescence intensity.
was conferred (data not shown). Additionally, because MM46 cells express no detectable MHC I, an effective T cell response was most likely not elicited. When Ab titers were checked in surviving mice that had received an induction implantation, one-half of the survivors and only mice that had received an untreated MM46 vaccine had very high anti-MM46 IgG and IgM levels (data not shown). The exact significance of these high anti-MM46 titers is not clear, but it is possible that they played some role in conferring immunity against MM46. The CTL response following vaccination, challenge, and induction implantation was, as expected, negligible (data not shown). It is thus probable that survival in these mice was elicited mainly through the action of non-T cell effector cells such as macrophages or activated NK cells.

It is evident from the results presented in this study that blockade of the Cry/p65 membrane complement-regulatory protein significantly affects tumor immunogenicity and mouse survival in the two models examined. Similar strategies could potentially be applied to immunotherapy of human cancer. For example, targeted blockade of membrane complement-regulatory proteins on tumors may enhance both the inductive and effector phases of an immune response and overcome tumor resistance to complement. This may be particularly effective for MHC class 1-negative tumors that are resistant to CTL-mediated lysis, such as MM46. In contrast, for tumor cells having the characteristics of Meth A, vaccination with complement regulator-neutralized irradiated tumor cells may induce an effective memory T cell and humoral immune response.

Therefore, the activity of membrane complement-regulatory proteins on tumor cells is an important factor to consider with respect to anticancer immunotherapy. By blocking the activity of membrane complement regulators on tumor cells, homologous complement deposition can readily occur, which can lead to tumor cell destruction and induction of a strong inflammatory response, thereby potentiating other antitumor effector mechanisms. In addition, as we demonstrate in this work, complement deposition can also enhance the induction of an antitumor immune response.

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References


