A Single Genetic Locus Determines the Efficacy of Serum Therapy Against Murine Adenocarcinoma 755a

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Introduction

We have recently described a unique model system for immunotherapy where immunological management of a highly lethal tumor, the ascites form of the Adenocarcinoma 755 (AD755a), could be carried out reproducibly and with remarkable efficiency [7]. Some of the basic features of this model are summarized in Table 1. The studies to be described in this report will concern themselves primarily with a more in depth analysis of the phenomenon of strain variation in relation to the protective capacity of the serum, and were done in an effort to gain some insight into the mechanism by which the serum mediates its powerful effect.

Preparation of Hyperimmune Anti-AD755a Antiserum

Ad755a was uniformly lethal in B6 mice after intraperitoneal (ip) inoculation of as few as 50 cells per mouse (Table 1). In contrast, when AD755a cells were injected subcutaneously (sc) into B6 mice in a dose range between 1×10^5 and 5×10^5 cells, a transient nodule appeared that was resorbed completely by week 2–3 after injection. Mice that had rejected AD755a cells inoculated sc were resistant to a later ip challenge with these cells. On this basis, B6 mice

Table 1. Characteristics of AD755a tumor system

AD755a is a "universal" tumor and is lethal in all mouse strains tested. Inoculation of fewer than 50 cells intraperitoneally gives 100% lethality and fewer than ten cells results in approximately 80% lethality. The tumor grows equally well in all strains tested.
Immunization in "syngeneic" C57B1/6J mice by subcutaneous inoculation of AD755a provides protection against an intraperitoneal challenge of 10^4 LD_{100} and this protection persists for greater than 90 days.
Serum or immune cells from mice hyperimmunized by multiple intraperitoneal injections after an initial subcutaneous immunization can transfer protection to a normal animal in quantities of 5–10 µl against a challenge of 1×10^5 AD755a cells.
Preliminary study of the protective factor(s) shows it is contained in the IgG fraction. It has an effective half-life in vivo of greater than 4.5 days and less then 9.0 days.
Studies in several mouse strains have revealed that the protective capacity of the serum is strain dependent.

were hyperimmunized against the AD755a cells by an initial sc inoculation of 10^4 AD755a cells followed by sc inoculations 2–3 weeks later of 1×10^5 and 5×10^5 cells respectively. Subsequently, increasing doses of AD755a cells from 1×10^4 to 1×10^6 were given ip over a period of seven weeks. After four additional ip inoculations with 1×10^6 cells, serum was taken. Animals were boosted and bled monthly thereafter.

Normal B6 mice were inoculated ip with various volumes of the immune sera at the same time that they were challenged with AD755a cells. Protection against 10^5 tumor cells was obtained with as little as 5–10 µl of B6 anti-AD755a serum. Subsequent experiments demonstrated that similar titration end points could also be obtained after sc or intravenous injection of the serum; this indicated that the serum and tumor cells did not need to be administered together for successful passive immunotherapy to be achieved. Finally, serum administered as late as 3 days after tumor inoculation was also protective, although larger quantities were required.

Protective Capacity of Immune Serum in Various Mouse Strains

Titration of B6 anti-AD755a immune serum against AD755a tumor cells given ip to other strains of mice revealed a significant strain specificity of the immune protective capacity (Table 2).

The results emphasize the variation observed earlier [7] where only a few strains were tested. The mice are arbitrarily subdivided into three groups based on their titration end point (see legend of Table 2). All standard C57B1 strains, as well as strains 129/J, CBA/J and A/J, were protected by low quantities of serum (10 µl) including strains congenic at H-2 (*) and Fv-2 (**).

At the opposite end of the spectrum were mouse strains which were not protected by at least ten (AKR) or more than 30 times (BALB/cJ) the quantity of serum used to protect C57B1/6J. Both Fv-1^n (NIH) and Fv-1^b (BALB) mice were members of this group, indicating no relationship to the Fv-1 gene. A variety of strains, however, could be protected by intermediate quantities of serum (group II).

