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Award Number: DAMD17-99-1-9325

TITLE: Complement Inhibitory Membrane Proteins and Their Role in
Tumorigenesis

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REPORT DATE: September 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGEForm Approved
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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE September 2000	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Sep 99 - 31 Aug 00)	
4. TITLE AND SUBTITLE Complement Inhibitory Membrane Proteins and Their Role in Tumorigenesis			5. FUNDING NUMBERS DAMD17-99-1-9325	
6. AUTHOR(S) Stephen Tomlinson, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) New York University School of Medicine New York, New York 10016 E-MAIL: tomlinss@musc.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) The purpose of this work is to determine the role of complement and complement inhibitors in tumorigenesis and to determine whether reversing the effects of tumor-expressed complement inhibitors will allow effective immune-mediated clearance of tumor cells. The work proposed is based on developing rodent models of human breast cancer in which human tumor cells express rodent complement inhibitors. Toward the project aims, we have: 1. Transfected various human breast cancer cell lines with rodent complement inhibitors and isolated stably expressing cell lines; 2. Shown that the expression of rat complement inhibitors on human breast cancer cells (and a neuroblastoma cell line) protects transfected cells from lysis by rat complement; 3. Established rat models of human cancer and demonstrated for the first time in vivo that a complement inhibitor expressed on a tumor cell surface can promote tumor growth. Our data strengthen the hypothesis that the modulation of complement inhibitors on a tumor cell surface will provide an effective therapy when combined with complement-activating anti-tumor antibodies. Neutralization of complement regulatory proteins may also enhance a normally ineffective cytolytic humoral immune response.				
14. SUBJECT TERMS Breast Cancer, Complement, Complement inhibitors, CD59, Crry, Decay accelerating factor, membrane cofactor protein.			15. NUMBER OF PAGES 17	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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INTRODUCTION

Complement is one of the major effector mechanisms of the immune system and membrane complement inhibitors on the surface of breast tumor cells, may play a crucial role in determining tumorigenesis and the outcome of mAb-mediated immunotherapy. The objective of this proposal is to determine the role of complement and complement inhibitors in tumorigenesis and to determine whether reversing the effects of tumor-expressed complement inhibitors will allow effective immune-mediated clearance of tumor cells. Complement inhibitors are species selective, and human complement inhibitors are not effective against rodent complement. The work proposed is based on developing rodent models of human breast cancer in which human tumor cells are transfected with rodent complement inhibitors. The models will be used to determine whether complement inhibitors expressed on the surface of breast tumor cells modulate tumor progression *in vivo*, both in the presence and absence of breast tumor-specific complement-activating antibodies, and whether blocking the function of tumor-expressed complement inhibitors will effect the growth of tumor cells *in vivo* of human breast cancer.

BODY

Training: During the period of the award the PI has been trained in and has gained hands on experience in rodent models of breast cancer, including methods of statistical analysis. The award has also provided for the PI to spend the time to become better trained in tumor immunology (theory and practice).

TASK 1: Months 0-6: Preparation of DNA constructs and vectors for expressing mouse and rat complement inhibitors Crry and CD59.

This task has been completed. Constructs containing rat CD59 and Crry were prepared in the mammalian expression vector pCDNA3.

TASK 2: Months 6-24: Transfection of breast cancer cell lines with cDNA encoding rodent complement inhibitors (Crry, and CD59), selection of high expressing clones, and determination of the susceptibility of transfected and untransfected cell lines to human and rodent complement *in vitro*.

This task has been largely completed and extended. In addition to the breast tumor cell lines MCF7 and BT474 (in proposal), an additional breast tumor cell line, SKBR3, and a neuroblastoma cell line, LAN-1, have been transfected. The neuroblastoma cell line was included to determine if complement-effector mechanisms against breast cancer cells is similar or different for other types of tumor, and may lead to a better understanding of complement mechanisms involved against breast tumor cells.

Cell populations stably expressing either rat CD59 or rat Crry have been isolated for each cell line. Flow cytometric analysis of rat CD59 transfected MCF7 cell is shown below, and similar profiles were obtained for other cell lines expressing CD59 and Crry. An MCF7 cell line expressing both rat CD59 and Crry has also been isolated.

anti-rat CD59

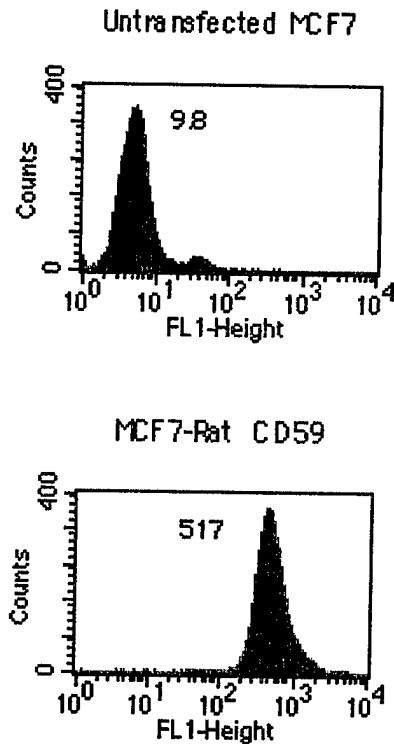


Fig. 1. Expression of rat CD59 by transfected MCF7. Stably transfected homogenous populations of MCF7 cells expressing rat CD59 were isolated by several rounds of cell sorting. Figure shows flow cytometric analysis of sorted populations. Cells were stained by immunofluorescence using anti-rat CD59 monoclonal antibody (6D1).

All cell lines (MCF7, BT474, SKBR3 and LAN-1) are resistant to lysis by human complement. The susceptibility of the transfected and untransfected cell lines to rat complement was determined. In every case, the expression of either rat CD59 or Crry on the human cell lines conferred resistance to lysis by rat complement. Complement susceptibility of transfected BT474 and SKBR3 is shown in fig. 2. These results were obtained using anti-membrane polyclonal antibodies to activate complement.

Further experiments using complement-activating monoclonal antibodies against breast tumor-associated antigens were also performed; BT474 is a HER2 positive cell line and MCF7 is a MUC1 positive cell line. Fig 3 shows that certain antibodies against the breast tumor-associated antigens HER2 and MUC1 are able to target breast cancer cell lines and activate complement. The anti-HER2 mAb used was from a commercial source. We have obtained an anti-HER2 IgG2b hybridoma, and will test this antibody (since commercial source is not available in high quantity for in vivo studies). The anti-MUC1 antibody has been designated C595 and was obtained from a collaborator (Dr. M. Price, University of Nottingham, UK). The transfected cell lines were protected from rat complement when these complement activating monoclonal antibodies were used, similar to that shown in fig. 2 using polyclonal antibodies. In addition to the breast tumor cell lines, the neuroblastoma cell line LAN-1 also became resistant to rat complement when expressing rat CD59 (fig. 4) and Crry (not shown).

