Nature of Hamster Complement-fixing Antibody to Adenovirus 12 Tumor Antigen

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The presence of complement-fixing (CF) antibody reactive with T antigen and with viral C antigen in hamsters bearing adenovirus 12-induced tumors has been confirmed. Antibody activity in serum obtained at a time when the host was bearing large tumors was found to be associated exclusively with 7S immunoglobulins. Two populations of 7S immunoglobulins showing CF reactivity were distinguished by electrical charge, as determined by diethylaminoethyl cellulose chromatography and immunoelectrophoresis. No antibodies of the 19S IgM type were detected in the serum of hamsters bearing large tumors.

It is well recognized that virus-induced tumors usually grow progressively despite the presence of humoral antibody in the tumor-bearing host. In relating these antibodies to tumor resistance, it is becoming increasingly evident that the class of antibody elaborated by the host must be considered. The demonstration by Borsos and Rapp (2) of the more efficient fixation of complement by 19S antibodies than by 7S antibodies, as well as recent evidence indicating that IgG antibodies can inhibit the bactericidal effects of IgM molecules (3), reveal functional differences that occur among the classes of immunoglobulins. In general, it has been assumed that 19S antibodies are more cytolytic than are 7S antibodies, and that the latter class even may interfere with the action of the former type.

In view of these considerations, an investigation of the nature of antibody molecules evoked in hamsters by developing tumors produced with adenovirus type 12 was undertaken.

MATERIALS AND METHODS

Animals. Newborn Syrian hamsters were inoculated subcutaneously with adenovirus type 12. After large tumors had developed, the animals were bled and the tumors were harvested.

Antigens. The neo-antigen (T antigen) induced by adenovirus type 12 was prepared by hand-grinding an approximate 50% suspension of viable tumor tissue in phosphate-buffered saline, pH 7.2 (PBS), in an ice-bath. The suspension was centrifuged at $1,200 \times g$ at 0°C to remove gross amounts of tissue, and then at $105,000 \times g$ for 2 hr at 2°C to clarify the supernatant fluid. This crude preparation of T antigen was partially purified by precipitation with ammonium sulfate, first at 0.8 M to remove nonreactive material,

and then at 1.6 M to precipitate active antigen. The precipitate was redissolved in PBS at one-fifth the original volume and was reactive in complement-fixation tests at dilutions greater than 1:2,000.

Crude adenovirus 12 was prepared by freezing and thawing the residue of virus-infected KB cells. Cell debris was removed by low-speed centrifugation.

Viral A and C antigens (10, 13, 14) were prepared from crude virus by diethylaminoethyl (DEAE) cellulose chromatography. The crude material was centrifuged at $105,000 \times g$ for 2 hr to remove whole virus particles, concentrated 10 times by pressure dialysis, dialyzed against 0.015 M tris(hydroxymethyl)-aminomethane (Tris) buffer (pH 8), and applied to columns of DEAE cellulose equilibrated in the same buffer. The antigens were eluted by means of a linear gradient from 0 to 0.25 M NaCl in the same buffer. The antigens were separately pooled and rechromatographed. Purity of the preparations was monitored with group-reactive human serum and rabbit anti-adenovirus 12 serum.

Antisera. Three pools of sera reactive with the T antigen were prepared, each containing sera from at least 14 hamsters bearing large tumors induced by the neonatal inoculation of adenovirus 12.

To obtain reagent antisera for immunoelectrophoresis, rabbits were immunized with normal hamster serum in complete Freund's adjuvant. Each animal received three weekly, subcutaneous inoculations of 0.25 ml of serum into each of two sites, and was bled 10 days after the last injection.

Reduction and alkylation. Whole and fractionated hamster sera were dialyzed against 0.2 M 2-mercaptoethanol overnight at room temperature, then against PBS, 0.02 M iodoacetamide, and PBS, in that order, each at 4°C overnight. Control samples were dialyzed at the same times and temperatures against PBS only.

Complement-fixation (CF) tests. CF tests were performed in microtiter plates by means of a modifica-

tion of the method of Sever (12). Dilutions of antisera were made in tubes and added to the plates with microtiter pipette droppers. Exactly 5 complement H_{50} units of guinea pig complement were used, and the optimal concentration of T antigen for the system was determined by means of an antigen-antibody checkerboard titration. Fixation was allowed to proceed at 4 C for 18 to 20 hr, after which optimally sensitized sheep erythrocytes at a concentration of 2×10^8 /ml were added. Antibody titers are expressed as the reciprocal of that dilution which fixed 4 of 5 complement H_{50} units in the presence of optimal antigen concentrations.