Some conclusions can be drawn from this analysis which indicates that protection did not seem to correlate with the H-2 type or with two well-known viral markers. Moreover, the variations observed cannot be explained by a difference in growth rate of the tumor since at the dosage given (10^5 tumor cells), the tumor was uniformly lethal within very nearly the same time period after administration in all strains tested.

Genetic Analysis of the AD755a System

The strain variation observed could be due to a number of factors including several immunological and virological parameters. Since some of the loci controlling these factors are known, a genetic analysis of the system might

Table 2. Strain analysis for protection against the AD755a tumor

Mouse strain	*Minimum protective volume (μl)*	
C57B1/6J	10	
C57B1/6J (male)	10	
C57B1/6By	10	
B6.C-H-2^{d}/By [a]	10	
B6.C-H-7^{b}/By [b]	10	
C57B1/10J	10	
B10.A/SgSn [a]	10	
B10-H-2^{a}-H-7^{b}Wts[a]	10	I
B10.129(21M)/SnJ	10	
B10.D2/nSn[a]	10	
C57B1/KsJ	10	
129/J	10	
CBA/J	10	
A/J	10	
B10.Br/SgSn [a]	35	
C3H/HeJ	35	
C3H/HeJ (male)	35	
C3H.SW/Sn [a]	50	
DBA/2J	50	
SJL/J	50	
C58J	50+	II
RF/J	50+	
NZB/BINJ	50+	
STU	50+	
P/J	75	
SEC/1ReJ	75	
DBA/1J	>75	
NIH/Sw	>75	
NIH/Sw (Nu/Nu)	>75	
PL/J	>75	
RII/2J	>75	
SM/J	>75	III
BUB/BnJ	>75	
BALB/c By	>75	
CD-1/Cr	>100	
AKR	>100	
BALB/cJ	>300	
BALB/cJ (male)	>300	

[a] H-2 congenics
[b] Fv-2 congenics

1. Unless indicated, females were tested
2. Same serum pool used in all strains tested
3. Strains titrated at 0, 10, 20, 35, 50, 75 or 100 and 300 μl where indicated. Four mice tested at each level

provide some clues as to the significance of this variation, and even illuminate the nature of the mechanism of protection itself. Using C57B1/6J (B6) (protected [P] at 10 μ) and BALB/cJ (C) (not protected [–] at 300 μl) mice, we carried out classical mating crosses F_1, F_2, F_1 backcrosses) to establish the number of genes involved in the phenomenon and their penetrance (dominant or recessive).

F_1 Generation

Both F_1 breeding combinations (C × B6), and the reverse (B6 × C) indicated that an intermediate quantity of serum (~75 µl) was required for protection of the F_1 hybrids and that there was no sex-linked effect (Table 3). The fact that these animals could be protected excludes the presence of a single dominant locus from BALB/cJ mice which blocks protection.

Table 3. Protection of parental and F_1 mice by immune serum

Mouse strain		*Minimum quantity of protective serum*
C57Bl/b	Parental	15 µl
BALB/cJ		>300 µl
(B6 × C) F_1		75 µl
(C × B6) F_1		75 µl

F_2 and F_1 Backcross Progeny Testing

The results of the second generation cross (C × B6) involving 313 mice, where protection was assayed at 15 µl of serum, are shown in Table 4. Since 22,4%, or one quarter of the mice resembled the B6 parent, this strongly suggests that a single locus is involved in the process. Studies to determine the percentage resembling the BALB/cJ parent confirm this notion (Table 4). For this study, 100 µl of serum was used; a quantity capable of protecting all F_1 hybrid mice and animals resembling the B6 parent, but not capable of protecting animals resembling the BALB/cJ parent. The observed values for the individual F_1 backcross generations are also in close agreement with the expected values for a single operative locus. A diagrammatic representation of the various crosses is presented in Fig. 1.