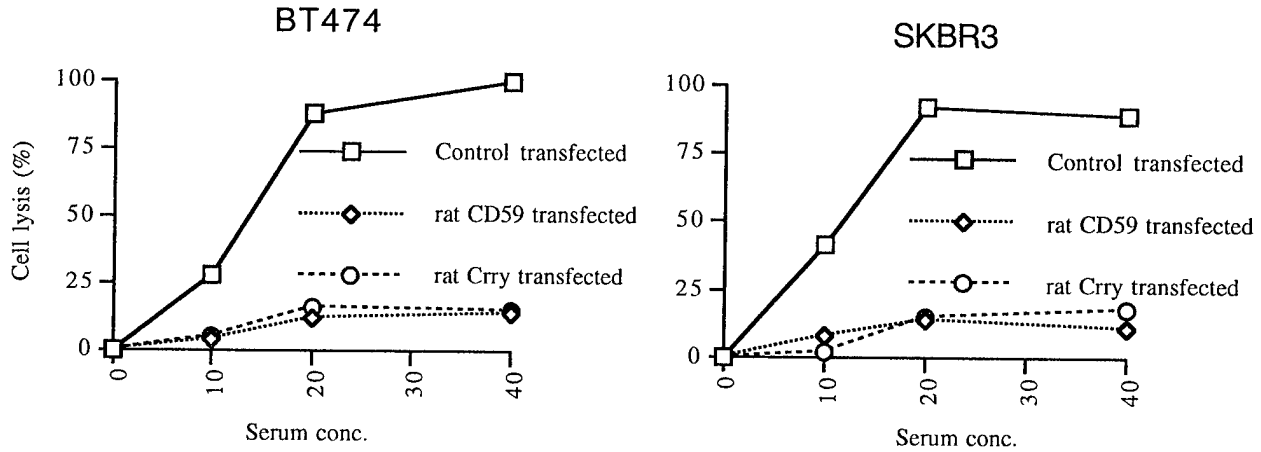


Fig. 2. BT474 and SKBR3 cells stably expressing rat CD59 or Crry were incubated in the indicated concentration of rat serum in the presence of anti-BT474 or anti-SKBR3 complement activating polyclonal antibody. Complement-mediated cell lysis was determined after 1 hour at 37°C. Antibodies were raised against cell membrane preparations.

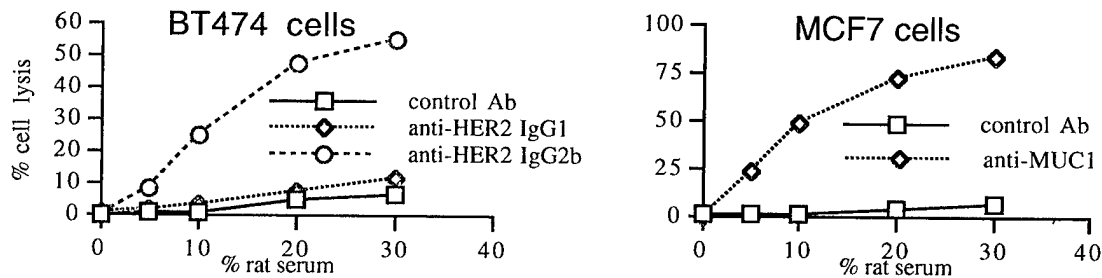
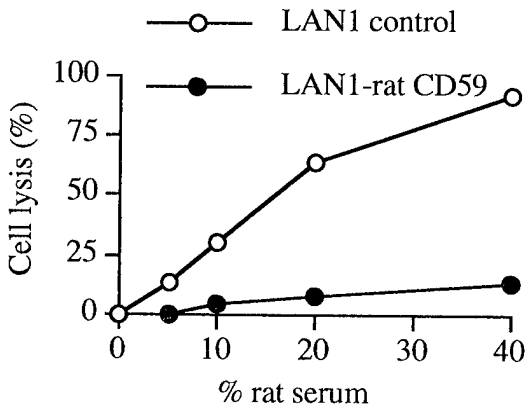


Fig. 3. Breast tumor cell lines can be sensitized to rat complement by antibody recognizing tumor-associated antigen. Standard assay procedures were followed (fig. 2). The antibodies were used at 20 ug/ml.

a. In presence of sensitizing antibody



b. In absence of sensitizing antibody

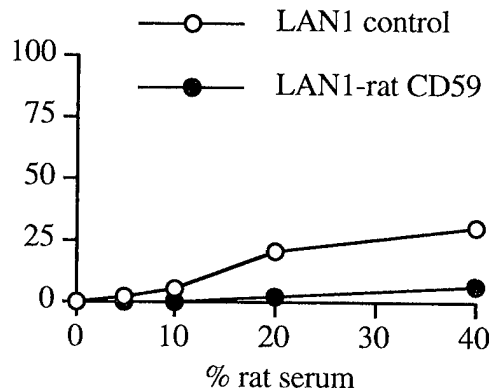


Fig. 3. Rat complement-mediated lysis of LAN-1 and LAN-1 expressing rat CD59. LAN-1 cells or LAN-1 cells stably expressing rat CD59 were incubated in the indicated concentration of rat serum either in the presence (panel a) or absence (panel b) of anti-GD2 (tumor associated antigen) complement activating antibody (3F8 mAb). Complement-mediated cell lysis was determined after 1 hour at 37°C.

TASK 3: Months 18-36: Determine the *in vivo* tumor growth rate in nude rats of untransfected breast cancer cells, and cells expressing various rodent complement inhibitors (estimate 100 rats required).

We are ahead of schedule have made progress on this aim. To determine whether complement inhibitors can promote tumorigenesis, we inoculated nude rats with MCF7 cells expressing rat Crry, rat CD59 or both and compared their growth *in vivo* with mock transfected control MCF7. A similar experiment has been performed using LAN-1 cells transfected with rat CD59. The data in figs 5 and 6 show that both rat Crry, but not CD59 significantly enhances MCF7 growth in nude rats. In contrast, CD59 significantly enhances growth of LAN-1. This is an indication that different complement-dependent mechanisms are involved in regulating the growth of these human tumors. The breast tumor experiments have been performed only once. Experiments using the LAN-1 cell line were begun earlier (tumors also grow faster), have been repeated several times, and are recently published (In appendix). These data represent the first *in vivo* study directly examining the effect of complement inhibitors on tumor growth, and fully support our hypothesis that inhibiting complement inhibitors on tumor cells will enhance antibody-mediated immunotherapy and immune-mediated clearance.

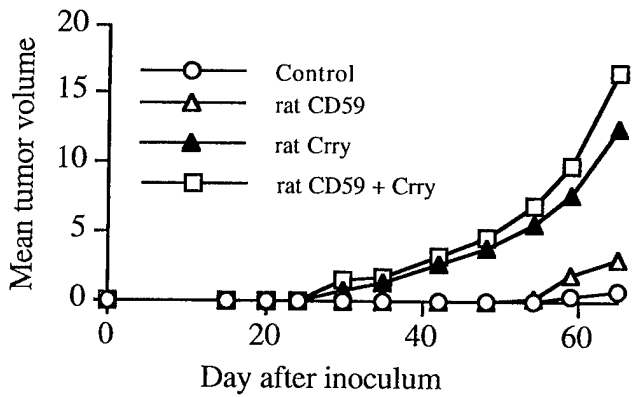


Fig.5. Growth curves of MCF7 breast cancer cells and MCF7 cells transfected with rat complement inhibitors in nude rats. Representative of 2 experiments where n=8/group.

