A STUDY OF THE ANTIGENS INVOLVED IN ADENOVIRUS 12 TUMORIGENESIS BY IMMUNODIFFUSION TECHNIQUES

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Recent studies (1–3) have shown that hamsters with primary or transplanted adenovirus 12 (Ad. 12)–induced tumors develop an immunologic response against a number of virus-specific antigens. These include the type-specific C antigen, an unidentified antigen in standard viral harvests, and one or more previously unrecognized antigens ("T" antigens) found in tumor extracts and suspensions of acutely infected cells (4). Of these, only the latter is directly demonstrable in tumors by either complement fixation (CF) or fluorescent antibody (FA) procedures (5); the others have as yet been identified in tumors only indirectly, by the antibody responses of tumor-bearing animals. All three of these antigens invoke type-specific (or 12–18 subgroup specific) CF antibody responses, although a more broadly group-reactive antibody has been detected by the FA procedures (5). To date, all attempts to demonstrate complete virus and the group reactive A antigen in the tumors have been unsuccessful (3, 6).

The immunological methods employed to date in the study of these tumor-produced viral antigens, *i.e.* CF, FA, and virus neutralization tests, are highly specific, but are unable to distinguish separate reactions in a complex system unless chemically purified antigens are used. We have therefore turned to another tool, immunodiffusion, in the hope of more clearly distinguishing the various viral antigens manufactured during the course of adenovirus oncogenesis.

Materials and Methods

The micro-Ouchterlony test was that described by Crowle (7). A plexiglas mold containing a pattern of wells was inserted on a thin layer of solidifying agar (1 per cent ionagar with 1 per cent Na azide) supported by a glass slide. After the addition of serum and antigen in 0.05 ml volumes, the plates were incubated in a moist chamber 48 to 72 hours at room temperature. All sera were tested undiluted and unheated.

The immunoelectrophoresis technique was Crowle's description of Scheidegger's technique (8). All tests were done by electrophoresing the antigen. 100 to 150 v current was run for 40 to 80 minutes through a field containing sodium barbital–buffered agar (1.2 per cent ionagar) at pH 7.9. After the serum was added, the plates were incubated in a moist chamber 24 hours and read.

The microcomplement fixation test was that described by Sever (9). The virus neutraliza-

tion tests were performed in Rhesus monkey kidney cultures by previously described techniques (10, 11).

Antigens.—The CF antigen titers and gel reactions of the more routinely employed antigen preparations are listed in Table I.

Crude concentrated viral antigens were prepared by inoculating KB cell monolayers or spinner cultures with high titer (10^6–10^7 $TCID_{50}$) Ad. 12. Cells and medium were harvested at complete cytopathic effect, then frozen and thawed several times. These crude lysates were concentrated 25- to 100-fold by polyvinyl pyrolidine (PVP) and carbowax dialysis. One preparation used in many of the tests, viral concentrate 3, was homogenized three times with fluorocarbon and concentrated 35-fold with PVP.

TABLE I

Serological Reactivity of Standard Antigens used in Gel Diffusion Tests

Antigens	CF titer and gel reaction *vs.* various sera					Antigen content (gel reaction)			
	Human convalescent pool	"Broad"* tumored hamster	"Narrow"* tumored hamster	Ad. 12 hyperimmune rabbit	Anti-KB (rabbit and/or guinea pig)	A	C	D	T
Ad. 12 viral concentrate 3	512/+‡	256/+	<4/−	>64/+	/+	+	+	+	
Ad. 12 C antigen C6B.1M	<8/−	32/+	/−	128/+	/+		+		
Ad. 12 A antigen C6B.05 M	32/+	<8/	/−	64/+	/+	+		?	
Ad. 12 tumor extract pool 6	/−	>128/+	>64/+	/−	/−				+
Ad. 12 cell pack 12BC3	>128/+	>512/+	64/+	/+	/+	+	+	+	+
Control KB antigen	/−	4/−	<4/−	/+	512/+				

* "Broad" serum reacts with viral and tumor antigen preparations, while "narrow" serum reacts only with tumor extract.