Table 4. Protection of F_2 and F_1 backcross (BC) progeny A: By 15 µl of immune serum

Cross	*Number of mice*	*Percent protected*	*Percent expected for one locus*
(C × B6) F_2	313	22,4	25,0
(B6 × F_1) BC	50	42,0	50,0
(F_1 × C) BC	52	0	0
B: By 100 µl of immune serum			
(C × B6) F_2	51	68,6	75,0

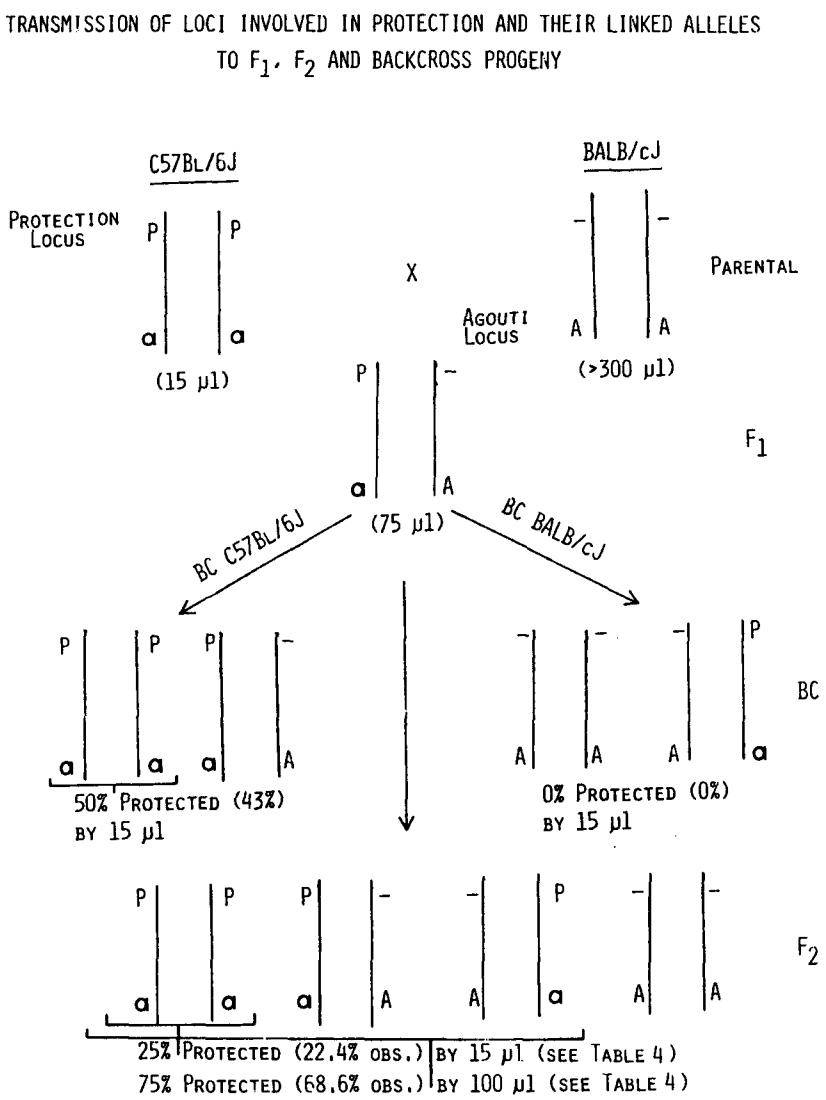


Fig. 1. Diagrammatic representation of genetic crosses between C57B1/6J and BALB/cJ mice and their offspring demonstrating the putative relationship between loci associated with protection and that of the agouti coat color

Linkage Studies to Determine the Position of the Locus Controlling Protection

During the course of these experiments, we noticed that certain alleles (agouti) controlling coat color might be linked to the protection locus. In crosses between BALB/cJ and C57B1/6J mice, multiple genes located on separate chromosomes determine coat color [11]. The a locus confers the agouti characteristic which is represented by hair with a yellow banding. The locus is dominant except in albino mice where melanin is not produced. The C57B1/6J parents are black mice, homozygous nonagouti (aa), while BALB/cJ mice are white (no melanin) but homozygous at agouti for the wild-type allele (AA).