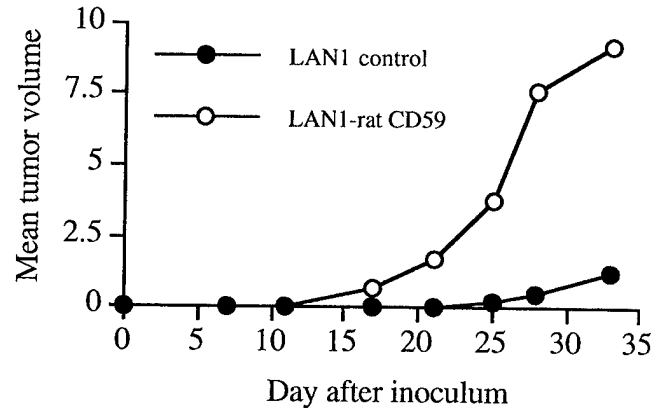


Fig 6. Growth curves of LAN-1 cells and rat CD59 transfected LAN-1 cells in nude rats. n=8.

TASK 4: Months 18-36: *In vitro* analysis of control and transfected tumor cells isolated from tumors grown in rats. Analysis of continued complement inhibitor expression, complement deposition, and *in vitro* susceptibility to complement.

TASK 5: Months 18-36: Determine the effect of blocking mouse Crry and CD59, expressed on human breast tumor cells, on the susceptibility of transfected human tumor cells to rat complement *in vitro*.

TASK 6: Months 24-48: Determine the effect of administering complement activating, tumor specific anti-HER2 and/or anti-MUC1 antibodies to rats bearing control and transfected tumors (estimate 60 rats required). Analysis of isolated tumor cells.

TASK 7: Months 30-48: Determine the effect of blocking mouse Crry and CD59, expressed on human breast tumor cells, on the growth of transfected human tumor cells in nude rats (estimate 100 rats required). Analysis of isolated tumor cells.

KEY RESEARCH ACCOMPLISHMENTS

- Shown that the expression of either rat CD59 and rat Crry on human breast cancer cells (and a neuroblastoma cell line) protects them from lysis by rat complement. Used transfected human tumor cell lines to show directly that complement inhibitors expressed on tumor cells provide effective protection from complement-mediated lysis.
- Confirmed the species selectivity of human complement inhibitors expressed on breast tumor cells.
- Established rat model of human cancer. Demonstrated for the first time in vivo that a complement inhibitor expressed on a tumor cell surface can promote tumor growth.

REPORTABLE OUTCOMES

Publications, abstracts and presentations:

- Caragine, T., Chen, S., Frey, A.F., Cheung, N.K. and Tomlinson, S. (2000). Expression of a complement inhibitor on the surface of MCF7 breast cancer cells promotes tumor growth. Era of Hope: Department of Defense Breast Cancer Research Program, Atlanta, June 8th-12th, 2000.
- Chen, S, Caragine, T., Cheung, N, K. V. and Tomlinson, S. (2000) CD59 Expressed on a Tumor Cell Surface Modulates DAF Expression and Enhances Tumor Growth in a Rat Model of Human Neuroblastoma. *Cancer Res.*, **60**, 3013-3018.

Cell Lines

- Breast cancer cell lines (MCF7, BT474 and SKBR3) and a neuroblastoma cell line (LAN-1) stably expressing rat CD59 and rat Crry complement inhibitors have been developed.

CONCLUSIONS

We have established rodent models of human cancer that are suitable for evaluating the role of complement and complement inhibitors in the growth and control of breast cancer. We have shown for the first time in vivo that a complement inhibitor expressed on a tumor cell surface can promote tumor growth. Our data strengthen the hypothesis that the modulation of complement inhibitors on a tumor cell surface will provide an effective therapy when combined with complement-activating anti-tumor antibodies. Neutralization of complement regulatory proteins may also enhance a normally ineffective cytolytic humoral immune response.

CD59 Expressed on a Tumor Cell Surface Modulates Decay-accelerating Factor Expression and Enhances Tumor Growth in a Rat Model of Human Neuroblastoma¹

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ABSTRACT

It has been hypothesized that complement inhibitors expressed on the surface of tumor cells prevent effective immune-mediated clearance. Whereas there are *in vitro* data to support this hypothesis, the species-selective activity of complement inhibitors has been a hindrance to investigating the role of membrane-bound complement inhibitors in rodent models of human cancer. The CD59-positive LAN-1 human neuroblastoma cell line was significantly more sensitive to lysis by rat complement than by human complement, illustrating the species selectivity of endogenously expressed complement inhibitors. Transfection of LAN-1 cells with rat CD59, an inhibitor of the terminal cytolytic membrane attack complex, effectively protected the cells from lysis by rat complement *in vitro*. When LAN-1 cells stably expressing rat CD59 were inoculated into immune-deficient rats, the onset of tumor growth and the rate of tumor growth were significantly enhanced compared with those of control-transfected LAN-1 cells. These data show directly that the expression of a complement inhibitor on a tumor cell promotes tumor growth. Flow cytometric analysis revealed that the endogenous expression of decay-accelerating factor (DAF), an inhibitor of complement activation, was up-regulated on the surface of cells after *in vivo* growth. Of further interest, higher levels of DAF were present on CD59-transfected cells than on control-transfected cells derived from tumors. Increased DAF expression correlated with decreased complement deposition on the tumor cell surface. These results show that expression of complement inhibitors on a tumor cell has functional consequences with regard to complement deposition *in vivo* and indicate that CD59 can indirectly effect complement activation and C3 deposition *in vivo* via a link between CD59 and DAF expression.

INTRODUCTION

Normal cells are protected from inappropriate complement attack by membrane-bound complement-inhibitory proteins that either prevent complement activation or block the formation of the terminal cytolytic MAC.³ Tumor cells also express complement-inhibitory proteins, sometimes at elevated levels, and provide tumor cells with protection from complement-mediated injury. Blocking the function of complement inhibitors expressed on the surface of tumor cells may allow effective immune-mediated clearance of some tumors and improve prospects for immunotherapy using complement-activating antitumor antibodies. Complement effector mechanisms that may be involved in host response to tumor cells include the activation and amplification of an inflammatory response, recruitment of immune effector cells, promotion and enhancement of cell-mediated lysis, and direct complement-mediated cytotoxicity. The major inhibitors of com-

plement activation on human cells are DAF and MCP. These proteins regulate complement enzymatic complexes that are involved in the amplification of the cascade and the resulting generation of C3/C4 opsonizing fragments and physiologically active C3a and C5a peptides. Formation of the cytolytic and proinflammatory MAC on host cell membranes is inhibited by CD59, a glycosylphosphatidylinositol-linked glycoprotein that binds to C8 and C9 in the assembling complex.

