Depletion of Total Hemolytic Complement in Sera from Hamsters Bearing Herpes Simplex Type 2-induced Tumors

Ernest D. Márquez, Joan M. Backenstose, and Janet L. Chapman

Department of Microbiology and Specialized Cancer Research Center, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

ABSTRACT

Total hemolytic complement (CHso) levels were compared in sera from normal hamsters and hamsters bearing tumors derived from herpes simplex virus type 2-transformed cells. CHso in normal sera ranged from 160 to 212 while CHso in tumor bearer sera ranged from 82 to 146. Preincubation of tumor bearer sera with cell surface proteins (CSP) from homologous herpes simplex virus type 2-derived tumor cells resulted in a 66% depletion of CHso whereas preincubation with heterologous herpes simplex virus type 1-derived tumor CSP resulted in a decrease of 26%. The depletion of CHso appeared to occur via the classic complement pathway. Similar results were seen using CSP from herpes simplex virus-infected cells although overall depletion of CHso was considerably less than that seen using tumor cell material. Using the complement subcomponent 1q (C1q)-binding test, tumor bearer sera and tumor bearer sera preincubated with homologous CSP were shown to contain increased levels of immune complexes not present in normal serum. These results indicate that the observed depletion of complement activity in sera from tumor-bearing hamsters could be the result of complement pathway activation by antigen-antibody complexes.

INTRODUCTION

Although there have been numerous attempts to correlate serum levels of complement with various neoplastic diseases, investigations of complement levels in the case of solid tumors have been limited. Drake et al. (4) have shown that hemolytic complement levels in tumor-bearing mice are progressively depleted as tumor burden increases. Evidence that complement may be associated in IC has been shown in human neoplasia (3) and in mice bearing mammary tumor (11). In the latter, mammary tumor virus-specific IC deposits were demonstrated in renal glomeruli. In addition, Heimer and de Vaux St. Cyr (7) have demonstrated the presence of circulating IC in sera of hamsters bearing SV40-induced tumors. In this report, evidence is presented which indicates that decreased complement titers in sera from tumor-bearing hamsters are due to activation of the classical complement system by formation of IC.

MATERIALS AND METHODS

Tumor Production. HSV-2 tumors were induced by HEF cells originally transformed by UV-irradiated HSV-2 strain 333 as previously described (5). These tumor cells were subsequently passaged twice in animals and 29 times in cell culture prior to use. Cell growth conditions were as described elsewhere (8). HEF cells originally transformed by UV-irradiated HSV-1 strain 14-O12 as described by Rapp and Duff (8) were used to induce HSV-1 tumors. Approximately 10⁶ cells were inoculated s.c. into syngeneic, weanling LSH/LAK hamsters (Lakeview Hamster Colony, Newfield, N. J.), and tumors were allowed to develop for approximately 4 weeks. The animals were exsanguinated, and all tumors were excised, measured, and weighed.

Reagents. Sheep RBC were purchased from Flow Laboratories, Rockville, Md. Anti-sheep RBC (19S) and whole guinea pig complement were purchased from Cordis Laboratories, Miami, Fla. Zymosan was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Bio-Rad protein assay and Enzymobeads were purchased from Bio-Rad Laboratories, Richmond, Calif. Sodium ¹²⁵I was purchased from New England Nuclear, Boston, Mass.

Preparation of CSP. CSP’s were prepared from tumor cells (HSV-2 TCSP), HEF cells (HEF CSP), and HEF cells infected with HSV-1 (HSV-1 CSP) and HSV-2 (HSV-2 CSP). The medium was removed, the cells were washed gently with 0.05 M phosphate-buffered saline (pH 7.4), and then the cell monolayer was vigorously agitated with phosphate-buffered saline which was removed and centrifuged to remove intact cells. Protein content of the supernatant was analyzed using the Bio-Rad protein assay according to manufacturer’s instructions.

Titration of CHso. Blood was removed from all hamsters by cardiac puncture. Immediately after clot formation (30 min at room temperature), the blood was placed at 5° for 1 hr to allow for clot retraction and then centrifuged. All sera were stored at −70° prior to use. Complement titrations were carried out as described by Rapp and Borsos (9).