Analysis of the (C × B6) F_1 to the B6 parental type backcross generation at the 15 µl protection end point in relation to coat color yields the results

outlined in Table 5. Assuming no linkage with any color marker, one would expect that the percentage of mice protected in the agouti and nonagouti categories would be the same as the overall percentage protected (ideally 50%, 43,4% experimentally). On the other hand, a close linkage to agouti should result in no protection for agouti mice since these would resemble the BALB/cJ parent; and complete protection of nonagouti animals (like B6 parents). The observed percentages represent significant deviations from either idealized situation suggesting a definitive, but distant, linkage of the protective function to the agouti allele. Similar analyses of the F_2 generation support the linkage to the agouti allele (data not shown).

Table 5. Linkage of protective function to agouti in the backcross of (C × B6) F_1 to B6 parental type

	Agouti	*Nonagouti*	*Total*
Number tested	70	36	106
Number protected	16	30	46
Percent protected	22.9	83.3	43.4
Percent expected without linkage	50.0	50.0	50.0
Percent recombination	$\frac{16 + (36 - 30)}{106} \times 100 = 20.8$		

Both agouti mice which are protected and nonagouti animals not protected represent recombinants and from the frequency of recombination (20,8%, Table 5), one can tentatively place the allele associated with protection about 21 map units from the agouti locus. A similar analysis of the F_2 generation is in close agreement with this conclusion (data not shown). Since the agouti locus has been mapped, the protective function is located in chromosome 2, linkage group V. Our preliminary data employing recombinant inbred strains suggests that the function maps toward the centromere from agouti (unpublished observations).

The Specificity of Protection is Dictated by Virus Associated Antigens

The mechanism by which AD755a tumor challenge is rejected and the identity of the antigens involved in the induction of immune transfer capacity remain to be defined. Possibly relevant to these questions were the observations of Brandes and Groth [1] that virus particles were present in both the solid and ascites form of Adenocarcinoma 755. We have recently demonstrated that this agent, termed ADV (Adenocarcinoma-755a virus) is a type-C virus closely related to the Friend and Rauscher murine leukemia agents [3]. Moreover, the B6 anti-AD755a serum could neutralize ADV and viruses of the FMR (Friend-Moloney-Rauscher) group, possessed a high antibody titer in radioimmunoassays with the major glycoprotein of Friend virus (gp71) and effectively lysed AD755a tumor cells or murine cells infected with Friend virus.

Indeed protection by this antiserum seems to correlate with an antigen associated with FMR viruses. Some of this information is summarized in Table 6. We have observed complete cross protection with universal tumors which likewise express FMR antigen, but none with tumors expressing unrelated viral antigens or no known viral antigens. Of considerable interest is that introduction of FMR viral antigens on a non virus-producing (NP) tumor (the Harvey sarcoma virus-induced C57B1 sarcoma [C57B1 (MSV HA)]) now renders this tumor ([C57B1 (MSV HA) FLV]) susceptible to rejection by an AD755a immune mouse.

Moreover, it has been possible to immunize mice with intact or disrupted Friend virus against challenge with AD755a. The viral specificity extends even to this parameter since AKR virus was not able to immunize against the tumor (Table 6).

Table 6. Specificity of protection seems to correlate with oncornavirus associated antigens

	Associated virus type
1. *Complete* cross-protection seen between AD755a and:	
S-180a	FMR
EAC	FMR
C57B1 (MSV HA) FLV	FMR
2. *No* cross-protection seen between AD755a and:	
6C3HED	Gross – AKR
EL-4	Gross – AKR
C57B1 (MSV HA) NP	None
3. Immunization against AD755a was possible with:	
Intact FLV	
Disrupted FLV	
But not with:	
Intact AKR	
Disrupted AKR	

Attempts to Achieve Protection Against AD755a Tumor Challenge with Anti-Viral Antisera

The concordance between protection and the presence of FMR viruses suggested that a viral component might represent the target antigen on the tumor surface. The most likely candidate for this was the major surface glycoprotein of the virus, not only because of its strategic location but also because the anti-AD755a serum possessed a high antibody titer against this virion component. Thus, it was reasonable to attempt protection with hyperimmune antisera to gp71 or whole virus. These antisera had anti-gp71 titers equal to or in some cases, much greater than those of anti-AD755a antisera.

