

Disruption of Cell-Cell Adhesion Enhances Antibody-dependent Cellular Cytotoxicity: Implications for Antibody-based Therapeutics of Cancer¹

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ABSTRACT

Resistance to antibody-based anticancer approaches has become of considerable interest because of the rapidly growing clinical use of several different monoclonal antibodies as therapeutic agents, coupled with the recent finding that their efficacy may be attributable in part to their participation in host antibody-dependent cellular cytotoxicity. In this proof-of-concept study, we demonstrate the novel ability of an antiadhesive antibody (SHE78–7), targeted at the potent homophilic cell adhesion molecule E-cadherin, to play a dual role as participant in, and sensitizing agent for, host immune-mediated antitumor activity. SHE78–7 disrupted preformed multicellular aggregates (spheroids) of HT29 colon carcinoma cells both *in vitro* and *in vivo* in an ascites tumor xenograft model, but had no direct antitumor effect *in vitro*. *In vivo*, however, i.p. injection of SHE78–7 significantly prolonged the survival of nude mice carrying established i.p. HT29 xenografts, most notably when injections were given biweekly. This antitumor effect was dependent on the antiadhesive effect of SHE78–7 and could be effectively recapitulated via treatment with a combination of nondisruptive anti-hMHC-I antibodies, capable of recruiting an F_c-mediated immune response but ineffective as a monotherapy and antiadhesive F(ab')₂ fragments of SHE78–7. Furthermore, additional therapy experiments using such F(ab')₂ fragments, or mice lacking activating F_cγRIII receptors or inhibitory F_cγRIIB, unequivocally indicated a role for host antibody-dependent cellular cytotoxicity, mediated by F_cγRIII and negatively regulated by F_cγRIIB. Taken together, the results suggest a possible means of improving antibody-based therapies of cancer, namely targeting antigens, selectively expressed or up-regulated by target cancer cells, which mediate cell-cell adhesive functions.

INTRODUCTION

Numerous studies, spanning a period of 3 decades, have demonstrated that human cancers, growing as three-dimensional multicellular masses (*i.e.*, solid tumors *in vivo*, spheroids *in vitro*, and so forth), exhibit adhesion-dependent multicellular resistance to a diverse array of anticancer therapeutic approaches, including many traditional chemo- and radiotherapies (1–9). In addition, cell-cell adhesion profoundly attenuates the efficacy of host defense mechanisms and immunotherapeutic modalities, including antibodies (10), immunotoxins (11, 12), lymphokine-activated killer cells (13, 14), IFNs (15), and complement (16, 17), at eliminating tumor cells grown in a three-dimensional context. Previous preclinical studies have shown that multicellular resistance to chemo- and radiotherapy can be pharmacologically reversed by using antiadhesive agents to disrupt cell-cell contacts, thereby disturbing the integrity of cancer cell spheroids *in vitro* or i.p. avascular tumor cell clusters *in vivo* (4). However, it

remains unclear whether this antiadhesive strategy could similarly sensitize tumor cells to other therapeutic modalities.

Whereas chemo- and radioresistance in spheroids may be because of a number of factors including those arising as a result of adhesion-mediated signaling events (6, 18, 19), adhesion-mediated resistance to immunotherapeutics has been shown to be largely, if not entirely, the result of restricted penetration of immune effectors into multicellular tumor masses (11–17). As such, we reasoned that antiadhesives might indeed be used not only as chemo- and radiosensitizing agents, but also as immunosensitizers, by providing increased accessibility of tumor cells for endogenous and/or exogenous immune effector challenges.

The increasing clinical use of chimeric or humanized monoclonal antibodies for cancer treatment has stimulated research endeavors aimed at improving the efficacy of such drugs (20, 21). This holds especially true in light of recent work in F_cγR³-knockout mice, which demonstrated that some antibodies approved for clinical use in cancer treatment, namely Rituxan (22) and Herceptin (23, 24), may owe much of their antitumor efficacy *in vivo* to their involvement in ADCC (25). It follows that antibodies that target cell adhesion molecules expressed by cancer cells could represent particularly promising antiadhesive agents for use as both immunotherapeutic and sensitizing agents, as they themselves could play an active role in any enhanced immune response (and coincidentally, as sensitizers to enhance the efficacy of other therapeutic modalities, including gene therapy, radiation, and chemotherapy).

We have reported previously the use of one such antiadhesive antibody, namely a particularly effective monoclonal antibody against E-cadherin, called SHE78–7 (26), to prevent spheroid formation of a number of E-cadherin-expressing human tumor cell lines when plated in three-dimensional culture *in vitro* (19). The purpose of the present study was to examine the ability of SHE78–7 to act as an immunotherapeutic antibody to prolong the survival of nude mice carrying i.p. xenografts of HT29 human colorectal carcinoma and to determine whether any such action is achieved by rendering the tumor cells more susceptible to host ADCC via the antiadhesive properties of the antibody.

By using a human tumor xenograft system, the antibody assumes tumor-specific qualities, and host toxicity is avoided. This would not be possible in a clinical situation, when targeting a molecule such as E-cadherin; as such, the experimental model we used was designed as a proof-of-concept preclinical model in which to demonstrate the possible antitumor efficacy and therapeutic advantages of exploiting cell adhesion molecules, selectively or preferentially expressed by tumor cells, for antibody-based therapeutics.

MATERIALS AND METHODS

Animals. Female NIH Swiss *nu/nu* mice used in antiadhesive disruption (of tumor aggregates) and survival experiments were obtained from Charles

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³ The abbreviations used are: F_cγR (IIB/III), F_c γ receptor (type IIB or III); (m)Ab, (monoclonal) antibody; ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; GFP, green fluorescent protein; hCG, human chorionic gonadotropin; polyHEMA, poly(2-hydroxyethylmethacrylate).

River (St. Constant, Quebec, Canada) or Taconic (Germantown, NY). Wild-type control mice used in $F_c\gamma R$ (IIB/III) $-/-$ experiments were female BALB/c-*nu/nu* mice (Taconic); corresponding $F_c\gamma R$ IIB $-/-$ and $F_c\gamma R$ III $-/-$ female BALB/c-*nu/nu* mice were made by Toshiyuki Takai (Tohoku University, Sendai, Japan) as described previously (27). Mice were approximately 6–10 weeks old at the time of tumor cell injections. Animal care was in accordance with institutional guidelines.

Antibodies. SHE78–7 monoclonal antibody (murine IgG2a) against E-cadherin (26) and $F(ab')_2$ fragments thereof were generously provided by Takara Shuzo Co., Ltd. (Shiga, Japan). $F(ab')_2$ fragments were generated by pepsin digestion of 10 mg of SHE78–7 antibody followed by passage through a protein A column; purity was verified by SDS-PAGE. Purified, preservative-free antihuman-MHC-I antibody (α -hHLA-ABC, clone W6/32; murine IgG2a) was obtained from Serotec (Raleigh, NC). Nonspecific murine myeloma IgG2a (Zymed, San Francisco, CA) was dialyzed in serum-free RPMI 1640 (Life Technologies, Inc., Burlington, Ontario, Canada) using Slide-a-Lyzer dialysis cassettes (Serotec, Raleigh, NC) to remove NaN_3 before use.