‡ CF antigen titer/gel reaction. Blank space indicates not tested.

Concentrated cellular viral antigens ("cell-pack antigen preparations") were prepared by infecting human embryonic kidney cells in monolayers and KB cells in monolayers and spinners with high titered adenovirus 12. The cells were harvested at earliest cytopathic effects (CPE) by scraping with a rubber policeman. The medium was removed by centrifugation and the cells were resuspended in small quantities of phosphate-buffered saline (10^6 to 10^7 cells/ml). Preparations were then frozen and thawed several times before use.

Partially purified viral antigens were obtained by DEAE cellulose column chromatography of crude concentrated viral preparations. Stepwise elution was carried out with phosphate buffers of increasing molarity. In agreement with earlier reported findings (12–16), on other adenovirus types, an A group-specific and a C type-specific antigen were demonstrated. However, there was no serologic evidence of a B (cell-detaching) antigen. As reported by Huebner *et al.* (2), the group antigen (A) eluted at 0.01 to 0.05 molarity and the type-specific antigen (C) at 0.1 to 0.15 molarity. The A and C antigens used in the gel diffusion tests had been doubly or triply chromatographed. Complement fixation tests showed significant antigen titers with little cross contamination of the specific antigens, although all fractions contained

some host cell protein (Table I). One preparation of C antigen, C6B.1M, had a >64 titer against an anti-C Ad. 12 rabbit serum[1] and a <8 titer against an anti-A Ad. 12 rabbit serum.[1]

Tumor antigen preparations were prepared by making 20 to 33 per cent extracts of viable adenovirus 12 tumors with a motor-driven tissue grinder. Both primary and transplant tumors were used. The tumors were debrided of necrotic tissue and the viable pieces washed several times in phosphate-buffered saline. The tissue suspensions were then ground to fine consistency and clarified at 2000 RPM for 20 minutes.

TABLE II

Serological Reactivity of Standard Sera Used in Gel Diffusion Tests

Sera	CF antibody titer and gel reaction *vs.* various antigens						Antigen reactivity (gel reaction)			
	Ad. 12 virus	Ad. 12 cell pack	Ad. 12 hamster tumor	Ad. 12 A	Ad. 12 C	Control KB	A	C	D	T
Human Ad. convalescent pool 33917	80/+*	>16/+	<10/−	>16/+	/−	/−	+		+	
Hamster 6155 (Ad. 12 tumor)	80/+	/+	640/+	/−	40/+	/−		+		+
Hamster 9205 (Ad. 12 tumor)	160/+	320/+	160/+	/−	40/+	/−		+	+	+
Hamster pool 2B (Ad. 12 tumor)	<10/−	>640/−	>160/−	/−	/−	/−				+
Guinea pig 7179 (hyperimmune Ad. 12)	160/+	/+	/−	/+	/−	10/+	+		+	
Rabbit 3-360 (hyperimmune Ad. 12)	320/+	/+	/−	/+	>80/+	/+	+	+	+	
Anti-KB sera 8135 (guinea pig) and 3-307 (rabbit)	/+	/+	/−	/+	/+	320/+				

* CF antibody titer/gel reaction. Blank space indicates not tested.

Sera.—The CF antibody titers and gel reactions of the commonly used sera are listed in Table II.

Hamster sera: Two hamster sera, 6155 and 9205, (both from animals with primary tumors) were used in most of the tests described. These had high CF titers (Table II) and virus neutralization titers of 80 and 40 respectively. Sera from many hamsters with large primary or transplanted Ad. 12 tumors were also screened against viral and tumor antigen preparations. Some of the sera were selected purely at random, and others were selected for high CF antibody titers.

Guinea pig hyperimmune serum (7179): This serum was prepared by inoculating 1 ml of high titered Ad. 12 intranasally, followed by 1.5 ml intraperitoneally 3 weeks later. The animal was bled out 1 week after the final inoculation. A control antihost cell serum (8135) was prepared by giving 2 and 1 ml doses of concentrated KB cell lysate in Freund's adjuvant 10 days apart. This guinea pig was bled out 9 days after the final inoculation.