The results of these studies (Table 7) were disappointing, however, since *none* of these antisera were able to mediate protection. Moreover, absorption of the protective anti-AD755a antiserum with Friend virus or purified gp71 under conditions where all of the anti-gp71 activity is removed, had no effect on its ability to reject AD755a tumor challenge.

Table 7. Effectiveness of anti-tumor vs. anti-viral antibodies in abrogation of AD755a tumor growth

Antiserum	*Specificities*	*Prevention of tumor growth*
Anti-AD755a	Anti-Tumor, Anti-Virus	+
Anti-AD755a abs. with FLV	Anti-Tumor	+
Anti-AD755a abs. with AD755a cells	?	+
Anti-FLV, anti-FLV gp71	Anti-Virus	–

Attempts to Identify a Non-Structural Viral-Induced Antigen Associated with AD755a Tumor Cells

The results presented in the previous section could be interpreted to signify the presence of a non-structural virus-associated antigen which was responsible for induction of the transferable protective antibody population, as well as serving as a target antigen. Such virus-induced non-structural proteins have been identified on MLV-induced YAC lymphoma [5,6,8] and on feline leukemia and sarcoma cells [9]. A direct attempt to immune precipitate an analogous component from surface labeled AD755a cells with various antisera was carried out. As is shown in Fig. 2, the major component precipitated from surface iodinated AD755a cells with both mouse anti-FLV (Fig. 2A) and mouse anti-AD755a (Fig. 2B) antisera is represented by gp71. In fact, except for a minor component of about 45000 MW, no other distinct molecular species are evident in the anti-AD755a antiserum immune precipitates. That the major component represents gp71 is substantiated by its nearly quantitative removal subsequent to absorption of the antiserum with purified Friend virus (Fig. 2C). Although a few minor components remain after this absorption, the results indicate that most of the reactivity of the anti-AD755a serum against iodinated species on the tumor cell surface is directed toward gp71. Similar results were obtained when the cell surface glycoproteins were labeled with galactose oxidase (data not shown). Thus these results indicate that antigens other than gp71 cannot be identified using these procedures. However, a second antigen could be either inaccessible to external labeling or inactivated following disruption of the cells prior to immune precipitation. Alternatively, gp71 may indeed represent the only relevant antigen involved in production and binding of protective antibody, as discussed below.