Cell Lines. HT29 and CaOV-3 cells were purchased from the American Type Culture Collection (Rockville, MD). HT29(-GFP/-hCG) and CaOV-3 cells were maintained in RPMI 1640 or DMEM (Life Technologies, Inc.), respectively, supplemented with 10% fetal bovine serum (Life Technologies, Inc.) in a humidified atmosphere at 37°C in 5% CO_2 . HT29 cells with stable expression of GFP, to aid in cell tracking *in vivo*, were generated by transfection of pEGFP-N1 (Clontech Laboratories Inc., Windsor, Ontario, Canada) into HT29 cells using Lipofectin (Life Technologies, Inc.) followed by selection in medium containing G418; stable GFP expression was verified after several passages in culture by flow cytometry (FACScan; Becton Dickinson, Mississauga, Ontario, Canada). HT29-hCG cells were generated by transfection of a pClneo-hCG expression vector (gift of Dr. Bert Vogelstein, Johns Hopkins Medical Center, Baltimore, MD) into HT29 with LipofectAMINE 2000 (Life Technologies, Inc.) followed by selection in G418. Medium was collected from isolated individual clones and screened for levels of the secreted β -subunit of hCG using the pathozyne-free β -hCG enzyme immunoassay kit (Omega Diagnostics, Aliso, Scotland, United Kingdom). Selected positive clones were injected i.p. into *nu/nu* mice to test for hCG secretion into the urine [for use as a soluble surrogate (molecular) marker of tumor progression and disease burden, as well as response to therapy; Ref. 28]; urine was collected weekly and assayed as above for the presence of hCG, normalized to levels of creatinine (creatinine detection kit; Sigma Diagnostics, Oakville, Ontario, Canada). A single clone (#20) was subsequently chosen for use in all of the experiments described below.

Spheroid Culture and Disaggregation *in Vitro*. Spheroids were made using the liquid overlay technique as described previously (1), although polyHEMA (Aldrich Chemical Co., Milwaukee, WI) was used to coat plates in place of agarose; the transient addition of 2% polyHEMA in 100% ethanol leaves, after evaporation of residual ethanol, a thin film that prevents cell adhesion to the plastic surface. For disaggregation experiments, 1,000, 5,000, 10,000, or 20,000 cells of HT29 or CaOV-3 were plated per well in polyHEMA-coated, round-bottomed, 96-well plates (Life Technologies, Inc.) in 100 μ l of complete medium and allowed to form spheroids for 5 days (75% of the medium in each well was changed at day 2 and day 4). After 5 days, SHE78–7 (or isotype-matched nonspecific murine IgG2a or anti-hMHC-I Ab) was added in 100 μ l of fresh medium to a final concentration of 1 μ g/ml; $F(ab')_2$ fragments of SHE78–7 were added in the same manner, although because they have a mass ~70% of their complete parent antibody, they were added at 0.7 μ g/ml to ensure exposure of cells to equimolar amounts of anti-E-cadherin binding sites. Disruption of preformed spheroids, or the lack thereof, was then observed and photographed at 6, 12, and 24 h from initial exposure.

***In Vivo* Disruption of Ascites Tumor Cell Clusters.** Xenografts were established by injecting *nu/nu* mice with 5×10^6 HT29 cells i.p.; within 2 days in the peritoneal cavity, injected single cells of HT29 spontaneously form free-floating multicellular aggregates of various sizes. Seven days after xenograft injection, mice were injected i.p. with 0.2 ml of PBS, either alone, or containing 10, 20, or 40 μ g SHE78–7 whole antibody, 20 μ g anti-hMHC-I mAb, or 14 μ g $F(ab')_2$ fragment of SHE78–7. After 48 h, the peritoneum of each mouse was flushed with 4 \times 5 ml washes of PBS, and the contents were collected into tubes for microscopic examination.

Survival Experiments. HT29(-GFP/-hCG) cells for injection were collected by trypsinization, rinsed free of fetal bovine serum, and prepared as

single-cell suspensions in PBS. Mice in all of the survival experiments were injected i.p. with 5×10^6 HT29(-GFP/-hCG) in 0.2 ml of PBS. After 1 week, mice were injected i.p. with 0.2 ml of PBS +/- antibody treatment [20 μ g SHE78–7 or 20 μ g α -hMHC-I Ab and/or 14 μ g SHE78–7 $F(ab')_2$; $F(ab')_2$ fragments were given at 70% of the dose of the complete antibody, reflecting the difference between their relative masses, as discussed above]. In three independent continuous therapy experiments, mice received additional antibody treatment, as described above, every 2 weeks. Mice were sacrificed (and their peritoneal contents examined) when: (a) profuse peritoneal hemorrhaging was observed; or (b) when transperitoneal tumors at the injection site(s) grew excessively large, in accordance with institutional guidelines. Mice that failed to develop tumors (as determined by postmortem examination on termination of each experiment) were excluded from subsequent analyses; average tumor take in the aforementioned models was ~6 of 7.

Detection of Urinary hCG as a Surrogate Marker of Tumor Progression. Pooled urine samples were collected from each group of mice (carrying HT29-hCG xenografts) once weekly and assayed for levels of hCG secreted by their respective xenografts using the Pathozyne-free β -hCG enzyme immunoassay kit (Omega Diagnostics), normalized to levels of creatinine (measured using creatinine detection kit from Sigma Diagnostics).

Experiments in $F_c\gamma R$ Knockout Mice. Wild-type *nu/nu*, $F_c\gamma R$ IIB $-/-$ *nu/nu*, or $F_c\gamma R$ III $-/-$ *nu/nu* mice were injected i.p. with a single cell suspension of 5×10^6 HT29-hCG cells in 0.2 ml of PBS. Treatments were given as follows: (a) $F_c\gamma R$ IIB $-/-$ single-standard dose (Fig. 6), 7 days after xenograft injection, mice were injected i.p. with 0.2 ml of PBS +/- 20 μ g SHE78–7; (b) $F_c\gamma R$ IIB $-/-$ biweekly treatment with subtherapeutic (1/10 of standard) dose (Fig. 7), beginning 7 days after xenograft injection, mice were injected i.p. with 0.2 ml of PBS +/- 2 μ g SHE78–7 every 2 weeks; (c) $F_c\gamma R$ III $-/-$ weekly treatment (Fig. 8), beginning 7 days after xenograft injection, mice were injected weekly i.p. with 0.2 ml of PBS + 20 μ g SHE78–7. Mice were sacrificed when profuse peritoneal hemorrhaging was observed or when transperitoneal tumors at the injection site(s) grew excessively large, in accordance with institutional guidelines.

Statistics. Survival curves were subjected to statistical analysis using the Mantel-Haenszel log-rank test. Transperitoneal tumor growth measurements [volumes approximated using Volume = (Length \times Width²)/2] were compared by paired *t* test. In all of the cases, statistical analyses, as well as best fit curve determinations for hCG data, were performed using the GraphPad Prism software package v3.0 (GraphPad Software Inc., San Diego, CA). Significance was set at $P < 0.05$.

RESULTS

A Neutralizing Antibody against E-Cadherin Disrupts Preformed Spheroids *in Vitro*. We have demonstrated previously the ability of SHE78–7 (26), a particularly effective antihuman E-cadherin neutralizing antibody, to prevent spheroid formation by a number of E-cadherin-expressing human cancer cell lines, including colon (HT29 and DLD-1), breast (MCF-7 and BT-20), and lung (L23) carcinoma lines (19). However, in a clinical setting it is critical that an antiadhesive can disrupt pre-existing adhesive interactions rather than simply prevent their occurrence. As such, we examined the ability of the SHE78–7 antibody to disrupt preformed spheroids of HT29 and CaOV-3, by allowing adhesive interactions to take hold for 5 days (Fig. 1, A and G) before exposing the spheroids to SHE78–7.

Within 6 h of exposure to 1 μ g/ml SHE78–7, the liberation of single cells from the periphery of intact spheroids could already be seen (Fig. 1B), and essentially complete disaggregation into a single cell suspension was achieved after 24 h in both cell lines (Fig. 1, D and H). Similarly, exposure to 0.7 μ g/ml SHE78–7 $F(ab')_2$ fragments resulted in complete disaggregation after 24 h (Fig. 1E); $F(ab')_2$ fragments were given at 70% of the dose of the complete antibody, reflecting the difference between their relative masses. Fig. 1 demonstrates this antiadhesive effect on small spheroids, comprised of 1000 cells; however, it should be noted that complete disruption of spheroids of up to 20,000 cells was achieved under the same experimental conditions (data not shown).