[1] Kindly supplied by Dr. H. G. Pereira.

Human adenovirus convalescent pool (33917): This was a collection of pooled human sera from military recruits convalescent from recent adenovirus infection.

Rabbit hyperimmune serum 3-360: This was a standard typing serum prepared by hyperimmunization with crude Ad. 12 KB tissue culture–grown virus by Microbiological Associates, Bethesda. A control rabbit antihost cell serum (3-307) was prepared in the same manner.

Controls.—Antigens which failed to give any positive gel reactions with tumored hamster serum included KB control cell-pack antigen, polyoma and SV40 hamster tumor extracts, and CAM-grown Rous sarcoma. Control sera which did not react to Ad. 12 viral and tumor antigens included Schmidt-Ruppin-Rous-tumor hamster serum, and Yaba facial tumor monkey serum. Three anti-KB cell sera, two rabbits (3-307 and L001) and one guinea pig (8135), were also prepared. These gave lines with Ad. 12 viral preparations which were identified with some of the reactions of the anti-Ad. 12 hyperimmune sera. In this way the actual antiviral lines of the hyperimmune sera could be distinguished. These anti-KB sera gave no reaction with Ad. 12 tumor antigen.

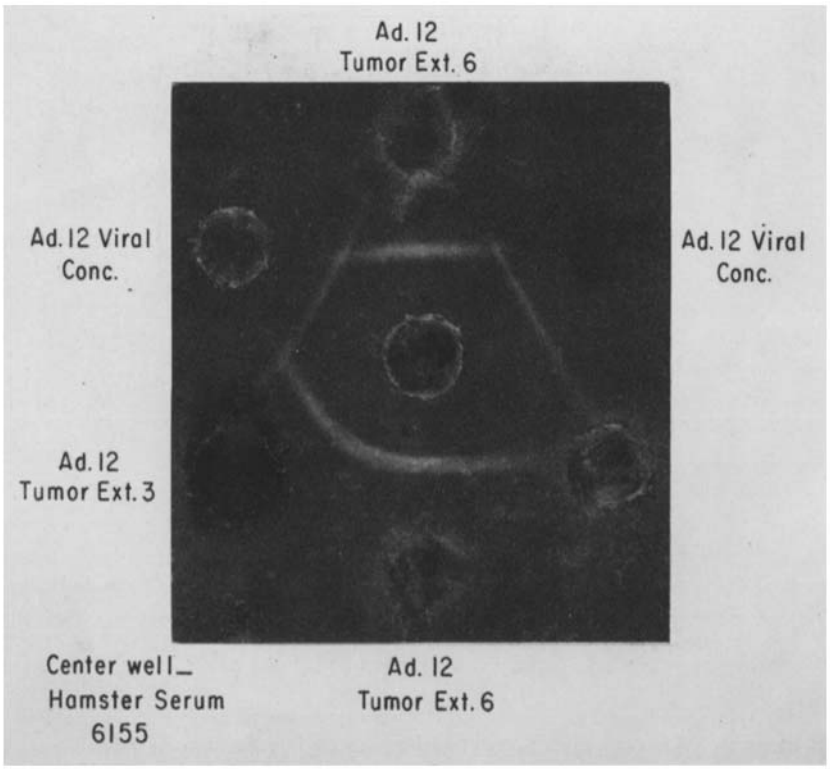

FIG. 1. Reaction of tumored hamster serum to adenovirus 12 viral and tumor antigens. A common reaction to two separate tumor extracts crosses and is distinct from the antiviral reaction.

RESULTS

Ninety-eight sera from hamsters carrying Ad. 12–induced tumors were tested in gel diffusion against both standard concentrated viral and tumor antigens. Of these, thirty-one were found to react positively with tumor antigen only, and six were found that reacted to both viral and tumor antigen. In all cases the reaction with tumor antigens was a single fairly broad line. The reaction with viral antigen was sometimes encountered as a double line, neither part of which was continuous with the anti-tumor reaction.