Chromatographic separation of serum. DEAE cellulose column chromatography with tumor-bearing hamster sera was performed essentially as described above for viral A and C antigens. Three separate pools of sera of 15, 19, and 25 ml were dialyzed against 0.015 M Tris buffer (pH 8.0), centrifuged at $35,000 \times g$ for 30 min to remove the precipitate formed at low ionicity, and applied to columns of cellulose, 2.5×40 , 2.5×45 , and 2.0×80 cm, respectively. The columns were eluted by means of a linear NaCl gradient at flow rates of about 60 ml/hr and 10-ml fractions were collected. Protein in the fractions from the columns was monitored by measuring the optical density (OD) at 280 m μ .

Gel filtration. A calibrated column (2.5×95 cm) of Sephadex G-200, equilibrated with 0.14 M NaCl in 0.01 M Tris buffer (pH 7.4), was used. After the application of 2 to 4 ml of serum, the column was washed with the same buffer at a flow rate of 20 ml/hr, and 5-ml fractions were collected.

Immunoelectrophoresis. Electrophoresis was performed by a modification of the method of Scheidegger (11) with the use of 1% Noble agar in pH 8.6 barbital acetate buffer, 0.015 M. Separation was carried out for 50 min at 2.5 v/cm. After the addition of reagent antiserum to the trough, the slides were allowed to stand in a humid atmosphere at room temperature for 24 hr, washed with PBS for 16 hr, and stained with 0.1% Buffalo black.

Sucrose density gradient centrifugation. The procedure followed for sucrose density gradient centrifugation was described previously (7).

Titration of the first component of complement ($C'1$). $C'1$ reactivity was measured in microtiter plates as described by Nelson et al. (8).

RESULTS

CF reactivity of hamster sera. Sera were collected from 100 tumor-bearing hamsters at a time when the tumors were large but before the animals became moribund. The actual time of bleeding after the appearance of the tumor varied owing to a difference in the rate of individual tumor growth. A sample of each serum was heated at 56 C for 1 hr and was titrated for CF reactivity with a 1:200 dilution of T antigen, which had been found to be optimal for the system. Of the serum samples, 69 were found to have titers of from 32 to 512 against T antigen.

No correlation could be made between the CF antibody titer and the length of time the tumor was observed to be in the animal. Three pools of sera were made according to titer, i.e., 32 to 48, 64 to 96, and 128 to 512.

DEAE cellulose chromatography. The results of column chromatography are presented in Fig. 1. With each of the three pools of sera, two peaks of protein were observed, one passing through the column into the 0.015 M Tris buffer effluent and the second eluting with the NaCl gradient which was started after no more protein could be detected in the effluent. All fractions were tested for CF reactivity with T antigen. As may be seen in Fig. 1, two peaks of antibody reactivity were found: one in the effluent, and one eluting at 0.03 to 0.05 M relative NaCl concentration. Active fractions from each peak were pooled and concentrated by pressure dialysis.

Sucrose density gradient centrifugation. The two concentrated preparations (designated DEAE I and DEAE II by order of their elution from the DEAE cellulose column) and the original pools of whole serum were subjected to sucrose density gradient centrifugation. Figure 2 shows that activity in each of the three samples was found to concentrate in identical fractions from the sucrose gradient in the zone shown to correspond to 7S IgG rabbit antibody. The anticomplementary activity found in the sample of unheated whole serum was in the zone known to correspond to $C'1$ activity.

FIG. 1. DEAE cellulose chromatographic separation of unheated whole serum from tumor-bearing hamsters. Those fractions showing specific CF activity with T antigen are indicated in the shaded area. The bar (SM) represents the CF antibody titer of the starting material that was applied to the column.

Reduction and alkylation. Concentrated DEAE I and DEAE II fractions and the corresponding original pool of whole serum were treated with 2-mercaptoethanol or with 2-mercaptoethanol plus iodoacetamide. The results of representative experiments are shown in Table 1. It may be seen that 2-mercaptoethanol alone did not affect the CF reactivity of any of the antibody preparations. However, reduction followed by alkylation with iodoacetamide markedly depleted the CF capacity of all sera and fractions.