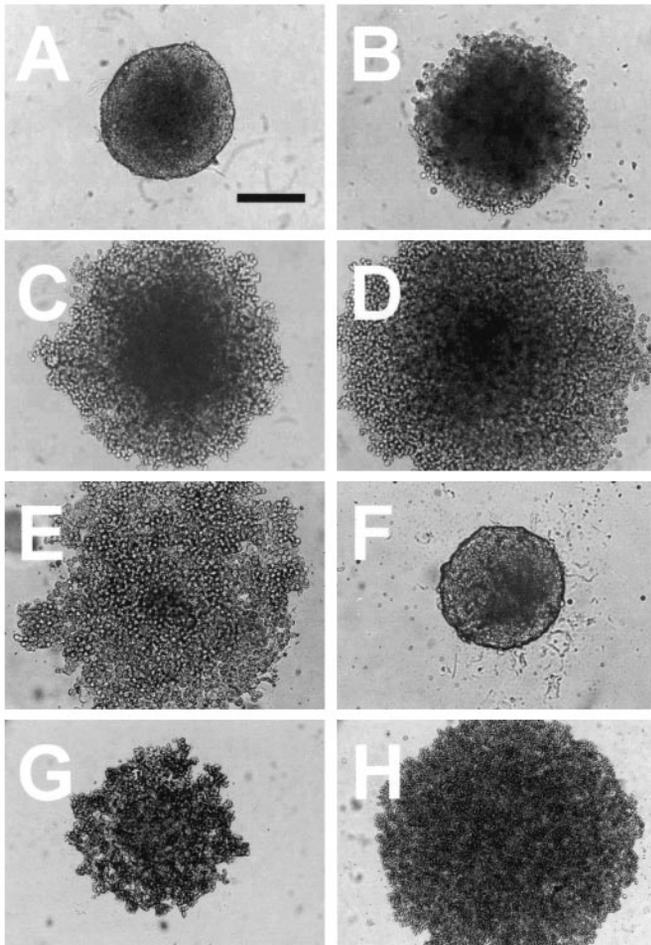


Fig. 1. Disruption of preformed spheroids of HT29 (A–F) and CaOV-3 (G and H) *in vitro* via exposure to SHE78–7. Five-day old, 1000-cell spheroids of HT29 (A) or CaOV-3 (G), in round-bottomed plates, were exposed to 1 µg/ml SHE78–7 (B–D and H), 0.7 µg/ml SHE78–7 F(ab')₂ fragments (E), or 1 µg/ml α-hMHC-I mAb (F), and photographed after 6 (B), 12 (C), or 24 h (D–F and H) under ×100 (A–F) or ×40X (G and H) magnification. Bar = 200 µm (A–F) or 500 µm (G and H).

In contrast, 24-h exposure of HT29 spheroids to an isotype-matched (IgG2a) nonspecific control antibody (not shown) or to an isotype-matched monoclonal antibody against the human MHC class I (which binds to the cell surface but should not affect cell-cell interactions) had no observable effect on adhesion (Fig. 1F). Finally, disruption of spheroids by SHE78–7 (whole Ab or F(ab')₂) did not cause cell death, confirming our previous finding that prevention of cell-cell adhesion by SHE78–7 led to a stimulation of growth in several human cancer cell lines, including HT29 (19).

A Neutralizing Antibody against E-Cadherin Disrupts Multicellular Aggregates of HT29 in an i.p. Ascites Tumor Xenograft Model *in Vivo*. Having established the ability of the SHE78–7 antibody to disrupt preformed aggregates of cells *in vitro*, we next sought to determine whether it could display similar antiadhesive properties *in vivo* in an i.p. ascites tumor model. i.p. xenografts were established in nude mice via i.p. injection of 5×10^6 HT29 cells in suspension, which spontaneously form free-floating multicellular aggregates in the peritoneal cavity within 2 days (Fig. 2A). Seven days after tumor cell injection, mice were injected i.p. with 0.2 ml of PBS, either alone or containing 10, 20, or 40 µg SHE78–7. Forty-eight h later, the peritoneum of each mouse was flushed with PBS, and the harvested contents were examined for evidence of disruption of cell-cell adhesion.

Mice treated with PBS alone showed no evidence of ascites tumor

cluster disruption (Fig. 2A). Injection of 10 µg SHE78–7 resulted in partial disruption, although the effect was incomplete (not shown); however, treatment with 20 µg SHE78–7 effectively disrupted the tumor cell-cell interaction, as few, if any, multicellular aggregates were recovered after 48 h (Fig. 2B). No additional antiadhesive effect was seen in mice treated with 40 µg SHE78–7; as such, 20 µg was chosen as an appropriate dosage for use in subsequent *in vivo* experiments. When 14 µg of the F(ab')₂ fragment (*i.e.*, 70% of 20 µg, the effective dose of intact SHE78–7) was injected in place of the whole Ab, i.p. tumor clumps were similarly disrupted (data not shown).

To ensure that the disruption was exclusively the result of targeting E-cadherin, this experiment was repeated, replacing 20 µg SHE78–7 with 20 µg of an isotype-matched α-hMHC-I Ab (which binds to HT29 but is nondisruptive *in vitro*). As expected, when peritoneal contents were examined 48 h after injection of the α-hMHC-I Ab, tumor cell clusters were indistinguishable from those of PBS-treated mice (data not shown).

SHE78–7-mediated Disaggregation of i.p. Ascites Tumor Clusters *in Vivo* in Nude Mice Prolongs Survival in an F_c- and Anti-adhesion-dependent Manner. Prevention of E-cadherin-mediated adhesion in HT29 grown in three-dimensional culture frees cells from

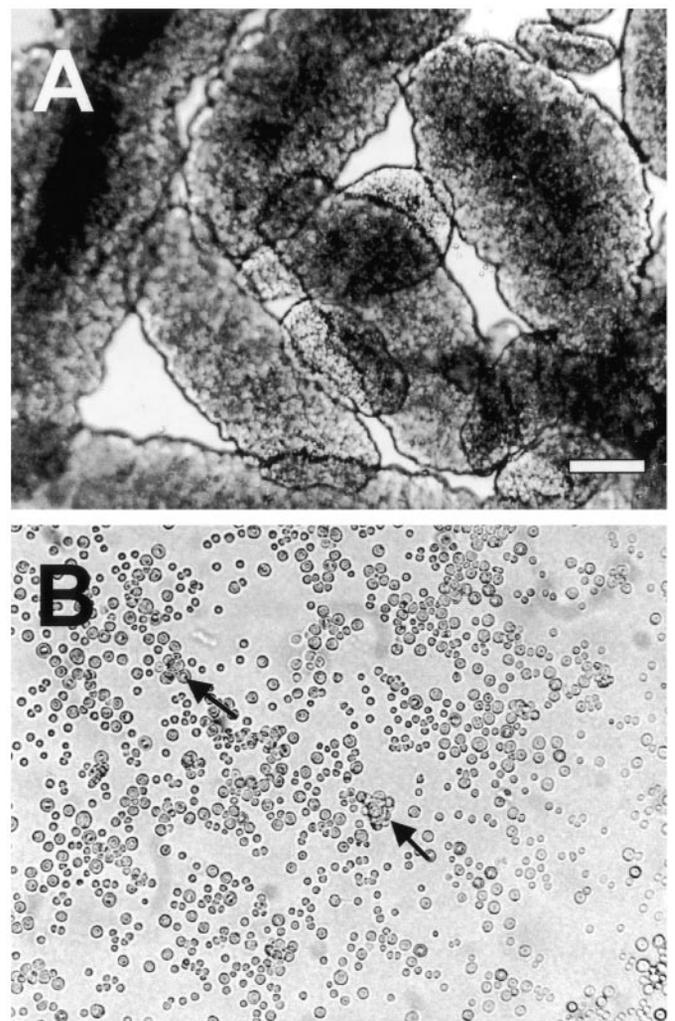


Fig. 2. Disruption of HT29 i.p. ascites tumor cell clusters *in vivo* via exposure to SHE78–7. A, multicellular aggregates of HT29, which spontaneously form in the peritoneum after i.p. injection of a single-cell suspension into *nu/nu* mice, rinsed from the peritoneum with multiple PBS washes, 7 days after xenograft injection; B, peritoneal contents of mouse with previously established (7 day) i.p. HT29 xenograft as in A, 48 h after i.p. injection of 0.2 ml PBS containing 20 µg SHE78–7; arrows indicate small residual multicellular aggregates. Bar = 250 µm.