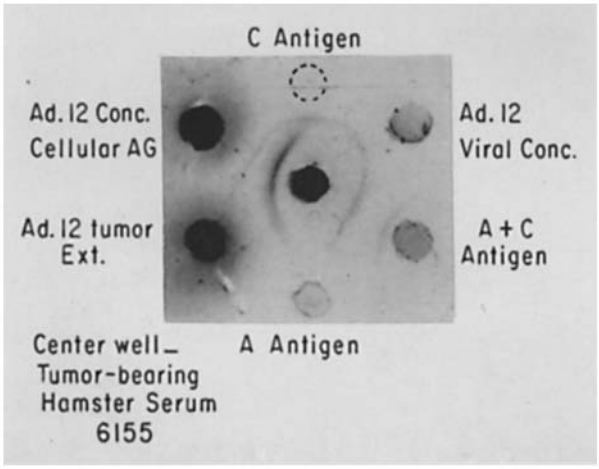

FIG. 2. Reaction of tumored hamster serum to various adenovirus 12 viral and tumor antigens. There is a common reaction line shared by cell-pack antigen, crude viral antigen, and purified C type–specific antigen. Another common reaction between cell-pack antigen and tumor extracts is distinct from the first line.

Fig. 1 shows the reaction of a high–titered hamster serum (No. 6155) against both viral and tumor antigens. The antitumor reaction appears as a single line which is continuous for two different preparations. The identity of this antigen line has held true for four different tumor extracts. The antiviral reaction in Fig. 1 also appears as a single line, which crosses, and is therefore distinct from, the antitumor reaction.

Fig. 2 shows the same hamster serum tested against additional viral and tumor antigens. There is a positive reaction against purified Ad. 12 type–specific (C) antigen which is continuous with the reactions against crude virus, an A plus C mixture, and a cell-pack preparation. A second reaction line, between hamster serum and tumor extract, is shared by the cell-pack antigen preparation; a third line shows a reaction only with the A plus C antigen fraction. The purified group (A) antigen shows no reaction.

The reactions shown in Figs. 1 and 2 confirm the CF antibody studies in showing the response to C but not to A antigen, the distinctness of the C reaction from that with tumor extract, and the presence of an antigen in acutely infected cells identical to that in tumor extracts. The third line may represent yet another antigen whose concentration in the chromatography fractions may have been enough to elicit a positive response with serum 6155. This is probably not the usual non-C antigen in viral harvests, which remains unidentified in this system.

Fig. 3 shows the reaction of hamster serum 9205 and human serum pool 33917 to crude Ad. 12 and purified C antigen. In contrast to serum 6155, this hamster serum reacted to two antigens in the crude virus preparation, one being the C

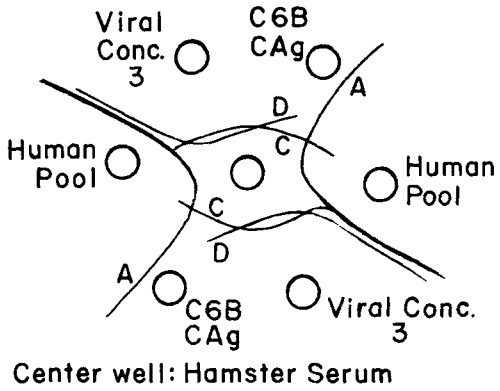

FIG. 3. Reaction of tumored hamster serum 9205 and pooled human convalescent serum 33917 to viral antigens. The hamster serum is reacting to two antigens, one of which is the type-specific C antigen (C6B CAg) and the other reaction, shared by the human pool, is to a previously undescribed antigen (D) which is distinct from the classical A and C antigens.

antigen. Surprisingly, the antigen present in crude virus and absent in the C fraction is also reacting with the human serum (line D). A third antigen forming a broad arc on the sides of the pattern is the A antigen, probably reacting only to the human serum. The presence of this line between the human serum and the C antigen may indicate contamination of the C fraction with A antigen, or migration of the A antigen in viral concentrate 3 past the C well.

