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# Production of High-Titer Antibody in Serum and Ascitic Fluid of Hamsters for a Variety of Virus-induced Tumor Antigens

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Methods not involving the use of viable tumors were developed for production of high-titer antibodies. The methods involved use of tumor homogenates. Homogenates from all tumor lines tested contained the characteristic tumor antigen. Attempts to isolate virus failed, and virus antigens could not be demonstrated. Immunization with tumor homogenates, in Freund's complete adjuvant, resulted in high antibody levels. None of the animals so immunized developed tumors.

The potential usefulness of immunization procedures for the production of antibody to the virus-induced tumor antigens of a number of oncogenic viruses has been shown in several studies (2, 9, 13). The advantages of obtaining large volumes of high-titer antibody from small laboratory animals are obvious, and ability to obtain serial bleedings from "unusual" or rare responders can be critical. In certain instances when the "classical" source, tumor-bearing animals, has proven unsuccessful, immunization has been the only method of obtaining high-titer reactive sera. Our methods do not involve the use of viable tumors at any point and thus differ in this respect from those reported by other workers (9, 13). Although current indications are that such procedures give reagents of equal utility to those obtained from tumor-bearing animals, the possibility of unexpected cross-reactions resulting from responses to non-tumor antigens must be kept in mind.

## MATERIALS AND METHODS

Tumor homogenates (20%, w/v, in buffered saline) were prepared from 15-mm (ca. 1 g) tumors in 10- to 14-day-old hamsters which had received tumor transplants at birth. Such tumor preparations consistently yield higher antigen titers in complement-fixation (CF) tests than do tumors obtained from weanling animals, and they are rarely anticomplementary. Periodic attempts to isolate adenoviruses, simian virus 40 (SV40), and polyoma from such hamster transplant lines have failed, and virion antigens have thus far not been demonstrated. Homogenates from all tumor lines contained the tumor (T) antigen characteristic of the particular virus type, although in variable titer. The individual samples of a given type with the highest titer were pooled and used for immunization within 24 hr after CF tests (12). Animals received 1 ml of a 1:1 mixture of homogenate (frozen and thawed several times and clarified by low-speed centrifugation) and Freund's complete adjuvant. Injections were given intraperitoneally or in the foot pads of weanling hamsters. Repeat injections were given with homogenates which had been either frozen at −70 C or freshly prepared; in both cases, CF tests were performed before injection. Blood samples were obtained from the orbital plexus. Ascitic fluid, withdrawn with an 18-gauge needle and generally free of blood, was dialyzed against phosphate-buffered saline (*p*H 7.2) to adjust *p*H and tonicity.

## RESULTS

*Human adenovirus group A (types 12, 18, 31).* Four weekly intraperitoneal injections of homogenate (CF titer, 1:8 to 1:32) in Freund's complete adjuvant regularly induced high-titer antibody responses for the three members of this group (5). Of 23 animals injected with type 12 antigen only 1 failed to respond; the remainder were strongly positive (Table 1). Approximately 50% of the animals developed ascitic fluid (15 to 25 ml) within the time period studied, i.e., 10 to 14 days after the final injection. These fluids were of high antibody titer but the magnitude of the titers was up to fourfold lower than those of the corresponding sera. None of the animals in this group or in any other immunized group developed tumors, thus demonstrating that a viable tumor is not essential for production of antibody to the tumor antigen. The reactions obtained appeared to be specific, since no reactions with nonadenovirus tumor antigens or group B adenovirus tumor antigens were encountered (Table 2).

TABLE 1. *Individual responses of animals inoculated with adenovirus type 12 tumor antigen in Freund's complete adjuvant*[a]

| Animal no. | Titer, reciprocal[b] | | Anticomplement activity[c] | |
|---|---|---|---|---|
| | Serum | Ascitic fluid | Serum (1:20) | Ascitic fluid (1:20) |
| C1-H 2 | 640 | 160 | 1− | |
| C1-H 3 | 640 | 160 | | |
| C1-H 4 | 320 | | | |
| C1-H 8 | 640 | 160 | | |
| C1-H 10 | 160 | | | |
| C1-H 11 | 80 | | 2− | |
| C2-H 1 | 320 | 160 | 3 | |
| C2-H 2 | 160 | | | |
| C2-H 3 | 640 | 320 | 1− | 1− |
| C2-H 4 | 160 | 40 | 4 | 2 |
| C2-H 5 | 40 | | | |
| C2-H 6 | 640 | 320 | | |
| C2-H 7 | <20 | | | |
| C2-H 8 | 320 | 160 | | |
| C2-H 9 | 320 | 160 | | |
| C2-H 10 | 160 | 20 | 2 | |
| C3-H 1 | 320 | 80 | | |
| C3-H 2 | 160 | | | |
| C3-H 3 | 320 | 160 | | |
| C3-H 4 | 320 | 160 | | |
| C3-H 5 | 640 | | 4− | |
| C3-H 6 | 320 | | | |
| C3-H 7 | 80 | | 2 | |

[a] Bleedings 10 to 14 days after the fourth injection.