Sephadex G-200 gel filtration. Samples of unheated whole serum from tumor-bearing hamsters were fractionated on Sephadex G-200 and yielded three peaks of protein (Fig. 3). The material in the first peak was markedly anticomplementary and was shown to contain the first component of complement (C'1). Table 2 shows the reduction of anticomplementary activity which resulted from heating the material at 56 C for 10, 20, 30, and 60 min. Since C'1 reactivity also was destroyed by heating, it was assumed that the anticomplementary effect was due primarily to the presence of activated C'1. Although there was a slightly higher titer in the presence of

TABLE 1. *Effect of reduction and alkylation on the complement-fixing reactivity of hamster immunoglobulin*

Sample	Complement-fixation titer		
	Whole serum	DEAE I	DEAE II
Control.....	64	48	48
2-Mercaptoethanol.....	64	48	48
Control.....	16	32	64
2-Mercaptoethanol + iodoacetamide.....	3	4	8

FIG. 2. *Sucrose gradient analysis: unheated whole serum from tumor-bearing hamsters (B), DEAE II (C), DEAE I (D), and 2-mercaptoethanol-treated rabbit IgG control (A). The broken line shows the area of anticomplementary activity in whole serum.*

FIG. 3. *Sephadex G-200 gel filtration of unheated whole serum from tumor-bearing hamsters. The broken line shows the zone of anticomplementary activity. Those fractions showing specific CF activity with T antigen are indicated in the shaded area. The bar (SM) represents the CF antibody titer of the starting material that was applied to the column.*

antigen after the material was heated for 30 and 60 min, it is unlikely that this was due to the presence of specific CF activity, as will be shown in the next experiment where the DEAE II fraction was subjected to gel filtration. Figure 3 also shows that specific CF reactivity was associated with only the second peak of protein, where 7S antibody is known to elute. More than 90% of the reactivity of the whole serum was recovered in this area. These reactive fractions were pooled, concentrated, dialyzed against 0.015 M Tris buffer (pH 8), and applied to a column (1.2 × 10 cm) of DEAE cellulose. Again, two peaks of antibody reactivity were found: one in the effluent and one in the 0.03 to 0.05 M NaCl eluate.

Initial electrophoresis revealed the DEAE II fraction of the serum to be impure, and an effort

was made to separate extraneous proteins by filtration through Sephadex G-200. As shown in Fig. 4, there was a reduction in the amount of protein in all peaks, but the overall profile remained similar to the one obtained from whole

TABLE 2. *Effect of heat on anticomplementary fractions from gel filtration of hamster serum*

Sample	Titer of hemolysis inhibition		C'1 titer
	Without T antigen	With T antigen	
Unheated control..	384	384	1280
56 C, 10 min.....	256	256	nd
56 C, 20 min.....	128	128	nd
56 C, 30 min.....	48	96	nd
56 C, 60 min.....	8	16	10

FIG. 4. *Sephadex G-200 gel filtration of unheated DEAE II fraction of serum from tumor-bearing hamsters. The broken line shows the zone of anticomplementary activity. Those fractions showing specific CF activity with T antigen are indicated in the shaded area. The bar (SM) represents the CF antibody titer of the starting material that was applied to the column.*

serum. Two pertinent findings emerged from this experiment. The anticomplementary activity in the first peak was virtually eliminated, presumably owing to the prior removal of most of the C'1 by low ionicity dialysis preceding the application to DEAE cellulose. In addition, all of the specific CF activity was eluted in the region corresponding to 7S immunoglobulin. No specific activity was found in the first peak, and only one fraction showed slight anticomplementary activity.

Composition of DEAE I and DEAE II. Immuno-electrophoresis of DEAE I and of the DEAE II preparation that had been partially purified by filtration through Sephadex G-200 first was performed by use of rabbit anti-whole hamster serum reagent. As shown in Fig. 5, DEAE I contained two proteins, the dominant one of which had a slow mobility characteristic of IgG. Even after filtration through Sephadex G-200, DEAE II was found to be a heterogeneous mixture yielding several lines of precipitation. Contained within DEAE II was an IgG-like protein which moved faster than the dominant protein in DEAE I.

A more distinctive demonstration of the globulin component in DEAE II was obtained in immunoelectrophoresis with rabbit anti-hamster γ -globulin reagent (Hyland Laboratories, Los Angeles, Calif.). The results are shown in Fig. 6, where it may be seen that the protein in DEAE II migrates faster than the major protein in DEAE I.

Range of reactivity and the effect of heat. The range of CF reactivity of each of the three original pools of serum and of the DEAE I and DEAE II fractions obtained from chromatographic separation of each of the pools was measured. Unheated material and samples heated at 56 C for 60 min were tested against T antigen, crude adenovirus 12, viral C antigen, and viral A

FIG. 5. *Immunoelectrophoresis of DEAE I (top well) and DEAE II after gel filtration (bottom well). Rabbit anti-hamster whole serum reagent was placed in the trough.*

FIG. 6. Immunoelectrophoresis of DEAE I (top well) and DEAE II (bottom well). Rabbit anti-hamster γ -globulin serum reagent was placed in the trough.