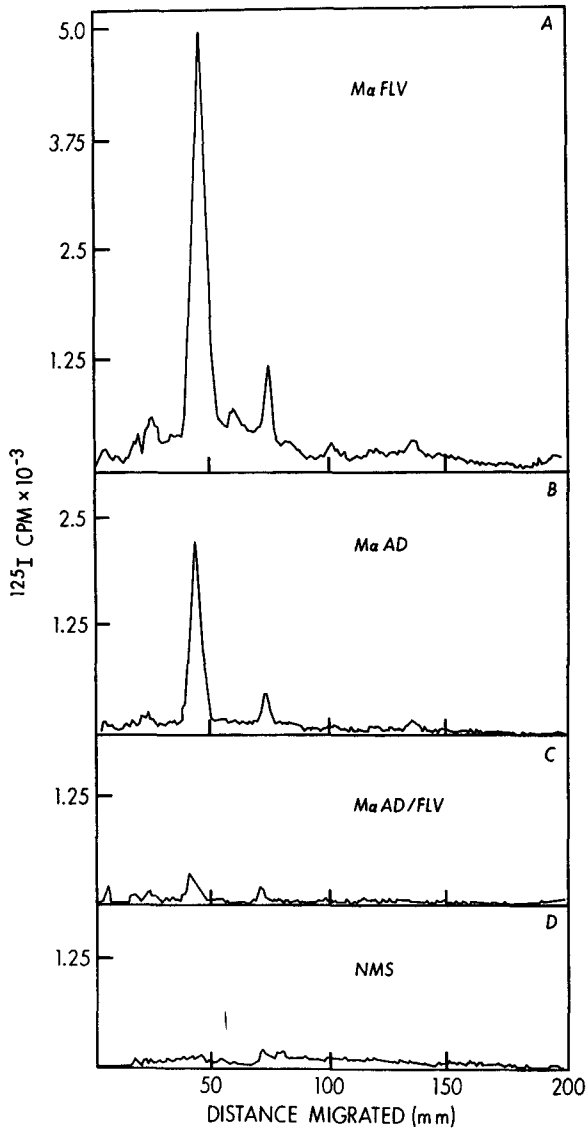


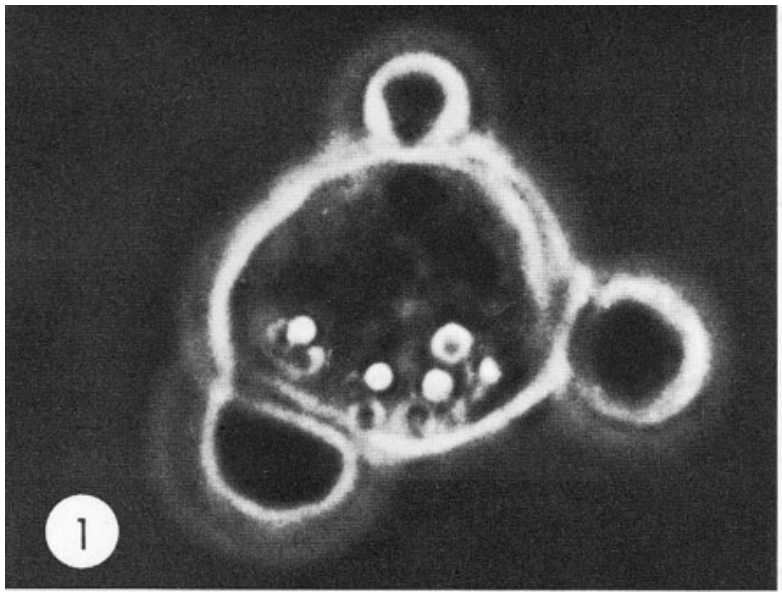
Fig. 2. Analyses of ^{125}I-labeled AD755a cells after immune precipitation with various mouse sera followed by electrophoresis on SDS polyacrylamide gels. (A) MαFLV (B6 serum raised against purified Friend leukemia virus); (B) MαAD (B6 anti-AD755a serum); (C) MαAD/FLV (B6 anti-AD755a serum absorbed with FLV); and (D) NMS (normal mouse serum)

Protection Appears to Require a Cytophilic Antibody

An observation which was made early in this study which bears heavily on the mechanism of protection is that sequential absorption of the hyperimmune anti-AD antiserum ten times with fresh AD755a cells *at its titer end point* had no effect on the protective capacity of the serum (see Table 7). Moreover, although antibodies absorbed to the cell surface under conditions of great excess could yield lysis in the presence of added complement; they were unable to provide protection when these antibody coated cells were inoculated as tumor challenge. Thus, the protective function was not easily absorbed by the target cells.

Along with the inability to directly absorb the protective serum with the target cell, the potent protective capacity of small volumes of the immune serum, as well as its apparent strain specificity, may be indicative of a cellular component in the passive serum transfer process. Phase contrast microscopic analysis of AD755a cells after interaction with the B6 immune serum and

normal B6 peritoneal exudate cells, both in vivo and in vitro, demonstrated the induction of large mononuclear cell attachment to the tumor cells (Fig. 3). This response was not observed when normal B6 mouse serum was substituted and suggests that the B6 anti-AD755a serum may be capable of activating macrophages or other mononuclear cells for tumor cell destruction. It is of interest in this regard that antisera directed against FLV or FLV-producing cells which are not protective, also do not have the capacity to form rosettes in vivo. It is thus possible that the protective factor is a cytophilic antibody with affinity for a host effector cell, presumably a macrophage. Because of the previously described viral specificity of the tumor rejection process, we tentatively postulate that a viral component remains the principal candidate for the target antigen, but that its function in this regard can only be demonstrated through a cooperative action between the antibody and the appropriate effector cell. Studies are planned to identify this component and the corresponding antibody population through in vitro and in vivo assays involving effector cells.