Complement inhibitors have been found on nearly all primary tumors and cancer cell lines that have been examined, and some studies indicate that complement-inhibitory proteins are up-regulated on tumor cells. DAF and the serum complement inhibitor factor H or related proteins have been identified as tumor-associated antigens (1, 2), and the overexpression of DAF confers a poor prognosis in colorectal cancer patients (2). *In vitro* studies have shown that complement inhibitors expressed on tumor cells can inhibit both complement opsonization and direct cytotoxicity by the MAC (for recent reviews of immune evasion and complement resistance of tumor cells, see Refs. 3 and 4). However, there is little information regarding how tumor-expressed complement inhibitors relate to complement deposition *in situ*, and the *in vivo* relevance of complement effector mechanisms and the importance of tumor-expressed complement inhibitors in controlling tumor growth remain largely unexplored. One reason for this is that complement inhibitor proteins (particularly CD59) are species selective, and human complement inhibitors are less effective against rat and mouse complement (5, 6). Thus, endogenous complement inhibitors expressed on the surface of human tumor cells will not provide the cells with effective protection from complement in rodent models of human cancer. Indeed, the species-selective activity of membrane complement-inhibitory proteins may be a basis for observations that complement-activating mAbs effective at causing regression of human tumors in rodents have, in most cases, proven ineffective in clinical trials.

When investigating the role of complement-inhibitory proteins in immune evasion of tumor cells *in vivo*, it is therefore relevant to study rodent complement inhibitors in rodent models of cancer. The ubiquitous and high level of expression of membrane complement inhibitors on normal tissues has not allowed for the targeted blocking of complement inhibitors (using current technologies) on tumor cells in syngeneic rodent models of cancer. In the studies described here, we investigated the effect of heterologously expressed rat CD59 on the growth of a human neuroblastoma cell line in nude rats. The neuroblastoma cell line endogenously expressed CD59, but we have previously determined on a quantitative basis that human CD59 is severalfold less effective at inhibiting rat complement compared to human complement (6). The data show for the first time *in vivo* that the complement inhibitor CD59 expressed on a tumor cell surface significantly promotes tumor growth. We also show that growth *in vivo* resulted in the up-regulation of DAF on the tumor cell surface and that the level of DAF expression was further up-regulated by the expression of functional CD59.

Received 2/14/00; accepted 4/17/00.

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¹ Supported by NIH Grant AI 34451 and Department of the Army Grants DAMD17-97-1-7273 and DAMD12-99-1-9325.

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³ The abbreviations used are: MAC, membrane attack complex; DAF, decay-accelerating factor; MCP, membrane cofactor protein; mAb, monoclonal antibody.

MATERIALS AND METHODS

Cells and DNA. The LAN-1 neuroblastoma cell line was obtained from Dr. Robert Seeger (University of California at Los Angeles, Los Angeles, CA) and maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS (Hyclone, Logan, UT), 2 mM glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Incubation was at 37°C in 5% CO₂. cDNA encoding rat CD59 and cDNA encoding murine Ly6E were the gifts of Drs. B. P. Morgan (University of Wales, Cardiff, United Kingdom) and U. Haemmerling (Memorial Sloan-Kettering Cancer Center, New York, NY), respectively. Stably transfected LAN-1 cell populations were selected by fluorescence-activated cell sorting after the cultivation of cells in the presence of G418.

Antibodies and Complement. mAbs to human (YTH53.1) and rat (6D1) CD59 and rabbit antirat C9 polyclonal IgG were the gifts of Dr. B. P. Morgan. Human MCP mAb M75 (7) was a gift of Dr. D. M. Lublin (Washington University, St. Louis, MO). Antihuman DAF mAb 1A10 was described previously (8), and anti-GD2 3F8 mAb (9) was described previously. Goat antihuman C3 IgG cross-reactive with rat C3 was obtained from ICN Pharmaceuticals (Aurora, OH). Anti Ly6A/E mAb D7 was purchased from BD Pharmingen (San Diego, CA). FITC-conjugated antibodies used for flow cytometry were purchased from Sigma (St. Louis, MO). Normal human serum was obtained from the blood of healthy volunteers in the laboratory, and rat serum was obtained from the blood of normal and immune-deficient rats. Serum was stored in aliquots at -70°C until use.

Preparation of LAN-1 Transfectants. Rat CD59 cDNA and Ly6E cDNA were subcloned into the multiple cloning site of mammalian expression vector pCDNA3 (Invitrogen, Carlsbad, CA). DNA was transfected into 50–75% confluent LAN-1 cells using LipofectAMINE according to the manufacturer's instructions (Life Technologies, Inc., Grand Island, NY). Stable populations of LAN-1 cells expressing either rat CD59 or Ly6E were isolated by several rounds of cell sorting using either antirat CD59 mAb 6D1 or anti-Ly6A/E mAb D7 as described previously (10).

Complement Lysis Assays. Complement-mediated cell lysis was determined by both ⁵¹Cr release (11) and enumeration after trypan blue staining (12), as described previously. Both methods gave similar results. Lysis assays of LAN-1 cells were performed using detached cells in both the absence and presence of antitumor complement-activating antibody. In assays in which cells were antibody-sensitized to complement, the anti-GD2 monoclonal antibody 3F8 was added at 15 μ g/ml, and cells were incubated for 30 min at 4°C before the addition of rat serum. Experimental details have been described previously (13).

Flow Cytometric Analysis. Analysis of cell surface protein expression and complement protein deposition was performed by flow cytometry using appropriate antibodies (see above), as described previously (10). Primary antibodies and isotype-matched irrelevant control antibodies were used at a concentration of 10 μ g/ml. Analysis was performed on cells removed from tissue culture using versene (Life Technologies, Inc.) for cell detachment and on cells isolated from excised tumors. Cell suspensions were obtained from tumors by gentle teasing of tumor tissue (in RPMI 1640/10% FCS) with scalpels, followed by low-speed centrifugation through Ficoll to remove tumor pieces and aggregates (14). Tumor-derived cells were then washed in RPMI 1640/10% FCS by centrifugation before use.

In Vivo Experiments. Four-week-old male athymic *nu/nu* (nude) rats were obtained from the National Cancer Institute (Frederick, MD). The rats were housed in a clean room, and food and water were sterilized. Rats were injected s.c. in the right flank with the indicated numbers of LAN-1 cells suspended in 0.2 ml of PBS. Groups of rats received either LAN-1 cells transfected with rat CD59 or control-transfected LAN-1 cells. Control cells were transfected with Ly6E (a structural but not functional homologue of CD59) or with empty plasmid. There was no difference in tumor growth between the different control LAN-1 cells. Tumor volumes were calculated using the formula $4/3\pi r^3$ (volume of sphere). Statistical analyses were performed using the SAS system (SAS Institute Inc., Cary, NC).

RESULTS

Expression of Rat CD59 on LAN-1 Cells Confers Resistance to Rat Complement. We have previously shown that LAN-1 expresses CD59, DAF, and MCP and that the sensitivity of LAN-1 and LAN-

1-derived clones to lysis by human complement can be significantly enhanced by blocking CD59 function. Blocking DAF function on LAN-1-derived clones only slightly enhanced sensitivity to human complement, whereas blocking MCP function had no effect (13). However, human CD59 is not an effective inhibitor of rat complement (6), and Fig. 1 shows that LAN-1 cells are significantly more sensitive to lysis by rat complement than lysis by human complement after sensitization by anti-GD2 3F8 mAb. LAN-1 cells express high levels of GD2 antigen, and the complement-activating properties of 3F8 mAb have been described previously (13, 15). Of note, LAN-1 cells are also lysed by rat complement in the absence of 3F8 mAb, albeit less effectively (Fig. 1b). These results confirm that endogenous expression of human complement inhibitors on LAN-1 cells does not provide effective protection from lysis by rat complement. Similar data were obtained with serum isolated from either normal or immune-deficient rats. Lysis of LAN-1 cells in the absence of sensitizing antibody may be due to the presence of natural endogenous complement-activating antibodies that bind to LAN-1 cells, and flow cytometric analysis of cells after incubation in heat-inactivated rat serum revealed that small amounts of rat immunoglobulin were deposited on the cell surface, supporting this possibility (data not shown).