[b] Tested with 4 units of homologous tumor antigen.

[c] Degree of complement fixation: 4 = complete fixation; only positive results recorded.

*Human adenovirus Group B (types 3, 7, 14, 16, 21).* Responses of tumor-bearing animals in this group (5) were very erratic, and only rarely were CF titers of 1:80 obtained. The extreme cases were types 3 and 14, with which even a low-titer response (1:20) was unusual. These tumor lines definitely contained tumor antigen readily detectable by reaction with type 7 or type 21 tumor antiserum. With type 14, over 100 transplant tumor-bearing animals were tested without encountering a single positive serum. In contrast, immunized animals did develop high-titer antibody, with several responding with reactions at dilution of 1:640 to 1:1,280; however, six to eight injections over 3 to 4 months were required to achieve this result. Positive sera reacted with tumor antigens from all members of the B group and not with nonadenovirus tumor antigens or group A adenovirus antigens (Table 3). Preliminary success with type 16 has also been achieved; to date, animals injected with type 3 have been essentially nonresponsive. Type 3 regularly yields the lowest antigen titers of the adenovirus B group (1:2 to 1:8), in contrast to the 1:8 to 1:32 titers obtained for types 7, 14, and 21.

*Polyoma.* The polyoma tumor line yielded results similar to those with adenovirus type 14. Repeated injections (CF titer, 1:8), over a period of 4 to 6 months were necessary to obtain uniformly high titer responses (1:160 to 1:640). Once positive, titers remained high over intervals of 4 to 6 weeks, and booster responses could readily be obtained. The antiserum produced appeared to be specific in that no reactions with nonpolyoma tumors were obtained (Table 4). Such serum has proven useful in detecting the polyoma tumor antigen (1, 3, 14) in infected cells

TABLE 2. *Specificity of adenovirus type 12 antitumor ascitic fluid*

| Test antigens[a] | CF titer, reciprocals |
|---|---|
| Adenovirus 12, 18, 31 (tumor) | 160–320 |
| Adenovirus 7, 14, 21 (tumor) | <10[b] |
| SV40 (tumor) | <10 |
| Polyoma (tumor) | <10 |
| Rous sarcoma (tumor) | <10 |
| Adenovirus group | <10 |
| Adenovirus "C" antigen | <10 |

[a] Antigens used at 4 to 8 units, based on homologous titrations.

[b] Partial reactions obtained at 1:10 with high concentrations (16 to 32 units) of group B antigens.

TABLE 3. *Reactions of adenovirus (types 14 and 16) tumor antisera produced by immunization*

| Test tumor antigens[a] | CF titer, reciprocals[b] | |
|---|---|---|
| | Type 14 | Type 16 |
| Adenovirus 3, 7, 14, 16, 21 | 80–640[c] | 20–80[c] |
| Adenovirus 12, 18, 31 | <10 | <10 |
| Rous sarcoma | <10 | <10 |
| SV40 | <10 | <10 |
| Polyoma | <10 | <10 |
| F.Sa.-3[d] | <10 | <10 |

[a] Homologous and other adenovirus antigens used at 4 to 8 units; nonadenovirus antigens used undiluted.

[b] Representative serum titers after eight spaced injections.

[c] Titers essentially similar with any group B antigen.

[d] Sabin et al. (10).

TABLE 4. *Specificity of polyoma tumor antisera produced by immunization*

| Test tumor antigens[a] | CF titer, reciprocals[b] |
|---|---|
| Polyoma | 160–640 |
| SV40 | <10 |
| Adenovirus 7 | <10 |
| Adenovirus 12 | <10 |
| Rous sarcoma | <10 |
| F.Sa.-3[c] | <10 |

[a] Homologous antigen used at 4 units (1:4 dilution of 20% extract); heterologous antigens used undiluted.

[b] Representative serum titers after 12 spaced injections.

[c] Sabin et al. (10).

TABLE 5. *Specificity of Rous sarcoma (Schmidt-Ruppin strain) tumor antisera produced by immunization*

| Test tumor antigens[a] | CF titer, reciprocals[b] |
|---|---|
| Rous sarcoma | 160–640 |
| SV40 | <10 |
| Polyoma | <10 |
| Adenovirus 7 | <10 |
| Adenovirus 12 | <10 |
| Adenovirus 12 | <10 |
| F.Sa.-3[c] | <10 |

[a] Homologous antigen used at 4 units (1:8 dilution of 20% extract); heterologous antigens used undiluted.

[b] Representative serum titers after 8 spaced injections.