Reaction of Immune Sera with Cellular Antigens. Acetone-fixed HEF, tumor cells, and HSV-2-infected HEF cells on coverslips were treated with tumor bearer serum or anti-HSV-2 antiserum (from rabbit) for 30 min at 37°. Control sera included normal hamster serum or normal rabbit serum. Anti-hamster IgG or anti-rabbit IgG labeled with fluorescein was then placed on the coverslips to visualize the attached antibodies. Relative degrees of reactivity of each serum were determined at the dilution of serum yielding minimal fluorescence on the control HEF cells.

Effect of CSP on CHso. To determine whether CSP could deplete the CHso levels in serum, 100-μl aliquots of CSP (containing 60, 100, or 200 μg of protein, respectively) were added to 100-μl aliquots of serum (1:25 dilution) from normal...
and tumor-bearing animals. The reaction mixtures were incubated at 37°C for 30 min, and the residual $CH_{50}$ was calculated. Confidence levels were established using Student's $t$ test.

To determine whether CSP could activate the classical or alternate complement pathways, a modification of the procedures described by Fine (6) was used. CSP (200 μl) was added to 200 μl of hamster serum. Serum containing 10 mM EDTA or EGTA as chelates was also used. As controls, zymosan and stroma from hemolysin-sensitized sheep RBC (EA) were included. All reaction mixtures were incubated at 37°C for 1 hr, cooled to 0°C, and centrifuged. To recalibrate the serum, 300 μl of supernatant were mixed with 300 μl of 10 mM CaCl2 and reincubated for 30 min at 37°C. Aliquots were removed and assayed for residual complement activity.

Detection of IC in Complement-depleted Serum. Detection of IC was carried out as described by Zubier et al. (12). $C1q$ was isolated from whole guinea pig complement using the precipitation technique described by Yonemasu and Stroud (10). The isolated $C1q$ was then radioiodinated using lactoperoxidase-glucose oxidase immobilized on beads (Enzymobeads) and following manufacturer's instructions. To provide a standard, antigen-antibody complexes were produced by mixing normal hamster serum with anti-hamster IgG (from goat) until a fine precipitate began to form. The precipitate was removed by centrifugation, washed, and resuspended in 0.05 M Veronal-buffered saline (pH 7.4), and the protein content was determined. Known aliquots of hamster antigen-antibody complexes formed by reaction of hamster IgG with anti-hamster IgG from goat were then added to normal hamster serum to create a standard curve. All experimental serum samples were then assayed and compared to the standard curve. The amount of IC present in each serum sample was calculated from the standard curve as μg hamster antigen-antibody complex equivalents per ml serum.

RESULTS

$CH_{50}$ in Normal and Tumor-bearing Hamsters. The results of $CH_{50}$ determinations are shown in Chart 1. Sera from normal and tumor-bearing hamsters showed a rather wide range of complement titers. Normal sera ranged from approximately 160 to 212 $CH_{50}$ while tumor bearer sera ranged from approximately 82 to 146 $CH_{50}$ ($p < 0.005$). An attempt was made to correlate the level of complement in tumor-bearing hamsters with variable tumor sizes. The results indicated that there was no direct correlation of tumor size with $CH_{50}$ (not shown).

Reaction of Immune Serum with Cellular Antigens. In accordance with earlier observations (5, 8), tumor bearer serum displayed greatest reactivity with homologous tumor cells and minimal reactivity with HEF cells. Some degree of fluorescence was also seen with virus-infected HEF cells. Anti-HSV-2 anti-serum displayed greatest reactivity against virus-infected cells but also displayed reactivity against tumor cells although with less intensity and on a lower percentage of cells. Normal control sera did not react to any extent with any cell type.

Effect of CSP on Complement Levels. As shown in Table 1, when CSP from tumor cells grown in vitro was added to homologous antiserum from a tumor-bearing hamster prior to the complement assay, the level of $CH_{50}$ decreased by approximately 66% ($p < 0.005$). When heterologous cell surface material was used, there was a lesser decrease of approximately 26% ($p < 0.025$). Addition of TCSP or HEF CSP to sera resulted in only slight decreases (<10%) in complement titer. Similar results were seen with VERO cells and SV40-transformed cells (not shown). Increased amounts of cell protein (100 μg) did not result in further substantial decreases in complement titer.