LAN-1 cells were transfected with rat CD59, and LAN-1 cells stably expressing CD59 were isolated by cell sorting (Fig. 2). As a control for *in vivo* studies (see below), LAN-1 cells were also transfected with murine Ly6E antigen, a structural but not functional analogue of CD59, and sorted as described for rat CD59 transfectants. Fig. 3 shows that the expression of rat CD59 on LAN-1 cells significantly enhanced their resistance to lysis by rat complement, both in the absence and presence of complement-activating 3F8 mAb.

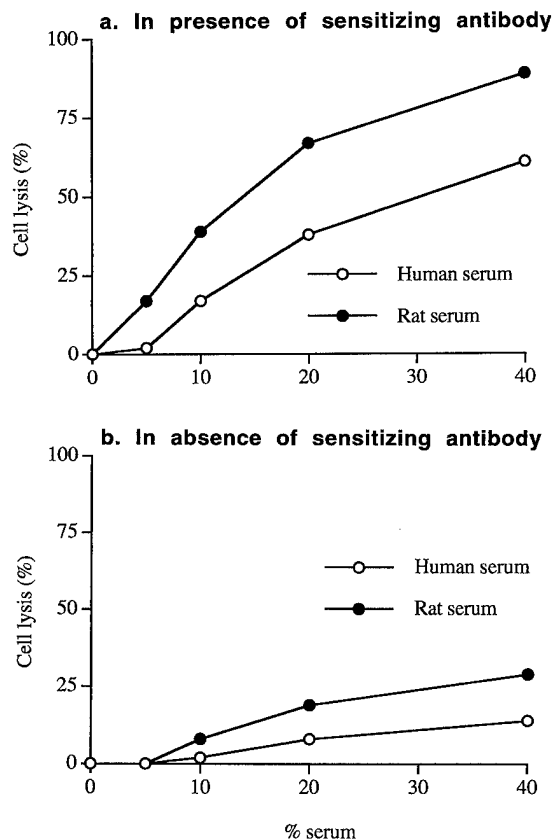


Fig. 1. Lysis of LAN-1 cells by human and rat complement. LAN-1 cells were incubated in the indicated concentration of rat or human serum in either the presence (a) or absence (b) of anti-GD2 complement-activating antibody (3F8 mAb). Complement-mediated cell lysis was determined after a 1-h incubation at 37°C. Representative data from at least three experiments are shown.

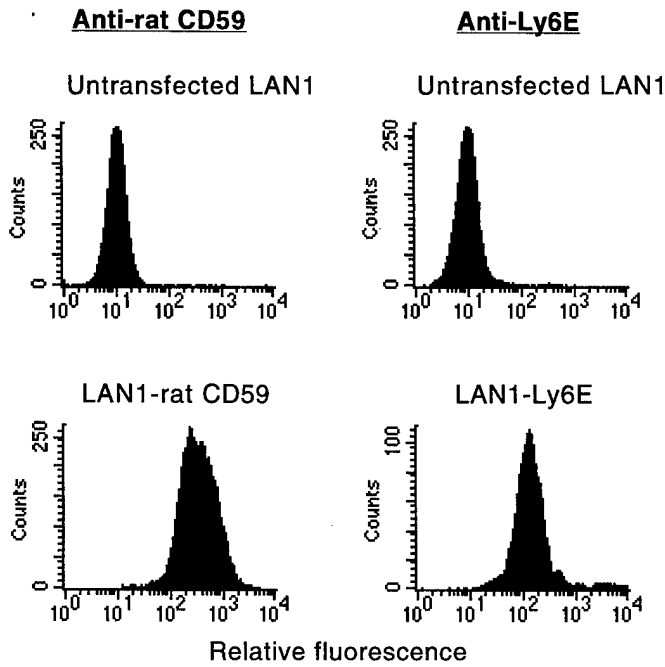


Fig. 2. Expression of rat CD59 and Ly6E on transfected LAN-1 cells. Stably transfected homogenous populations of LAN-1 cells expressing rat CD59 or murine Ly6E were isolated by several rounds of cell sorting. The figure shows flow cytometric analysis of sorted populations. Cells were stained by immunofluorescence using antirat CD59 mAb (6D1) or anti-Ly6E mAb (D7). Histograms of the relative fluorescence intensities are shown.

Expression of Rat CD59 on LAN-1 Enhances Tumorigenicity in Nude Rats. We first determined the tumorigenicity of LAN-1 cells in immune-deficient rats. The result of a dose-response experiment after s.c. injection of LAN-1 cells into the flank of nude rats is shown in Table 1. To investigate the effect of CD59 expression and increased complement resistance on *in vivo* tumor growth, control-transfected LAN-1 cells and LAN-1 cells stably expressing rat CD59 were injected separately into nude rats, and tumor growth was monitored. Groups of nude rats were inoculated with either 8×10^6 cells, a dose resulting in almost 100% tumor take for untransfected LAN-1 cells, or 4×10^6 cells, a dose determined to result in tumor growth in approximately 50% of animals (Table 1).

When LAN-1 cells expressing rat CD59 were injected into nude rats at a dose of 4×10^6 , 100% of rats grew tumors, and the onset of tumor growth was earlier than that seen for control-transfected LAN-1 cells ($P < 0.01$, χ^2 analysis). Regression analysis showed that the rate of tumor growth was also significantly faster in rats inoculated with rat CD59-transfected cells ($P < 0.01$). In addition, analysis of the mean difference in tumor size on each day of tumor measurement between the two groups of rats showed that tumors growing in rats inoculated with rat CD59-transfected cells were significantly larger, with $P_s < 0.01$ and an average P value of 0.0021 (Student's t test; Fig. 4a).

Increasing the inoculation dose to 8×10^6 cells resulted in almost 100% tumor take with both rat CD59-transfected cells (19 of 19 rats) and control cells (19 of 21 rats), as expected from the dose-response data shown in Table 1. The onset of tumor growth, however, occurred significantly earlier in rats inoculated with rat CD59-transfected cells (Fig. 5); the mean day of tumor onset was day 13 for rats inoculated with control LAN-1 cells and day 7.4 for rats inoculated with rat CD59-transfected LAN-1 cells. One week after inoculation, 7 of 21 rats inoculated with control LAN-1 cells contained tumors, whereas 15 of 19 rats inoculated with rat CD59-transfected LAN-1 cells contained tumors. This is a highly significant difference ($P = 0.001$,

χ^2 analysis). Similar to the data obtained with an inoculum of 4×10^6 cells, there was also a highly significant difference in the mean tumor size between rats inoculated with either control or rat CD59-transfected cells at each day of tumor measurement, with $P_s < 0.01$ and an average $P < 0.001$ (Student's t test; Fig. 4b).