[c] Sabin et al. (10).

and in tumor cells by indirect immunofluorescence (Fogel, Gilden, and Defendi, *in press*).

*SV40.* As previously reported (2), two to four injections of SV40 tumor homogenate in Freund's complete adjuvant sufficed to induce regular high-titer specific responses.

*Rous sarcoma (Schmidt-Ruppin strain).* Extensive immunization with tumor homogenates (CF titer, 1:16) induced high-titer antibody, with CF titers reaching 1:160 to 1:640. The suitability of such sera for use in the COFAL (6, 11) test is under study. Some nonspecific reactions with supposedly resistance-inducing factor (RIF)-free chick embryo cells have been detected with certain sera, although the generality of this observation is not certain. Certain hamster sera, regardless of origin, react with chicken embryo fibroblasts in CF tests; thus, suitable sera are necessarily selected on the basis of well-controlled tests (Huebner, *unpublished data*). However, certain of our pools do not react with third passage "RIF-free" chick embryo fibroblasts (Kimber Farms, Fremont, Calif.). Regardless of suitability for the COFAL test, i.e., detection of avian leucosis viruses in chick embryo fibroblasts, these sera are specific within the hamster tumor systems (Table 5).

*Anticomplement activity.* In our experience, sera obtained from tumor-bearing animals rarely fix complement. In contrast, sera from immunized animals did exhibit anticomplement activity in variable frequency in the different groups. In some groups, no anticomplement activity was encountered; in others, over 50% of the animals eventually developed anticomplement activity.

## DISCUSSION

The value of immunization procedures for production of tumor antibody can be emphasized from three different empirical findings: (i) the production of relatively large volumes of ascitic fluid and serum from previously well-documented tumor lines, e.g., SV40 and adenovirus 12; (ii) the production of reactive serum for tumor groups in which tumor-bearing animals have been poorly or nonresponsive, e.g., adenovirus 14 and polyoma; and (iii) the ability to obtain repeat samples from rare or early responders in groups in which current immunization methods do not induce uniform antibody responses.

The methods employed do not require the use of viable tumors at any time, and thus differ from those used by Takemoto and Habel (13). In the absence of viable tumors, positive animals may be maintained indefinitely and bled or given booster injections as desired. Our observations indicate that once animals become positive, be it after 2 or 10 injections, antibody titers tend to remain high or can be maintained at high levels by intermittent reinjection.

The variability in responsiveness generally observed follows the same pattern seen in tumor-bearing animals. Within the A group of adenoviruses, tumor-bearing animals are regularly positive, with titers of 1:80 to 1:160 being common; group B tumor-bearing animals are generally poorly or nonresponsive. In parallel fashion, immunization with group A adenovirus homogenates induced uniformly high-titer responses; group B adenovirus tumor homogenates induced erratic responses, although, after multiple injections, the majority of animals usually became positive. The essential point is that this immunization method has proven successful with certain group B adenovirus-induced tumors, whereas negative results were obtained with tumor-bearing

animals. In terms of weak antibody responses, polyoma represents the extreme case, since as many as 12 injections were needed to induce fairly uniform high-titer antibody responses; again, tumor-bearing animals were uniformly nonresponsive (CF titers <1:10).

The single known distinguishing factor between the immunogenically potent and poor antigens (if CF titers are equivalent) is their relative heat lability (Gilden et al., *unpublished data*). Adenovirus group A antigens are (or contain) a relatively thermostable (56 C) component (8); group B antigens are rapidly inactivated at this temperature, and are gradually inactivated at somewhat lower temperatures (45 C). Polyoma tumor antigen is extremely heat-labile and is inactivated within 24 to 72 hr at 5 C and quite rapidly at 37 C. Our recent studies with bovine adenovirus type 3 tumor antigens (Gilden et al., *in press*) have shown that, although tumor-bearing animals are regularly poorly or nonresponsive, immunization does induce tumor antibody in a high percentage of animals. The bovine adenovirus type 3 antigen is also extremely labile at 56 C. In vitro heat lability may well reflect an inherent susceptibility to in vivo degradative processes which decrease immunogenicity.

Originally, it was thought that immunization with tumor homogenates in Freund's complete adjuvant might result in extensive responses to nontumor iso-antigens (10). In our experience, such responses have not been evident. Broader reactions with "normal" antigens from heterologous sources were also anticipated; however, adenovirus antisera produced by immunization did not react with normal KB or HEK cells, in which T antigens for these viruses are generally produced (4, 7). Serological tests with adenovirus T antigen preparations did, however, give results (titer and specificity) similar to tumor preparations. Similarly, the SV40 immune sera did not react with uninfected green monkey kidney cells, but did react specifically with SV40 infected cells. These findings indicate that sera produced by immunization procedures are of general utility within carefully defined systems.

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