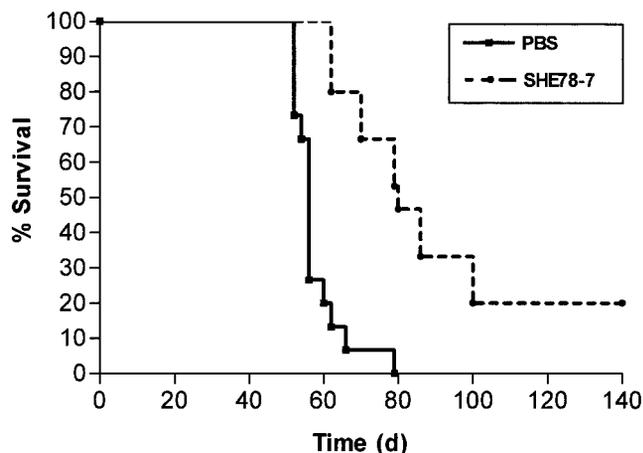


Fig. 3. SHE78-7-mediated disaggregation of i.p. HT29 ascites tumors *in vivo* in nude mice prolongs survival. *Nu/nu* mice ($n = 15/\text{group}$) were injected i.p. on day 0 with a single cell suspension of 5×10^6 HT29. On day 7, mice were injected i.p. with 0.2 ml PBS alone or containing 20 μg SHE78-7.

contact-mediated growth inhibition, thereby causing an increase in cell proliferation (19). As such, in the absence of a host immune response, one might expect SHE78-7-mediated disaggregation of i.p. HT29 ascites tumor clusters *in vivo* to accelerate disease progression by causing the liberated cancer cells to reproduce more quickly than they would otherwise.

However, as seen in Fig. 3, a single i.p. treatment with 20 μg

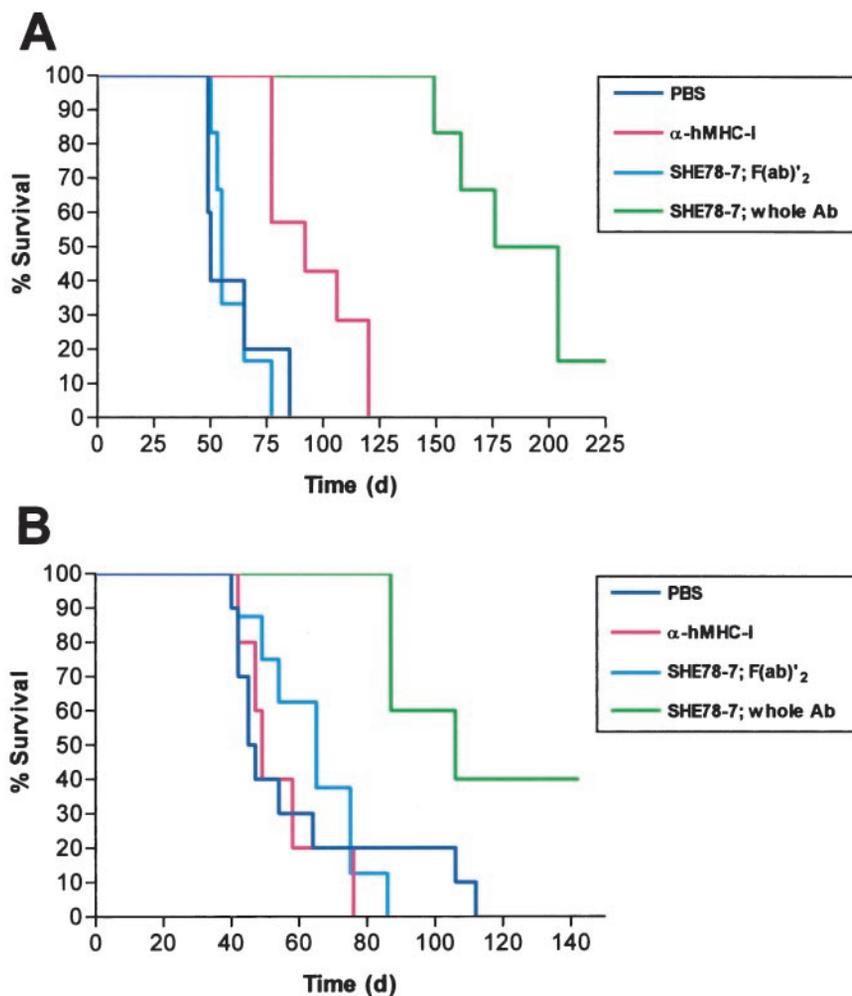
SHE78-7, 1 week after establishment of an i.p. HT29 xenograft, caused a significant ($P < 0.0001$) increase in survival in nude mice when compared with treatment with PBS alone ($n = 15$). Median survival times for the PBS and SHE78-7-treated groups were 56 and 80 days, respectively (Fig. 3), and were effectively reproduced in two independent experiments.

Subsequent experiments (Fig. 4) demonstrated that this increase in survival is enhanced additionally with repeated treatments (every 2 weeks) of SHE78-7 (Fig. 4, A and B; SHE78-7; whole Ab *versus* PBS). In i.p. xenograft models using two independent clonal derivatives of HT29, namely HT29-GFP (Fig. 4A) and HT29-hCG (Fig. 4B), biweekly administration of 20 μg SHE78-7 led to significant increases in survival over PBS-treated controls ($P = 0.0007$ and $P = 0.0198$, respectively), with median survival times of 190 days (Fig. 4A) and 106 days for SHE78-7-treated mice (Fig. 4B) compared with 50 and 47 days in the corresponding PBS-treated mice.

Also, at the time of sacrifice, 80% of PBS-treated mice in each experiment displayed profuse peritoneal bleeding, indicative of advanced i.p. disease, whereas no HT29-GFP-carrying mice and only 1 of 5 HT29-hCG-carrying mice treated with SHE78-7 developed peritoneal bleeding at any time. This suggests that the antitumor activity of SHE78-7 is particularly effective with respect to *bona fide* i.p. disease.

We hypothesized that this antitumor effect of SHE78-7, seen only *in vivo*, was attributable, at least in large part, to the ability of the antibody to recruit and/or enhance a host antitumor immune response. Although nude mice lack functional T-cell immunity, they maintain

Fig. 4. Survival of nude mice carrying i.p. xenografts of HT29 is prolonged via biweekly treatment with SHE78-7 whole antibody, but not with its F(ab')₂ fragment or an isotype-matched α -hMHC-I antibody. *Nu/nu* mice were injected i.p. on day 0 with a single cell suspension of 5×10^6 HT29-GFP (A) or HT29-hCG (B). Seven days later, and every 2 weeks thereafter, mice were injected i.p. with 0.2 ml PBS alone [$n = 5$ (A), 10 (B)] or containing 20 μg SHE78-7 whole antibody [$n = 6$ (A), 5 (B)], 20 μg α -hMHC-I antibody [$n = 7$ (A), 5 (B)] or 14 μg SHE78-7 F(ab')₂ fragments [$n = 6$ (A), 8 (B)].



populations of ADCC effector cells, such as natural killer cells and macrophages, and a functional complement cascade. As such, nude mice are capable of mounting an F_c -dependent immune response to antibodies of murine origin, such as SHE78-7, and the cells to which they bind.