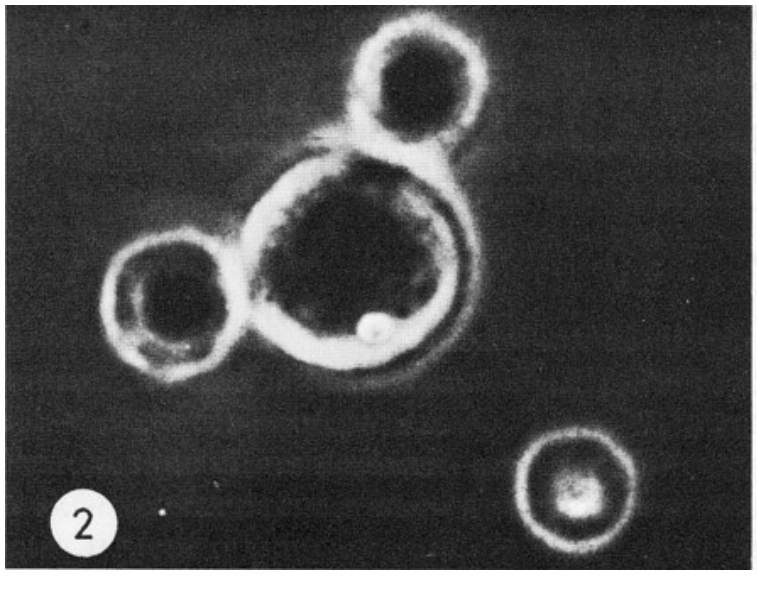


Fig. 3

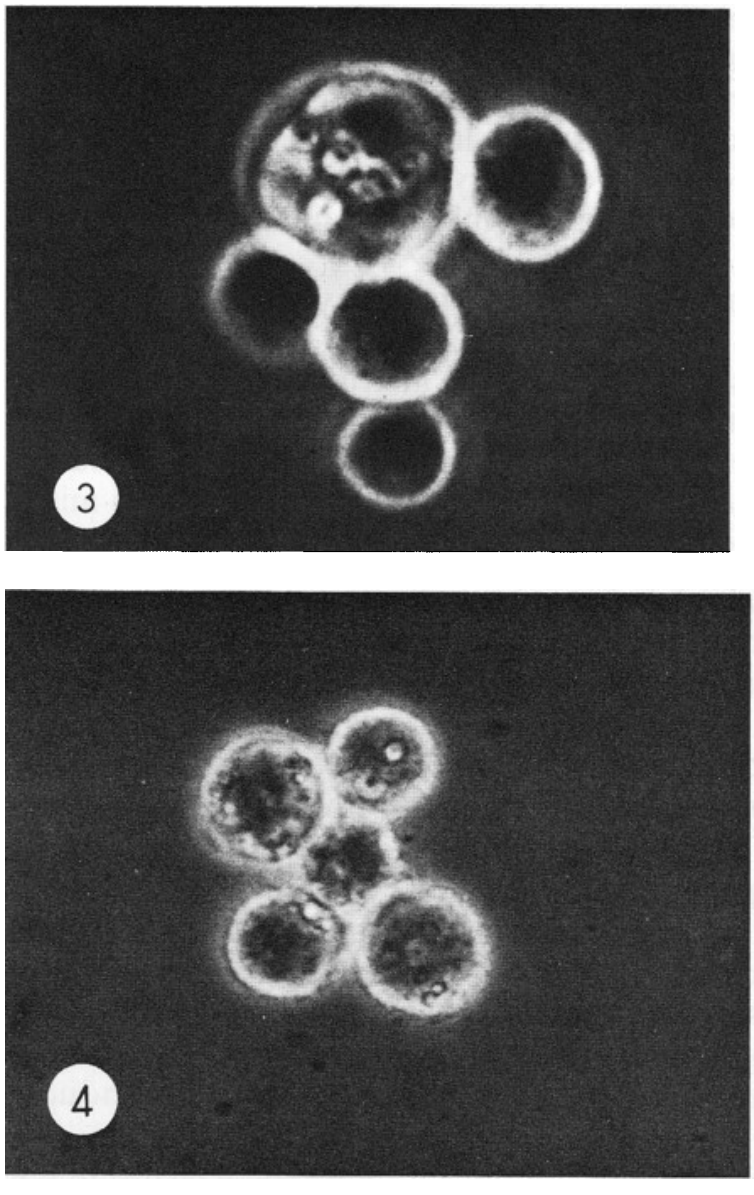


Fig. 3.1–4 Phase-contrast microscopic analysis of mouse peritoneal aspirates collected 2 hr (1), 3 hr (2) or 6 hr (3) after ip inoculation of AD755a cells and B6 mouse anti-AD755a serum. Note that tumor cells (large granular cells) are bound to large mononuclear cells. Similar analyses were carried out in vitro by mixing AD755a cells. normal B6 mouse peritoneal exudate cells and B6 mouse anti-AD755a serum for 1 hr (4). Note binding of tumor cells to a large mononuclear cell in a rosette formation. reverse of binding pattern seen in vivo (see 1–3). This rosette reaction was not observed when normal B6 mouse serum was substituted for immune serum. ×400

Concluding Remarks

In summary, AD755a can be used as an animal model system of tumor rejection that involves lymphoid cell and serum factors. This system can serve in the examination of the immune recognition and immune response mechanisms participating in tumor rejection, as well as in the study of mouse strain-specific interactions between serum factors and lymphoid cells, which may possibly mediate the observed transfer of tumor immunity.

The strain dependence of serum transfer protection was found to be controlled by a locus linked to agouti which itself is situated on linkage group V, chromosome 2. No function has been mapped in this region which could account for the phenomenon observed suggesting that the locus controlling protection by serum transfer is a new discovery. Extensive fine mapping using appropriate congenic and recombinant inbred strains is in progress to better establish its location.

Several parameters thus far noted in this system may reflect the function of this locus. These include

1. genes controlling various activities related to the virus associated with the AD755a tumors, particularly those which might affect the immune system of the recipient; or 2. immune response functions which regulate the cooperative effects between the protective antibody and the host effector cells, which might include factors such as Fc receptor specificity for the protective antibody.