These patterns demonstrate a third antigen of adenovirus 12 reactive with tumored hamster serum; this antigen is also reactive with the human serum and is distinct from the classical A and C antigens. Although we cannot exclude the possibility that this antigen corresponds to the B antigen of other adenoviruses or the non-C viral antigen demonstrated in CF with tumored hamster sera, our present feeling, based primarily on chromatographic separation and stability studies, is that the new antigen is distinct. For this reason, we will refer to it here as D antigen.

Data in Fig. 4 indicate that the D antigen is also reactive with hyperimmune anti-Ad. 12 sera. In this test, two hamster sera and two hyperimmune Ad. 12 sera were reacted against a concentrated virus preparation. All sera except the guinea pig (7179) react against the C antigen, and all sera except one hamster (6155) react against the D antigen. Neither hamster serum reacts with the

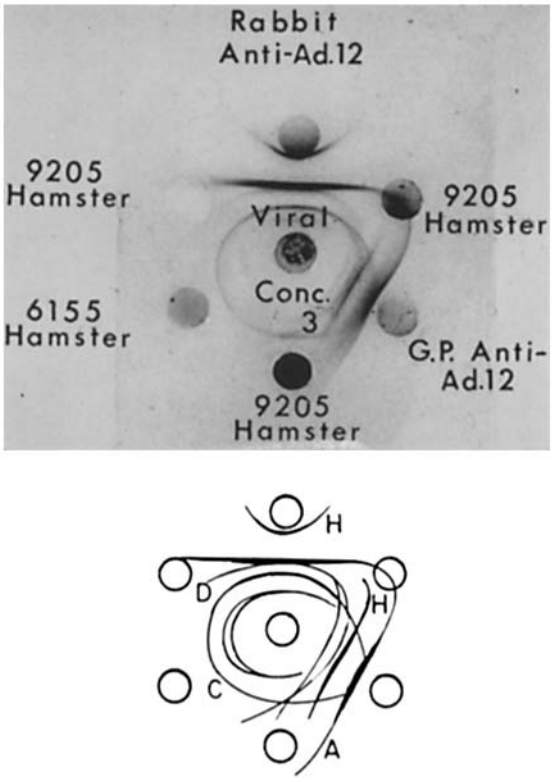

FIG. 4. Reaction of concentrated crude adenovirus 12 viral antigen to tumored hamster and hyperimmune sera. Both hamsters react to the C antigen, one reacts to the D antigen, and neither reacts to the A antigen.

classical A antigen. The two lines marked H are different separate reactions given by the hyperimmune sera. One of these has been identified as antihost cell reaction, and the other is thought to be antihost as well. Some other faint lines, (not visible in the photograph) were also present as reactions of the hamster sera. Whether these represent other antigens, or a splitting of the lines already described, is not clear.

Immunoelectrophoretic Studies.—Most of the reactive combinations of antigens and antisera discussed above were also tested in immunoelectrophoresis, in attempts to further separate components of the antigens. Repeated tests demon-

strated only one line with hamster serum and concentrated virus, while the human convalescent serum pool gave at least three distinct lines. One of these lines, which migrated toward the positive electrode, appeared to correspond with the hamster antiviral line, and hence, probably represented the D antigen. Repeated tests of hamster serum against tumor antigen yielded only two positive tests; in each case there was a single line showing essentially no migration of the antigen at pH 7.9.