To test whether the SHE78-7 antibody was, in fact, eliciting an F_c -dependent immune response, we included an additional group of mice in each of the aforementioned survival experiments (Fig. 4), which were treated biweekly with 14 μg of the $F(ab')_2$ fragment of SHE78-7. Because the $F(ab')_2$ fragment of SHE78-7 is capable of causing complete disaggregation of i.p. ascites tumor cell clusters *in vivo*, it would provide a survival advantage equivalent to that conferred by the intact antibody only if treatment of i.p. xenografts of HT29 with SHE78-7 was causing tumor cell death in an F_c -independent manner. However, as shown in Fig. 4, treatment with the $F(ab')_2$ fragment provided no significant survival advantage over PBS treatment in mice carrying xenografts of either HT29-GFP (Fig. 4A; $P = 0.984$) or HT29-hCG (Fig. 4B; $P = 0.6408$); median survival times of $F(ab')_2$ -treated mice were 55 and 65 days, respectively.

We hypothesized additionally that SHE78-7 is uniquely effective in its role of recruiting a host immune response because of its potent antiadhesive effects, which may enable it to act simultaneously as an immunosensitizer, enhancing any such response by increasing accessibility of target cells for immune effectors. To test this hypothesis, we included a group of mice in each survival experiment that were treated biweekly with 20 μg of an isotype-matched, HT29-binding, non-disruptive, α -hMHC-I monoclonal Ab. As predicted, SHE78-7 was significantly more effective than the α -hMHC-I Ab at prolonging survival of mice carrying i.p. xenografts of either HT29-GFP (Fig. 4A) or HT29-hCG (Fig. 4B). However, it should be noted that treatment with α -hMHC-I Ab did provide a significant survival advantage over PBS treatment in mice carrying HT29-GFP xenografts (Fig. 4A), confirming that this Ab is still capable of recruiting a limited F_c -dependent immune response. This was not the case in mice with HT29-hCG xenografts, likely because the relative antitumor capabilities of both the α -hMHC-I and SHE78-7 Abs were less pronounced in this model system, perhaps as a result of clonal differences between the hCG- and GFP-expressing HT29 lines. Nonetheless, the therapeutic superiority of the SHE78-7 Ab over the α -hMHC-I Ab in both systems strongly suggests that the antiadhesive ability of SHE78-7 is important, if not critical, to its efficacy as an immunosensitizer for an F_c -dependent immune response.

We subsequently reasoned that if, indeed, both the presence of an F_c portion and antiadhesive activity were necessary for SHE78-7 to act as a highly effective immunosensitizing agent, a similarly notable anticancer effect should, in theory, be achievable through the use of α -hMHC-I antibodies, which would enable recruitment of an F_c -mediated immune response, in combination with antiadhesive $F(ab')_2$ fragments of SHE78-7. As shown in Fig. 5A, this "combination therapy" regimen does indeed provide an enhanced survival advantage to mice carrying HT29-hCG i.p. xenografts that differs significantly from that conferred by PBS treatment ($P = 0.0334$), or monotherapy with either α -hMHC-I Ab ($P = 0.0020$) or SHE78-7 $F(ab')_2$ fragments ($P = 0.0013$) alone. The increased survival conferred by this combination therapy was statistically similar to that resulting from treatment with intact SHE78-7 antibodies.

In addition, pooled urine samples were collected weekly from each group of mice for tumor marker evaluation; levels of hCG secreted by the transfected tumor cells into the urine of xenograft tumor-bearing mice were normalized to urinary creatinine as a marker of renal function and used to measure tumor progression (28). As shown in Fig. 5B, the levels of hCG detected were indicative of substantially delayed disease progression in mice treated with either intact

SHE78-7 antibodies or the combination of α -hMHC-I Abs + SHE78-7 $F(ab')_2$ fragments, relative to all of the other treatment groups. Together with the survival data, this clearly demonstrates the importance of antiadhesion to the antibody-mediated, F_c -dependent antitumor immune response recruited by SHE78-7.

SHE78-7-mediated Antitumor Activity Is Enhanced in Mice Lacking the Inhibitory $F_c\gamma$ RIIB and Drastically Reduced in Mice Lacking the Activating $F_c\gamma$ RIII. F_c -dependent host immune responses in nude mice include those mediated by effector cells (ADCC) and those involving complement (CMC). Whereas complement is capable of binding directly to the CH2 domain of the F_c region of an antibody via complement protein C1q (29), ADCC effectors interact with antibodies via $F_c\gamma$ Rs on their cell surfaces. There are three known $F_c\gamma$ Rs, designated types I, II, and III, of which $F_c\gamma$ RII is the most widely expressed (30). Previous work has shown that antibodies that participate in ADCC do so much more effectively in the absence of the inhibitory receptor $F_c\gamma$ RIIB, although their efficacy is greatly compromised in mice lacking activating receptors, as in $F_c\gamma$ R $^{-/-}$ mice (25).

To determine which of these F_c -dependent processes, ADCC or CMC, factor most prominently into SHE78-7-mediated antitumor activity, we performed additional *in vivo* experiments, using an i.p. xenograft model as described above, comparing the therapeutic efficacy of SHE78-7 in wild-type nude mice *versus* nude mice that lacked expression of either the inhibitory receptor $F_c\gamma$ RIIB or the activating receptor $F_c\gamma$ RIII.

In the first of these experiments (Fig. 6), a single i.p. administration of 20 μg SHE78-7 was given to wild-type or $F_c\gamma$ RIIB $^{-/-}$ nude mice carrying i.p. xenografts of HT29-hCG, 1 week after xenograft injection. As shown in Fig. 6A, whereas SHE78-7 treatment significantly prolonged survival of wild-type mice compared with a group of PBS-treated controls ($P = 0.0136$), this effect was greatly enhanced in $F_c\gamma$ RIIB $^{-/-}$ mice ($P = 0.0246$), with all of the mice surviving beyond 100 days; median survival times for PBS- and SHE78-7-treated wild-type mice were 54 and 71 days, respectively. In addition, we found that tumor growth through the peritoneal wall at the xenograft/treatment injection site was significantly slower in $F_c\gamma$ RIIB $^{-/-}$ mice than in wild-type mice ($P = 0.0105$; Fig. 6B). However, such measurements fail to take into account any i.p. tumor growth that cannot be seen from the exterior of the mice. For this reason, levels of hCG, secreted by tumor cells into the urine of tumor-bearing mice, were measured to give an indication of complete tumor progression. In so doing, we found that the levels of hCG detected were notably greater in wild-type mice *versus* $F_c\gamma$ RIIB $^{-/-}$ mice (Fig. 6C), indicating more advanced disease progression in the former. The apparent exponential increases in hCG levels (Fig. 6C), in contrast to the linear growth curves associated with injection site tumor growth (Fig. 6B), suggest that the former may, in fact, be more reflective of true disease progression and/or imminent death.

In a separate experiment (Fig. 7), wild-type and $F_c\gamma$ RIIB $^{-/-}$ nude mice carrying HT29-hCG xenografts i.p. were treated biweekly, rather than once only as in Fig. 6, although the treatments used were 10-fold less than the established therapeutic dose of SHE78-7, *i.e.*, 2 μg instead of 20 μg . The rationale for the use of such subtherapeutic dosing is based on the observation that treatment of mice carrying s.c. xenografts of BT474MI breast carcinoma with subtherapeutic doses of Herceptin (*i.e.*, 10-fold less than the dose shown to be effective in wild-type mice), was sufficient to achieve a remarkable tumor growth delay in $F_c\gamma$ RIIB $^{-/-}$ mice but not in corresponding wild-types (25). Similarly, we have found that biweekly administration of 2 μg of SHE78-7 significantly delayed the growth of HT29-hCG tumors in $F_c\gamma$ RIIB $^{-/-}$ mice compared with wild-type ($P = 0.0134$; Fig. 7A). As in the previous experiment, levels of urinary hCG were notably