Although there was a highly significant difference in the rate of tumor growth between rat CD59- and control-transfected LAN-1 cells when rats were inoculated with 4×10^6 cells, there was a less pronounced difference in rats inoculated with a higher number of cells (compare Fig. 4, a and b). In this context, our data indicate the presence of low concentrations of natural endogenous antibodies in nude rats that bind to LAN-1 cells (see above), and when a high cell inoculum or after a threshold tumor size is reached, it is possible that endogenous antitumor antibodies may become depleted. At this point, complement may no longer be effectively activated at the tumor cell surface, and complement-sensitive (control-transfected cells) and -resistant cells (rat CD59-transfected cells) may grow at similar rates.

Complement Deposition and Expression of Complement Inhibitors on Tumor-derived LAN-1 Cells. Cells isolated from tumors after 28 days of growth were initially analyzed for deposition of

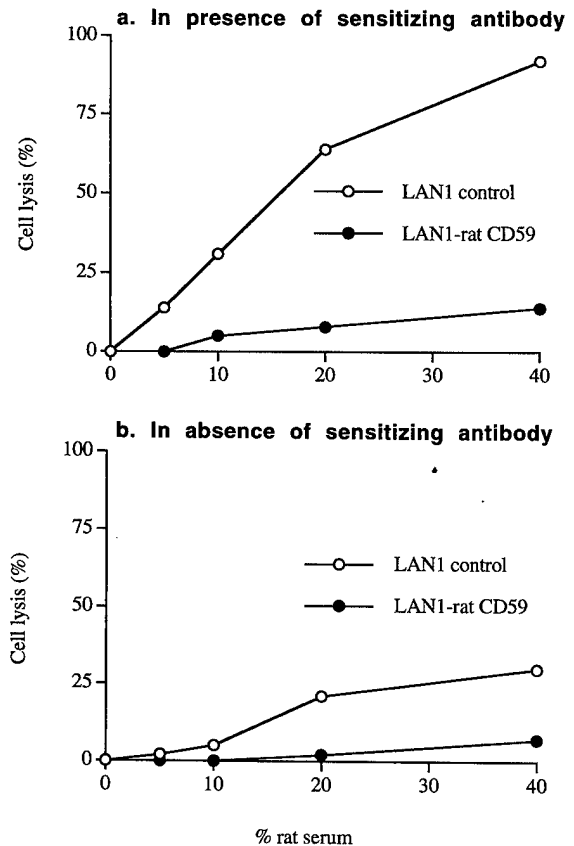


Fig. 3. Rat complement-mediated lysis of LAN-1 cells and LAN-1 cells expressing rat CD59. LAN-1 cells or LAN-1 cells stably expressing rat CD59 were incubated in the indicated concentration of rat serum in either the presence (a) or absence (b) of anti-GD2 complement-activating antibody (3F8 mAb). Complement-mediated cell lysis was determined after a 1-h incubation at 37°C.

Table 1. Tumor incidence of LAN-1 cells in immune-deficient rats

No. of cells injected ^a	No. of rats with tumor/ no. of rats inoculated
1×10^7	8/8
8×10^6	9/10
4×10^6	4/10
1×10^6	0/6

^a Rats were inoculated s.c. in the flank and examined for tumor growth for up to 30 days.

complement and the continued expression of transfected rat CD59 by flow cytometry. As shown in Fig. 6, expression of rat CD59 was maintained on the tumor cells at a level similar to that seen in *in vitro* cultured cells used for inoculation. Interestingly, the level of Ly6E expression on control-transfected LAN-1 cells was not maintained during *in vivo* growth. This finding may be the result of selective pressure exerted by rat complement on rat CD59 expression.

As shown above (see Fig. 1), unsensitized LAN-1 cells are lysed by rat complement *in vitro*, and, as anticipated, complement proteins C3 and C9 were both deposited on LAN-1 tumors *in vivo*. Less deposited C9 was detected on rat CD59-transfected tumor-derived cells than on tumor-derived control LAN-1 cells (Fig. 6), consistent with the known function of CD59. More surprising was the finding that rat CD59-transfected tumor cells also had lower levels of C3 deposited on their surface as compared with control cells; the difference was small but consistent (Fig. 6 shows the results from a representative analysis). This was surprising because CD59 does not inhibit complement activation and is not expected to influence C3 deposition. An explanation for these data was provided, however, when we analyzed the endogenous expression of complement inhibitors on LAN-1 cells. We compared the relative levels of endogenously expressed DAF, MCP, and CD59 between *in vitro* cultured LAN-1 cells and LAN-1 cells isolated from tumors. Fig. 6 shows that DAF expression was up-regulated on the surface of tumor-derived control LAN-1 cells by about twofold compared with *in vitro* cultured cells. The relative level of DAF expressed on rat CD59-transfected cells derived from tumors was even further up-regulated compared with that in cells grown *in vitro* (about threefold). Thus, the increased level of DAF expression is

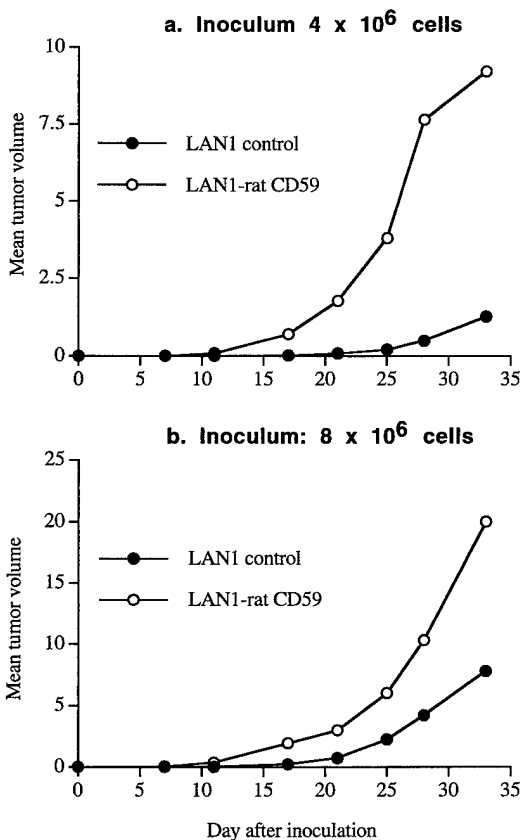


Fig. 4. Growth curves of control LAN-1 cells and rat CD59-transfected LAN-1 cells in nude rats. Either 4×10^6 cells (a) or 8×10^6 cells (b) were injected s.c. into the flank of nude rats. Growth was measured at intervals for 33 days. For experiment with a 4×10^6 inoculum (a), $n = 8$ rats/group; for the 8×10^6 inoculum (b), $n = 19$ rats/group.