Relation of Gel Diffusion Reactivity to CF and Neutralizing Antibody.—The serologic reactions of the ninety-eight Ad. 12–tumored hamster sera tested in gel diffusion are summarized in Tables III and IV. Of the six sera which reacted with viral antigen in gel diffusion, all were positive against tumor antigen as

TABLE III

Virus Neutralizing and CF Reactions of Ad. 12–Tumored Hamster Sera in Relation to Gel Diffusion Reactions

Ouchterlony reaction	No. of sera	Neutralization		Complement fixation					
				Viral antigen			Tumor antigen		
		No. pos.*	Per cent	No. pos.‡	Per cent	Average titer§	No. pos.‡	Per cent	Average titer§
Virus and tumor	6	6	(100)	6	(100)	101	6	(100)	252
Tumor only	31	7	(23)	30	(97)	94	31	(100)	187
Negative	61	9	(15)	28	(46)	30	50	(82)	108

* Test of undiluted serum.

‡ A reaction of at least 3+ at a 1 to 10 dilution.

§ Geometric mean.

well, and all were positive for neutralizing antibody and CF reaction to viral and tumor antigens. Some of the antiviral gel diffusion reactions were quite faint, and it was not possible to determine if they were against C or D antigen; the presence of neutralizing antibody indicates that these sera contained CF antibody to C antigen (2).

The sera which reacted in gel diffusion only against tumor antigen showed CF reactivity comparable to the virus-reactive group, but differed markedly in the frequency of neutralizing antibody, and, by inference, C antibody.

The frequency of gel diffusion reaction in relation to magnitude of CF and neutralizing antibody titer is shown in Table IV. High titer antiviral CF antibody was the most consistent indicator of antitumor gel diffusion reactivity; this may be due to the fact that animals developing high antiviral titers have had high antitumor CF antibody for a longer time (1).

TABLE IV

Gel Diffusion Reactions of Sera Titered in CF and Virus Neutralization

Test	Antibody level		No. of sera	Ouchterlony reaction			
				Viral		Tumor	
				No. pos.	Per cent	No. pos.	Per cent
Viral CF	High	≥80	34	5	15	28	82
	Medium	20–40	23	1	4	8	35
	Low	≤10	41	0	0	1	2
Tumor CF	High	≥80	72	6	8	34	47
	Medium	20–40	11	0	0	3	27
	Low	≤10	15	0	0	0	0
Neut.	High	≥20	9	3	33	5	56
	Low	1–10	13	3	23	8	62
	Neg.	0	76	0	0	24	32
Ouchterlony totals			98	6	6	37	38

DISCUSSION

The above data indicate that there are at least four distinct antigens associated with adenovirus type 12, and at least three of these are manufactured in the tumor cells.

Indirect evidence from this and preceding papers (1, 2), indicates that the A group–specific antigen is not associated with the tumor cells. However, the demonstration of virus-like particles in tumor extracts by electron microscopy (17), together with the association of A antigen with capsomere substance in other adenovirus types (18), is difficult to reconcile with this finding.

The best known of the viral antigens definitely associated with the tumors is the type–specific C antigen. CF reactions of tumored hamster sera with chromatographically purified viral material have previously demonstrated the association of this antigen with the tumors (2), and the gel diffusion reactions confirm this. The association of the antiviral gel reaction and the neutralizing antibody response also confirm the findings of Huebner *et al.* (2). Recent findings have shown the C antigen to be a complex structure, possibly a viral subunit (18, 19).

As yet, little is known about the previously undescribed D antigen. Chromatographic separation of Ad. 12 virus preparations sometimes yields a third peak of CF antigen activity (in addition to A and C) with crude antisera (2, 20, 21). This material may correspond to the D antigen, as the reaction is obtained

with the convalescent human serum pool, which is reactive to the D antigen in gel diffusion. The fact that the human serum gives this reaction indicates that it is not host cell antigen. The chemical and physical nature, the distribution among adenovirus types, and the breadth of reactivity of this antigen have yet to be determined.

The most abundant of the viral antigens associated with the tumor cells is the so-called "tumor" or "T" antigen(s). This antigen was first described in the tumors (1) and later during the early stages of the lytic cycle in viral-infected cells (4). The identity of the antigen from these two sources has been confirmed in gel diffusion. In addition, these studies showed the identity of tumor antigen derived from different tumors. The fact that this antigen is the only antigen so far to be directly demonstrated in the tumors, and that it is the most common by far of the hamster antibody responses, indicates that it is the most abundant of all the viral antigens in the tumor.