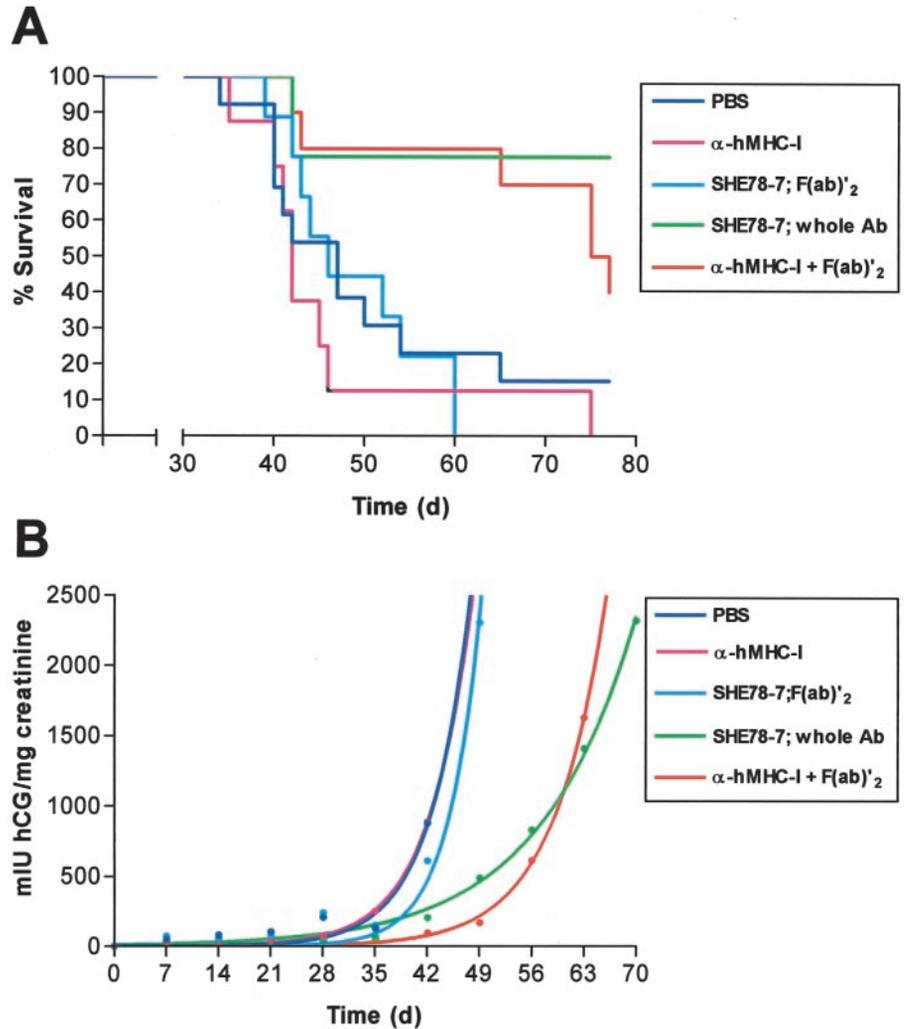


Fig. 5. Therapeutic efficacy of intact SHE78-7 mAb can be recapitulated via treatment with a combination of isotype-matched α -hMHC-I mAb + SHE78-7 F(ab)'₂ fragments. *Nu/nu* mice were injected i.p. on day 0 with a single cell suspension of 5×10^6 HT29-hCG. Seven days later, and every 2 weeks thereafter, mice were injected i.p. with 0.2 ml PBS alone ($n = 13$) or containing 20 μ g SHE78-7 whole antibody ($n = 9$), 20 μ g α -hMHC-I antibody ($n = 8$), 14 μ g SHE78-7 F(ab)'₂ fragments ($n = 9$), or a combination of 20 μ g α -hMHC-I antibody + 14 μ g SHE78-7 F(ab)'₂ fragments ($n = 10$). *A*, survival of mice in each treatment group. *B*, pooled urine samples were collected weekly from each group of mice and assayed for levels of hCG (normalized to creatinine levels) secreted by their respective xenografts; data points are shown with best fit curves.

different between the two groups (Fig. 7B) and were reflective of *bona fide* i.p. tumor growth delay in F_c γ RIIB $-/-$ mice compared with wild-types. This variable response to treatment with SHE78-7 is additionally illustrated by the fact that by week 9, 100% of wild-type mice, but no F_c γ RIIB $-/-$ mice, showed signs of peritoneal bleeding, which is an indication of advanced i.p. disease; on termination of the experiment at week 14, only 1 of 5 F_c γ RIIB $-/-$ mice had such peritoneal bleeding.

Whereas these experiments in F_c γ RIIB $-/-$ mice seem to implicate ADCC as the predominant F_c-dependent immune response ultimately responsible for the antitumor effect of SHE78-7, we sought to verify our findings by using a different, yet complimentary, knockout model; specifically, we used mice that retained expression of F_c γ RIIB, but instead lacked expression of an activating receptor, F_c γ RIII. Groups of wild-type or F_c γ RIII $-/-$ nude mice carrying i.p. HT29-hCG xenografts were treated weekly with PBS +/- 20 μ g SHE78-7, rather than biweekly, to maximize any F_c-mediated responses to the mAb. In so doing, we found that the response of wild-type mice to weekly treatment with SHE78-7, as assessed by transperitoneal tumor growth (Fig. 8A), was significantly ($P = 0.0238$) greater than that of F_c γ RIII $-/-$ mice; in fact, in F_c γ RIII $-/-$ mice, SHE78-7 was no more effective than PBS alone ($P = 0.4578$; data not shown) at slowing tumor growth. Again, we observed a vast difference in the levels of hCG secreted by xenograft cells in each of the two groups (Fig. 8B), demonstrating a profound

difference in therapeutic efficacy with respect to complete (*i.e.*, including disseminated, externally unobservable) disease.

Taken together, these data suggest that SHE78-7 does indeed mediate its antitumor effects predominantly via enhancement of ADCC, through F_c γ RIII. Furthermore, F_c γ RIIB was shown to negatively regulate this γ response, as in the absence of this receptor, the antitumor effect of SHE78-7 was greatly enhanced.

DISCUSSION

We have investigated the use of an antiadhesive mAb as a means of specifically targeting an effector immune response to human cancer cell xenografts *in vivo*, while simultaneously acting as an immunosensitizer by disrupting the multicellular aggregates in which the cells grow. We show that an antiadhesive Ab targeted at the cell adhesion molecule E-cadherin is capable of prolonging the survival of nude mice bearing ascites aggregates of HT29 human colorectal carcinoma, in a manner that is dependent on both its involvement in ADCC and its antiadhesive properties.

Antiadhesives have been championed previously as putative therapeutic agents in cancer treatment for a number of reasons, including their potential to interfere with adhesion critical for metastatic spread (31, 32), and their ability to cause chemo- and/or radiosensitization of therapy-resistant tumors (4, 7). However, to the best of our knowl-