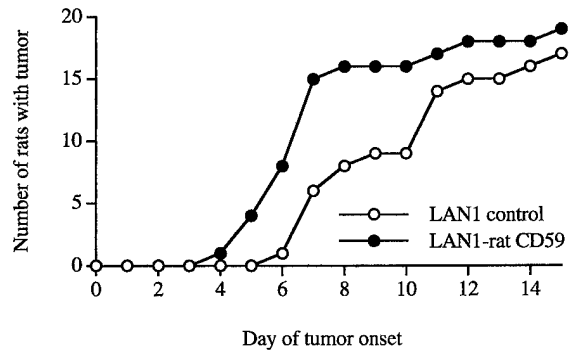


Fig. 5. Effect of rat CD59 expression on the onset of LAN-1 tumor growth. Control LAN-1 or rat CD59-transfected LAN-1 cells (8×10^6) were injected s.c. into the flank of nude rats, and the rats were examined daily for the appearance of a tumor (tumor recorded at a minimum diameter of 0.25 cm). $n = 21$ for the control group, and $n = 19$ for the rat CD59-transfected group.

likely to account for the decreased level of C3 deposited on the rat CD59-transfected tumor-derived cells. Of relevance to this finding, human DAF is known to inhibit rat complement, albeit less effectively than human complement (see "Discussion"). Multiple tumors from separate experiments were analyzed by flow cytometry, and the data shown in Fig. 6 are representative of at least six determinations for particular antigen groups. Transfection of LAN-1 with rat CD59 did not alter the level of endogenous DAF expression on cells cultured *in vitro*, and the level of endogenous CD59 and MCP expression on LAN-1 cells was unchanged after *in vivo* growth (Fig. 6). It is unlikely that the increased levels of DAF on LAN-1 cells after *in vivo* growth are due to selection because populations expressing higher-than-normal amounts of DAF could not be selected by cell sorting *in vitro*, and selection is not consistent with the finding that even higher levels of DAF are seen on rat CD59-expressing cells grown *in vivo*.

DISCUSSION

It has been hypothesized that complement inhibitors on the surface of tumor cells present a barrier to immune-mediated clearance of tumor cells by contributing to the ineffectiveness of humoral immune responses observed in some cancers or by preventing effective mAb-mediated immunotherapy. Nearly all human tumor cells examined express membrane complement-inhibitory proteins, and most display a high level of resistance to lysis by human complement *in vitro*, even in the presence of antitumor complement-activating antibodies. On the other hand, human tumor cell lines are more susceptible to lysis by heterologous complement. We show here that the LAN-1 human neuroblastoma cell line is highly susceptible to lysis by rat complement, despite the endogenous expression of complement-inhibitory proteins. Of relevance to this finding, we have shown previously that human CD59 is not an effective inhibitor of rat complement (6). Here, we established a LAN-1 neuroblastoma cell line stably expressing rat CD59 for use in a rat model of human cancer relevant for studying the role of complement and complement inhibitors. Using this model, we demonstrate directly that a complement inhibitor expressed on the surface of a tumor cell can influence tumor growth. We also found that DAF was up-regulated at the LAN-1 tumor cell surface after growth *in vivo* and that DAF was even further up-regulated on tumor cells expressing functional (rat) CD59 when grown *in vivo*. Increased DAF expression was associated with decreased C3 deposition. These data demonstrate that the expression of complement inhibitors on a tumor cell has functional consequences with regard to complement deposition and tumor growth.

The expression of membrane-bound complement-inhibitory pro-

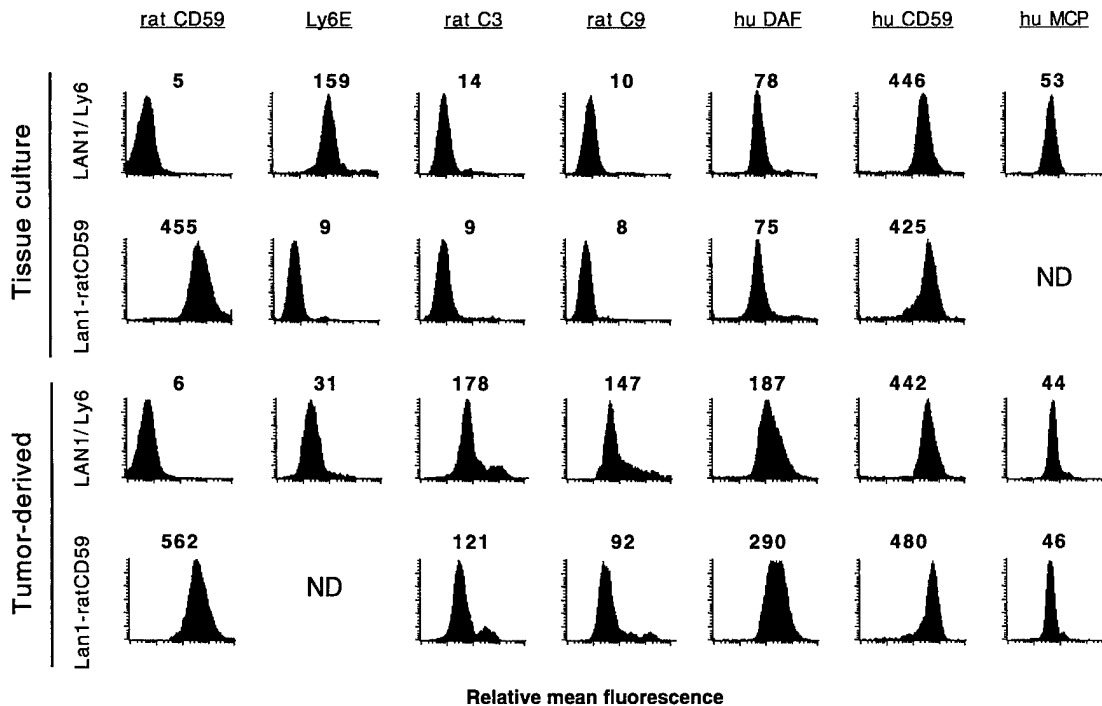


Fig. 6. Flow cytometric analysis of LAN-1 and rat CD59-transfected LAN-1 cells. Control or rat CD59-transfected LAN-1 cells grown in tissue culture (*top two rows*) or cells isolated from tumors (*bottom two rows*) were analyzed for expression of complement inhibitors and for the deposition of complement proteins as indicated. Cells were stained by immunofluorescence using appropriate antibodies (see "Materials and Methods"). The figure shows histograms of relative fluorescence, with numerals indicating the relative mean fluorescence intensities. Representative data are shown from at least six separate analyses for each antigen.

teins may benefit tumor cells for several reasons. Complement activation products (particularly C5a and the MAC) are powerful mediators of inflammation and may promote the recruitment of immune effector cells to the site of tumor growth. Cell-bound C3 activation products can promote and enhance antibody-dependent cell cytotoxicity and natural killer effector systems, and formation of the MAC can be directly cytolytic. Therefore, at least conceptually, it is reasonable to consider that up-regulation of complement inhibitors, as we observe here for DAF, may represent a mechanism by which some tumors can escape immune destruction. DAF is an inhibitor of complement activation and will inhibit the generation of C3/C5 activation products as well as the terminal MAC, whereas CD59 inhibits only MAC assembly. Because of the effect of CD59 on DAF expression, the current data do not provide information on the relative roles that these mechanisms may play in controlling tumor growth. However, the data do clearly establish that complement is involved in controlling tumor growth in this model and that CD59 promotes tumor growth whichever complement-associated mechanism(s) is operative.