There is indirect evidence of another viral antigen in the tumors, namely the antigen referred to as the non-C antigen in viral harvests. In view of the low degree of reactivity of hamster sera against C and D antigens in gel diffusion, it seems unlikely that these two antigens can account for the bulk of the anti-viral CF response. Various antigen treatments such as dialysis at pH 10.5, trypsin, and heat can destroy the reactivity of viral preparations with hamster sera with high antiviral and low anti-C titer, but will not affect their activity with hamster sera with high viral and high anti-C activity (20). However, the above procedures will also inactivate the tumor antigen, and it may be possible that this other viral antigen is an altered form of the tumor antigen that can not give a precipitin reaction or fix complement with low avidity serum. This concept is supported by the fact that we not infrequently observe that the so-called "narrow hamster sera" (reacting only with tumor antigen) give partial CF reactions with standard viral antigen preparations through high serum dilutions.

Fig. 4 and the immunoelectrophoresis studies indicate that there may be other undescribed adenovirus 12 antigens. Recent studies with disc-electrophoresis (22) have shown at least nine proteins associated with disrupted adenovirus. It is conceivable that most of these could act as antigens.

Table V summarizes the presently established adenovirus 12 antigens and their sources. It is interesting that hyperimmune sera may demonstrate antibodies to any of the above antigens with the exception of the tumor antigen. With one exception[2] only tumored hamster sera have been found to contain the "antitumor" antibody. Limited attempts to hyperimmunize with cell-free tumor extracts have been unsuccessful (23).

[2] One Rhesus monkey inoculated with adenovirus 12 at birth was found to contain CF antibody to the adenovirus 12 tumor antigen 2 months later. This animal never developed a tumor, however.

The results of these studies indicate some of the advantages in the use of tumored animal sera in gel diffusion. Most previous attempts (24–30) to utilize immunodiffusion techniques in the study of cancer immunology were always fraught with difficulty because of the use of hyperimmune sera, which have a number of antihost cell reactions which usually have to be absorbed out. Tests were frequently based on the appearance or absence of one line in a sea of reaction. The use of tumored sera provides a direct, clear-cut, specific tool in the detection of specific tumor antigens. There is no doubt that the CF and FA tests are more sensitive than the gel diffusion, as evidenced by the above results. Also some antibody molecules may be incapable of giving a precipitin reaction (31). However, the gel diffusion is highly specific and is capable of dissecting out complex reactions. Also, gel diffusion may be particularly useful with species whose antibodies do not fix complement.

TABLE V

Antigenic Content of Adenovirus 12 Viral and Tumor Preparations

Type of preparation	A	C	D	Other viral antigen	"Tumor antigen"
Standard concentrated viral harvests	+	+	+	Probable	
Cell-pack viral antigens	+	+	+	Probable	+
Tumor extracts		(±)*	(±)*	(?)	+

* Presence of these antigens indicated by antibody response only.

SUMMARY

The use of the agar gel diffusion technique has established the presence of three distinct antigenic reactions in the sera of Ad. 12 tumor–bearing hamsters. Only one of these antigens is directly demonstrable in the tumor. This "tumor" antigen is also formed during early stages of the infectious cycle in tissue culture cells. Other antigens present in the tumor, but only demonstrable indirectly with the use of antibody-containing serum of tumored hamsters, are the classical type–specific C antigen, and a new antigen, termed D.

Of ninety-eight Ad. 12 tumored hamster sera, six reacted in gel diffusion with virus and tumor preparations, and thirty-one with tumor only.

Sera which reacted in gel diffusion with viral antigen uniformly had neutralizing antibody and high titers of CF antibody against viral and tumor antigens; however, many sera with comparable antibody titers did not react with the virus in gel diffusion. Sera which reacted in gel diffusion only with tumor antigen also had high CF antibody titers, but there was no correlation with neutralizing antibody.

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