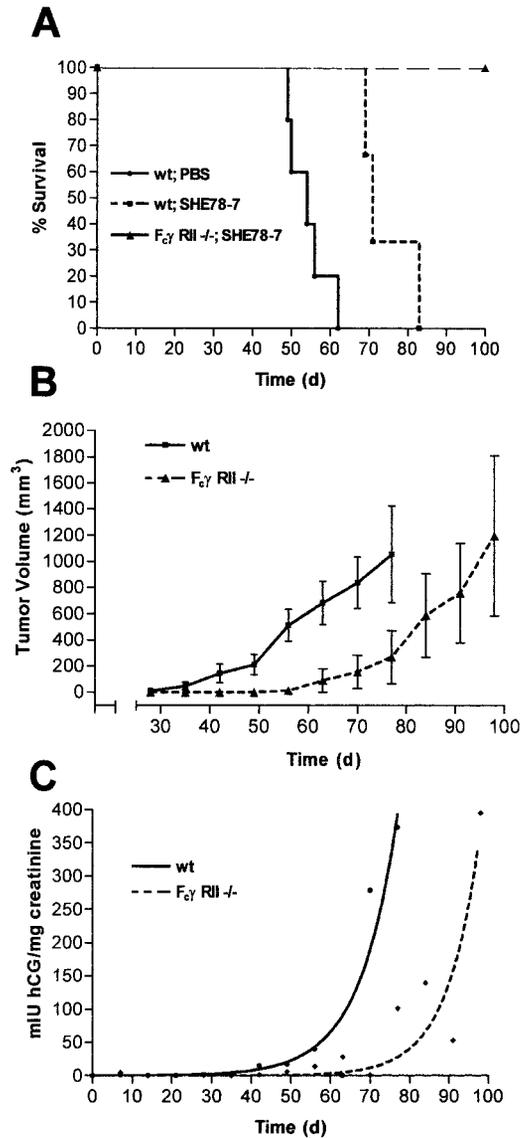


Fig. 6. SHE78-7-mediated anti-tumor effect is enhanced in $Fc\gamma RIIB^{-/-}$ nude mice. Wild-type (*wt*) *nu/nu* and $Fc\gamma RIIB^{-/-}$ *nu/nu* mice were injected i.p. on day 0 with a single cell suspension of 5×10^6 HT29-hCG. Seven days after xenograft injection, *wt* ($n = 3$) and $Fc\gamma RIIB^{-/-}$ mice ($n = 3$) were injected i.p. with 20 μ g SHE78-7 in 0.2 ml PBS, or (A) *wt* *nu/nu* mice ($n = 5$) were injected with 0.2 ml PBS alone. Tumor progression was assessed as follows: survival was monitored (A); transperitoneal tumors were measured weekly [Volume = (Length \times Width²)/2] (B); pooled urine samples were collected weekly from each group of mice and assayed for levels of hCG, normalized to creatinine levels, secreted by the xenografts; data points shown with best fit curves (C).

edge, this report is the first demonstration of the use of an antiadhesion strategy to sensitize cancers to immune-mediated destruction.

To date, many efforts to improve antibody-mediated treatment of cancer have centered on finding means by which to avoid immune neutralization of exogenous antibodies by, for example, engineering chimeric or humanized antibodies (20). However, recent work by Clynes *et al.* (25) showed that two mAbs approved for clinical use in cancer treatment, namely Rituxan (rituximab) and Herceptin (trastuzumab), were significantly less effective as antitumor agents when tested in $Fc\gamma R^{-/-}$ mice (incapable of ADCC) compared with wild-type mice (25). This finding illuminated the possibility of additionally improving the therapeutic efficacy of mAbs by addressing the extent to which they are capable of participating in certain host immune responses. Such a notion is strongly supported by another recent study, which showed an association between the $Fc\gamma RIIB$ -

158V/F genotype of non-Hodgkin lymphoma patients and their clinical response to rituximab (33). This suggests that, indeed, pharmacogenetic approaches may be used to design therapeutic antibodies tailored to take full advantage of anticipated immune responses in a given patient population, thereby giving rise to improved clinical outcomes.

However, such recruitment of natural host defenses may be hindered in tumors by the limited ability of immune effectors, including large molecular weight antibodies (10) and immunotoxins (11, 12), and especially lymphocytes (13, 14), IFNs (15), and complement (16, 17), to penetrate through a three-dimensional cancerous mass. Therefore, we reasoned that antiadhesives might provide a means to minimize, or overcome, such penetration or diffusion barriers by disrupting cell-cell contacts, thereby providing immune effectors with improved access to tumor cells. Furthermore, if an antibody was to be used as an antiadhesive agent, it would have additional putative therapeutic value as it could not only passively sensitize target cells to host defense mechanisms, but could become actively involved in immune-mediated antitumor activity.

However, it should be noted that high interstitial pressure gradients within a large solid tumor mass (Ref. 10; as opposed to a disseminated cancer as modeled in the experiments described herein) may sufficiently hinder the diffusion of an antibody, even one with antiadhesive function, as to seriously limit its therapeutic value in such a context. Whereas we feel that our data suggest that antiadhesives could aid, at least in part, in overcoming such diffusional constraints, additional experiments, *i.e.*, using solid tumors, would be necessary to fully elucidate the extent to which this may or may not occur.

We have found that SHE78-7 (26), a monoclonal antibody raised

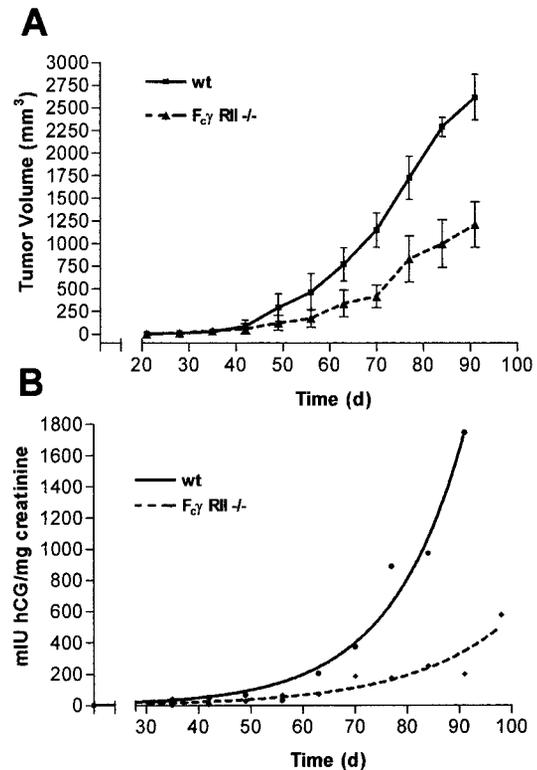


Fig. 7. Tumor response to subtherapeutic dosing with SHE78-7 is enhanced in $Fc\gamma RIIB^{-/-}$ nude mice. Wild-type *nu/nu* ($n = 4$) and $Fc\gamma RIIB^{-/-}$ *nu/nu* mice ($n = 5$) were injected i.p. on day 0 with a single cell suspension of 5×10^6 HT29-hCG. Both groups were injected i.p. on day 7, and every 2 weeks thereafter, with 2 μ g SHE78-7. A, transperitoneal tumors were measured weekly [Volume = (Length \times Width²)/2]. B, pooled urine samples were collected weekly from each group of mice and assayed for levels of hCG, normalized to creatinine levels, secreted by the xenografts; data points shown with best fit curves; bars, \pm SD.

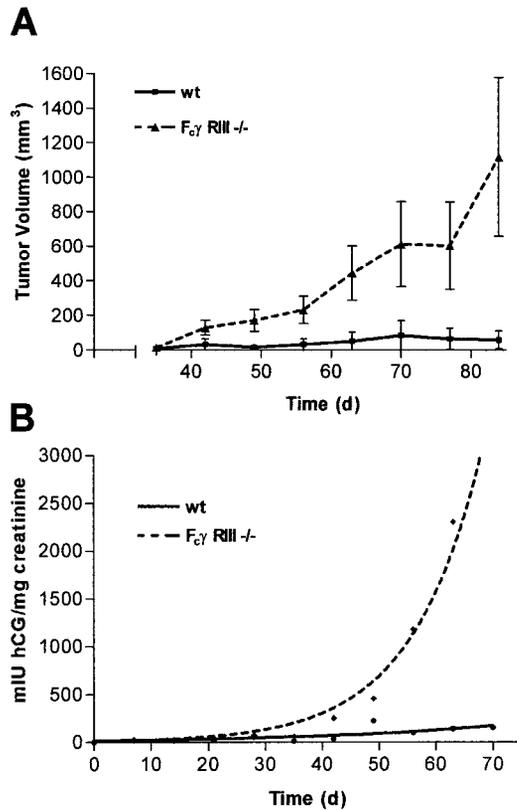


Fig. 8. Anti-tumor efficacy of SHE78-7 is drastically reduced in $F_c\gamma RIII^{-/-}$ nude mice. Wild-type nu/nu and $F_c\gamma RIII^{-/-}$ nu/nu mice were injected i.p. on day 0 with a single cell suspension of 5×10^6 HT29-hCG. Groups ($n = 7$) of each were injected i.p. on day 7, and every week thereafter, with 0.2 ml PBS + 20 μ g SHE78-7. A, transperitoneal tumors were measured weekly [Volume = (Length \times Width²)/2]. B, pooled urine samples were collected weekly from each group of mice and assayed for levels of hCG, normalized to creatinine levels, secreted by the xenografts; data points shown with best fit curves; bars, \pm SD.