So how does *in vivo* growth and, in particular, the expression of functional CD59 modulate DAF expression? Complement activation products and various cytokines have been reported to modulate complement inhibitor expression *in vitro*, although the effects appear to be variable, particularly for DAF (3, 4, 16–21). Also, a recent *in vitro* study reported that assembly of the MAC on endothelial cells directly up-regulated DAF expression and that expression was enhanced by cytokines (16). Similar mechanisms may be responsible for the up-regulation of DAF on tumor cells *in vivo*, as reported here. To explain the higher levels of DAF observed on rat CD59-expressing LAN-1 cells derived from tumors, it is conceivable that CD59-expressing cells may be able to survive higher levels of MAC that are initially deposited on the cell surface, thus enhancing the signal for DAF expression. CD59 limits the number of C9 molecules bound per MAC, and complexes containing bound C9, but with abrogated lytic function, may still be able to provide the signal for DAF up-regula-

tion. It is also possible that the signal for induction of DAF expression is delivered via rat CD59 after its engagement by assembling complement complexes. This notion is consistent with the demonstration that CD59 is a signal transducing molecule (22–26). Increased endogenous DAF expression on LAN-1 cells correlated with decreased rat C3 deposition, and in this regard, human DAF is able to inhibit rat complement, although it is a less effective inhibitor of rat complement than human complement.⁴

We show that LAN-1 cells activate rat complement in the absence of exogenously added complement-activating antibody both *in vitro* and *in vivo*. This is probably due to the presence of natural endogenous xenogeneic antibodies because rat immunoglobulin is deposited on the LAN-1 cell surface after the incubation of cells in nude rat serum. It may be that tumor cell lines that do not "spontaneously" activate rodent complement will require the administration of exogenous complement-activating antitumor antibodies for an effect of complement inhibitors on tumor growth to be observed in rodent hosts. Indeed, human tumor cell lines transfected with rodent complement inhibitors and grown in rodents may represent good preclinical models relevant for evaluating tumor-specific mAbs. For our studies, we chose to use a rat model because the rat complement system appears to be more robust than the murine complement system and may represent a better model. It is difficult to isolate hemolytically active mouse complement, and there are reports documenting low complement levels in common laboratory mouse strains and nude mouse strains as compared with complement levels found in humans and rats (27, 28).

In summary, our results show that a membrane complement inhibitor expressed on the surface of a tumor cell plays a role in determining tumorigenesis and that reversing the effects of tumor-specific complement regulators is likely to enhance immune-mediated clear-

⁴C. L. Harris, O. B. Spiller, and B. P. Morgan, personal communication.

ance of some tumors. The widespread expression of membrane-bound complement inhibitors presents technical difficulties for the selective blocking of complement inhibitors on tumor cells. However, it may be possible to adapt current and developing technologies to permit targeted delivery of antibodies, peptides, or perhaps antisense DNA to block the effects of endogenous complement inhibitors expressed on tumor cells.

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POSTER TEXT. Era of Hope, Atlanta, June 2000.

Title

Expression of a Complement Inhibitor on the Surface of MCF7 Breast Tumor Cells Promotes Tumor Growth

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Abstract

CD59, decay accelerating factor (DAF), and membrane cofactor protein (MCP) are widely expressed cell surface glycoproteins that protect normal host and tumor cells from the effects of homologous complement attack. Human complement inhibitors are unable to effectively control rodent complement, and this has been a hindrance to testing the role of complement and complement inhibitors in tumorigenesis in rodents. Using MCF7 breast tumor cells transfected with rodent complement inhibitors, we have established a rodent model of human cancer that is clinically relevant for studying the role of complement and complement inhibitors. We show directly for the first time in vivo that a complement inhibitor expressed on the surface of a breast tumor cell has functional consequences with regard to complement deposition and tumorigenesis.

Introduction

Complement is one of the major effector mechanisms of immunity. Complement effector systems involved in the immune response to tumor cells include direct complement-mediated cytotoxicity, amplification of inflammatory response, recruitment of immune effector cells and the promotion and enhancement of cell-mediated lysis. One or more complement regulatory proteins are expressed by all primary breast tumors and tumor cell lines that have been examined, and many primary tumor cells have been found to express higher than normal levels of complement inhibitors (up to 10-fold). The neutralization of CD59, DAF or MCP by function blocking monoclonal antibodies enhance complement-mediated lysis of tumor cells in vitro. These findings have lead to the hypothesis that complement regulatory proteins expressed on the tumor cell surface can promote tumorigenesis and present a barrier to effective monoclonal antibody immunotherapy. We propose that the reversal of the regulatory effects of these proteins will permit the effective immune clearance of tumor cells.

Objectives

- Prepare and characterize in vitro human breast tumor cell lines expressing rodent complement inhibitors, in order to develop a relevant model of human cancer.
- Determine whether complement inhibitors expressed on the surface of human breast tumor cells modulate tumor progression in vivo.

Experimental Design

- CDNAs encoding rat CD59 and rat Crry (the functional and structural analogue of human DAF and MCP) were transfected into MCF7 cells. Cell populations expressing high levels of rodent CD59 and/or Crry were isolated.
- Cells were incubated with sensitizing anti-tumor antibody and susceptibility of transfected cells to rat complement was determined. Cell lysis and C3 deposition were determined by ⁵¹Cr release assays and flow cytometry, respectively.
- 4 week old Rowett nude rats implanted with 90 day release pellets of 5 mg 17_estradiol were subcutaneously inoculated with 5 million control or transfected cells. Tumor growth was monitored and tumor-derived cells were analyzed by flow cytometry.

Results

1. Various tumor cell lines were examined for their susceptibility to human complement and expression of complement inhibitory proteins. The level of expression of endogenous complement inhibitors was directly related to their resistance to complement-mediated cytotoxicity (fig. 1)
2. Human tumor cell lines were significantly more susceptible to lysis by rat complement, illustrating the species selectivity of the endogenously expressed complement inhibitors. Human tumor cell lines (MCF7, breast and LAN-1, neuroblastoma) transfected with rat CD59 and Crry were resistant to rat complement (fig. 2). MCF7 cells expressing both rat Crry and CD59 were significantly more tumorigenic in nude rats than control transfected cells (fig. 3). Preliminary data show that Crry alone, but not CD59 alone is effective at promoting MCF7 growth in nude rats (data not shown). LAN-1 (neuroblastoma cell line) expressing a rat complement inhibitor was also significantly more tumorigenic in nude rats, but in apparent contrast to MCF7 data, rat CD59 was effective at promoting LAN-1 tumor growth (not determined for Crry). (fig. 4)

Conclusions

We show here that human tumor cell lines are susceptible to lysis by rat complement, despite the endogenous expression of complement inhibitory proteins. We established MCF7 and LAN-1 cell lines stably expressing rat

complement inhibitors for use in a rat model of human cancer that is relevant for studying the role of complement and complement inhibitors. Using this model, we demonstrate directly for the first time *in vivo* that complement inhibitors expressed on the surface of a tumor cell can influence tumorigenesis. These data show that the expression of complement inhibitors on a tumor cell has functional consequences with regard to complement deposition and tumor growth. These studies support the hypothesis that enhancing complement deposition on tumor cells *in vivo* will improve the outcome immunotherapies using complement activating anti-tumor antibodies, and may also enhance an ineffective cytolytic immune response against tumor cells in therapy which does not involve administration of exogenous antibodies.