TABLE 3. Complement-fixing activity of hamster immunoglobulin with various antigens and the effect of heating at 56 C for 1 hr

Sample	Complement-fixation titer				
	Whole serum, heated	DEAE I		DEAE II	
		Un-heated	Heated	Un-heated	Heated
T antigen	128	96	64	128	96
Crude adeno-virus 12.	256	96	96	372	256
Viral C antigen.	32	24	32	48	32
Viral A antigen.	4	4	16	32	4
Antibody-complement (control)	4	<4	16	32	4

antigen. Table 3 shows the results of a representative experiment. It may be noted that: (i) there was little to no loss of antibody reactivity with T antigen or with crude adenovirus upon heating; (ii) there was low, but consistent, reactivity of whole serum and both fractions with viral C antigen; and (iii) there was no reactivity with viral A antigen. The anticomplementary activity, as evidenced in the antibody-complement control, consistently was reduced by heating the DEAE II fraction and the whole serum (not shown), whereas it consistently was increased by heating the DEAE I fraction.

DISCUSSION

Based on the results of gel filtration, centrifugation, and treatment with 2-mercaptoethanol, the antibody present in the sera of hamsters bearing adenovirus 12-induced tumors belongs to the 7S class of immunoglobulins. The electrophoretic migration indicates that the antibody most likely is associated with IgG. These data

are in accord with a report by Hollinshead et al. (5), who found CF reactivity with T antigen in the "7S gamma globulin fraction" from a sucrose gradient run with serum from hamsters bearing transplanted adenovirus 12 tumors.

Although the presence of IgM has not been unequivocally excluded, particularly from the first protein peak obtained by gel filtration of unheated whole serum (Fig. 3), gel filtration and sucrose density gradient centrifugation of the heterogeneous DEAE II fraction failed to reveal antibody of this class. The absence of antibody activity corresponding to γ -M immunoglobulin may provide at least one explanation for the failure of the host to cope with the growing tumor. We recognize that the specificity of the immunoglobulins studied is not directed against the so-called transplantation antigen, and the antibodies, therefore, may not be expected to be cytolytic. Nevertheless, these studies may well reflect the predominant class of immunoglobulin elaborated by the host. It also must be kept in mind that no attempt has yet been made to search for 19S antibody early in the course of tumor growth and that the CF test may not be sufficiently sensitive for its detection. The immunological studies with tumor antigens are restricted to a few serological methods. To better assess the various physicochemical properties of hamster antibody, we have commenced work with other antigen-antibody systems with the intention of applying a larger array of serological techniques.

Although 2-mercaptoethanol alone did not destroy the CF antibody activity, reduction followed by alkylation did bring about a substantial diminution in titer. This finding does not gainsay the conclusion that the antibody belongs to the 7S IgG class, for it was demonstrated by Nussenzweig et al. (9) that this kind of antibody in the mouse also is modified by the alkylation

step, in the sense of losing its complement-fixing capacity without losing hemagglutinating activity.

The chromatographic and immunoelectrophoretic analyses indicate the presence of two populations of 7S antibody molecules differing in electrical charge. This finding is analogous to the demonstration of the γ -1 and γ -2 7S antibodies in guinea pigs (1) and mice (4, 9). The present results indicate, however, that both kinds of antibody in the hamster are capable of fixing complement, in contrast to the behavior of the mouse and guinea pig γ -1 and γ -2 immunoglobulins where the γ -1 does not fix complement. Classification of these proteins must await results of passive cutaneous anaphylaxis tests, and perhaps better electrophoretic resolution.

The whole sera and both DEAE fractions reacted with crude adenovirus 12 as well as with T antigen. No reactivity was found with viral A antigen, but consistent, low reactivity was observed in whole serum and in each of the two DEAE fractions with viral C antigen. The finding of reactivity of whole serum from tumor-bearing animals with viral C antigen confirms the original findings of Huebner et al. (6).

An interesting aspect of the experiments was the effect of heating on the anticomplementary activity of the globulin(s). It consistently was reduced in the DEAE II fraction and in whole serum by heating. This reduction may be due to the destruction of hamster C'1. The gel filtration experiments showed that the major anticomplementary activity was associated with C'1 and that removal of the component substantially reduced anticomplementary activity. It is known that activated C'1 will destroy C'2 and C'4 in the fluid phase. Presumably, hamster C'1 in the unheated sera exerts such activity on guinea pig C'2 and C'4 during the overnight incubation in the CF test procedure. On the other hand, anticomplementary activity consistently was increased by heating DEAE I. At present, this is not understood. However, the aggregation of γ -globulin by the concentration and heating procedures, or the presence of T antigen-antibody complexes in the circulation of the host, are possibilities that have been considered. The latter point is being investigated.

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