against the human homophilic cell adhesion molecule E-cadherin, is one such antibody capable of acting as an antiadhesive. We have shown that not only can SHE78-7 prevent cell-cell adhesion by a number of human cancer cell lines in spheroid culture *in vitro* (19), but can in fact disrupt preformed spheroids of HT29 and CaOV-3 ovarian carcinoma *in vitro*, bringing us closer to a clinically relevant scenario in which an intact cancerous mass could be disrupted. Such disruptive ability is not unique to this particular mAb; for example, exposure to 1 mg/ml of MoAF-6D6, a mAb raised against a cell surface-associated adhesive factor isolated from rat ascites hepatoma AH136B cells, caused disruption, *in vitro*, of preformed multicellular aggregates of AH136B grown *in vivo* i.p. in Donryu rats (34). Naturally, such antiadhesive ability must also be functional *in vivo* before any claims can be made with respect to therapeutic potential. Our results show that the disruptive capacity of SHE78-7 can indeed be recapitulated *in vivo*, where, remarkably, a single i.p. injection of 20 μ g SHE78-7 is sufficient to cause disaggregation of an established HT29 xenograft growing as a lethal malignant i.p. ascites tumor in nude mice.

The ascites tumor xenograft model is characterized by free-floating multicellular aggregates of various sizes in the abdominal cavity, closely resembling those seen clinically in colon carcinomatosis (13) or advanced human ovarian cancer (35, 36). Therefore, the choice of HT29 and CaOV-3 (colon and ovarian cancer, respectively) for use in an ascites tumor xenograft model was a conscious effort to use a clinically relevant model on which to initially test the disruptive abilities of SHE78-7. Unfortunately, only the former was suitable for

use in an *in vivo* context, as CaOV-3 proved to be nontumorigenic when injected i.p. into nude mice.

Using this *in vivo* model, we have shown that 20 μ g of SHE78-7, injected i.p., confers a significant survival advantage on nude mice carrying HT29 ascites xenografts. In addition, we have shown that an α -hMHC-I mAb, though seemingly able to induce a partial F_c -dependent immune response, was significantly less effective than SHE78-7 at prolonging survival in two independent sets of experiments using distinct transfected clones of HT29. The observed partial antitumor effect of α -hMHC-I, seen only in mice bearing HT29-GFP, but not HT29-hCG, was most likely because of the ability of this mAb to participate in F_c -dependent host immune responses. However, it should be noted that repeated administration of 20 μ g SHE78-7 or α -hMHC-I appeared to cause a more robust antitumor effect when used against HT29-GFP than with HT29-hCG, suggesting that clonal differences in the two lines may necessitate minor therapeutic adjustments, *i.e.*, modification of the quantity of antibody and/or dosing schedule used, to more readily observe the differences in the therapeutic efficacies of the two antibodies with respect to the latter cell system. Nonetheless, we have demonstrated that the immunotherapeutic efficacy of an α -hMHC-I mAb against i.p. xenografts of HT29-hCG can be greatly enhanced, leading to delayed tumor progression and prolongation of survival when combined with antiadhesive $F(ab')_2$ fragments of SHE78-7. As such, we feel that the data as a whole strongly support the conclusion that the antiadhesive effect of the SHE78-7 mAb is instrumental in its potent antitumor activity.

It has been shown that prevention of E-cadherin-mediated adhesion causes apoptosis of HSC-3 oral squamous cell carcinoma in suspension culture (37), and that cell-cell contact can provide protection against apoptosis in colonic epithelial cells (38) and LIM1863 colon carcinoma cells (39). However, the antitumor effect of SHE78-7 against HT29 is unlikely the direct result of an induction of apoptosis via the disruption of cell-cell contacts, for two main reasons: (a) we have found that neither the prevention (19) nor disruption of E-cadherin-mediated adhesion induces apoptosis of HT29 growing in three-dimensional culture *in vitro*; and (b) the $F(ab')_2$ fragment of SHE78-7, although equivalent to the parent antibody with respect to antiadhesive activity, was unable to confer any survival advantage on mice under the same experimental conditions in which the whole antibody demonstrates antitumor activity. Therefore, we concluded that antiadhesion was necessary, but not sufficient, for the observed antitumor activity of SHE78-7. In fact, the failure of the SHE78-7 $F(ab')_2$ fragment to provide any significant survival advantage, unless coupled with an intact α -hMHC-I mAb, directly implicated an F_c -dependent host immune response in the antitumor activity of the whole antibody.

Nude mice, although lacking in functional T-cell-mediated immunity, retain the ability to mount F_c -dependent immune responses mediated by effector cells (ADCC) and complement (CDC). Recent work has implicated $F_c\gamma RIIB$ as an essential modulator of ADCC involving therapeutic monoclonal antibodies (25). However, this or any other $F_c\gamma$ -receptors are not required for CDC. As such, to determine which of these mechanisms was predominantly responsible for the antitumor activity of SHE78-7 *in vivo*, we tested the impact of the antibody on the growth of i.p. xenografts of HT29-hCG in $F_c\gamma RIIB^{-/-}$ and $F_c\gamma RIII^{-/-}$ nude mice, in comparison to wild-type nudes, reasoning that if ADCC was the more important of the two mechanisms in this case, the antibody should demonstrate added potency in mice lacking $F_c\gamma RIIB$, whereas its efficacy should be compromised in mice lacking $F_c\gamma RIII$.

Our results show $F_c\gamma RIIB^{-/-}$ mice do indeed mount an increased response to SHE78-7 treatment, both at full and subtherapeutic doses, compared with corresponding wild-type mice. In both treatment sce-

narios, tumor growth at the injection site(s), a characteristic feature of this model system, was significantly delayed in $F_c\gamma RIIIB^{-/-}$ mice. When mice received only a single treatment with 20 μg SHE78-7 (Fig. 5), this growth delay was accompanied by a significant prolongation of survival of $F_c\gamma RIIIB^{-/-}$ mice.

In addition, SHE78-7 treatment proved to be ineffective in $F_c\gamma RIII^{-/-}$ mice, despite being administered at twice the frequency required to see a profound antitumor effect in wild-type mice. Indeed, tumor growth proceeded seemingly unimpeded in the knockouts, in direct contrast to the growth hindrance caused by SHE78-7 treatment in wild-type mice.

Overall, this study supports the hypothesis that antiadhesives may be used as immunosensitizers with which to improve the efficacy of monoclonal antibody-mediated therapy of cancer. One important, and obvious, caveat to such a claim concerns the use of human E-cadherin as a target molecule in the present study. Numerous studies have shown that E-cadherin is down-regulated or absent in many human cancers (40), and, as such, it would presumably make a poor therapeutic target in such cases, in terms of both host toxicity because of damage of E-cadherin-expressing normal cells and reduced antitumor efficacy. One notable exception in this regard is ovarian cancer: the normal ovarian surface epithelium, the modified mesothelium from which ovarian carcinomas arise, is devoid of E-cadherin expression, whereas many ovarian cancers have been found to gain significant expression thereof (41, 42, 43). Indeed, it was for this reason that we chose an *in vivo* model for this work that mimicked, in some respects, advanced ovarian cancer. However, more importantly, this work may be viewed as a demonstration of the principle that cell adhesion, be it mediated by cadherins, integrins, or other adhesion molecules, may represent a hindrance to the therapeutic efficacy of monoclonal antibodies and should, therefore, be taken into account when investigating means by which to improve monoclonal antibody-mediated anticancer therapy. In this regard, there are certain cell adhesion molecules that are strongly up-regulated in cancer cells, compared with their normal cell counterparts, *e.g.*, ICAM-1 and MUC-18 in melanoma (44), or N-cadherin or CD44 in a number of malignancies (45, 46), among others. This differential expression may provide a therapeutic window for the use of antibodies directed against such targets, provided that the molecules mediate, in a significant way, tumor cell-cell adhesion.

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