When sera were preincubated with CSP from herpes simplex virus-infected HEF cells (not shown), the greatest activity was seen when higher concentrations of cell material (100 to 200 μg) were used. As seen previously with tumor cell material, the greatest decrease was observed in the homologous system. CSP from HSV-2-infected cells decreased the complement titer in tumor bearer serum by approximately 28% while HSV-1 CSP decreased the complement level by approximately 18%. Further increases in protein concentration did not result in further substantial decreases in hemolytic complement titer. When CSP was added to either normal or tumor-bearing hamster serum, the decrease in hemolytic complement was less than 7% in all cases.
Activation of Classical Complement Pathway. The results (not shown) were consistent with previous observations in that homologous CSP in unchelated serum produced the greatest decreases in CH₅₀ (approximately 72%). EA also depleted CH₅₀ in unchelated serum (approximately 86%). As expected, EDTA effectively blocked all decreases in complement activity indicating both pathways were blocked. In EGTA-chelated serum, HSV-2 TCSP depleted the homologous serum CH₅₀ by only 10% while depletion was not seen with EA or with other CSP. However, almost complete depletion was seen with zymosan, the alternate pathway activator, indicating the presence of an active alternate pathway not activated to any great extent by EA or CSP.

Detection of Immune Complexes in Hamster Tumor-bearing Serum. The results of the Cl₉-binding assays are shown in Table 2. Normal serum contained an average of 2 μg of IC whereas tumor bearer serum contained an amount approximately 20 times as great. Addition of HSV-2 TCSP to normal serum caused a slight increase in detectable IC. Its addition to tumor bearer serum caused an increase of over 100% (p < 0.01). Addition of HEF CSP did not increase the level of IC in normal serum but increased the level of IC in tumor bearer serum by approximately 18% (p < 0.25).

**DISCUSSION**

Soluble tumor-associated antigens are released by malignant tumor cells. These soluble tumor antigens which have been detected as immune complexes (1) can presumably also complement with as has been shown in other immune complex-forming disorders (2). The decreased levels of complement found in tumor-bearing hamsters are in accord with this explanation. Tumor antigen, antibody, and complement as complexes formed at the cell surface could be shed into the serum, or soluble antigens could complex with antibody and complement at a site away from the tumor. In any case, the decrease in complement might be expected to parallel the quantity of antigen as reported previously (4). Therefore, the reason for the lack of correlation between tumor size and complement levels is unknown although it has been observed in other tumor-bearing hamsters (7). It is possible that overall tumor cell activity, including metastases that were active to various degrees in these animals, is the determining factor and one that cannot be quantified.

The observations that tumor bearer serum and anti-HSV-2 antiserum react with cellular antigens and that depletion of CH₅₀ occurred in sera from tumor-bearing hamsters after addition of tumor cell surface components suggest that formation of antigen-antibody complexes initiates the complement cascade and results in decreased complement levels. Also of importance is the observation that there appeared to be some degree of specificity with regard to tumor type. Although there is some cross-reactivity, the greatest decrease in hemolytic complement levels was observed in homologous systems (Table 1). Similar results were also seen with virus-infected cell material. There appeared to be more reactivity, however, using the tumor cell material rather than the virus-infected cell material. In addition, the limited reactivity seen with SV40-transformed cells would also indicate a degree of specificity for herpes simplex virus-related antigenicity.

The results indicate that the alternate pathway was active in normal hamster serum. However, the observation that EA and homologous TCSP depleted CH₅₀ to the greatest extent in unchelated serum and very little to nil in EGTA-chelated serum suggests that primary depletion of complement occurred via the classical pathway.

From the data presented in Table 2, it appears that immune complexes were produced in tumor bearer serum after preincubation with TCSP and that these are formed in addition to the immune complexes already present in tumor bearer serum. Thus, the formation of immune complexes would appear to be the major factor in depletion of CH₅₀ in vitro and quite possibly in vivo. Further studies are currently under way to determine the exact nature of the antigens involved in complement depletion and whether specific complement components are depleted or altered.

**ACKNOWLEDGMENTS**

We greatly appreciate the cooperation and support of Dr. F. Rapp and the helpful advice of Dr. J. Kreider and Dr. S. S. Tevethia.

**REFERENCES**

10. Yonemasu, K., and Stroud, R. M. C1₉: rapid purification method for prepa-
E. D. Márquez et al.


Depletion of Total Hemolytic Complement in Sera from Hamsters Bearing Herpes Simplex Type 2-induced Tumors

Ernest D. Márquez, Joan M. Backenstose and Janet L. Chapman


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/40/3/713

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.