The question of antigenic specificity remains elusive. The data obtained thus far strongly suggest a viral related component as the antigen involved in both the generation of protective antibody and as a target for tumor rejection. The best candidate for the antigen at present is the gp71 surface glycoprotein of ADV. However, we would have to postulate that two forms of antibody to this antigen are produced during our immunization procedure. One is a classical antibody and can be measured in virus neutralization, cytotoxicity and radioimmunoassay analyses and can be efficiently absorbed with virus or target cells. The other is an antibody with little or no affinity for the target cell. In the presence of both lymphoid cells and target cells, however, this antibody induces rosette formation, linking the target and effector cells very efficiently. Antibodies with similar functions have also been described by Haskill and colleagues [12, 13]. Studies are now in progress to determine the nature of this antibody subclass and the mechanism by which it induces rosette formation.

An alternative explanation is that our immunization protocol results in the formation of an anti-idiotype antibody. Such antibodies can actively stimulate the host immune response in a very specific fashion [4], particularly if administered in the presence of antigen [2, 10]. Thus, their existence would explain both the potency and specificity of the protective function, as well as the relative inability of the protective factor to bind to the target cell. Studies are in progress to determine if such antibodies are present and their relationship to the viral gp71 antigen.

Having noted the powerful protective function of antiserum prepared as described against AD755a tumor challenge, we have prepared similar antisera against other murine tumors. Such sera also demonstrate strong antitumor effects, particularly against virus associated sarcomas (see Table 6). Application of this principle to sarcomas in cats induced by the Snyder-Theilen strain of feline sarcoma virus caused a dramatic regression of lethal tumors in 8 of 9 cats at a relatively late stage of tumor growth, where the primary tumors were more than 7 cm in diameter and the animals were near death. Studies to determine whether similar factors are involved in this form of tumor rejection are also in progress (de Noronha and Bolognesi, in preparation).

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