SUPPRESSION OF IN VIVO TUMOR FORMATION INDUCED BY SIMIAN VIRUS 40–TRANSFORMED CELLS IN MICE RECEIVING ANTIIDIOTYPIC ANTIBODIES

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The idea that the immune response to an antigen (Ag) can be regulated by an idiotype (Id)-antiidiotype network was first proposed by Jerne (1). Id located on or close to the Ag-binding site of both antibody molecules and lymphocyte Ag receptors are components of this network. Numerous studies have implicated Id networks in regulating the immune response to a large variety of haptens and protein or carbohydrate Ag (reviewed in 2 and 3). The injection of anti-Id before Ag exposure has resulted in either suppression of the Id+ Ag-binding molecules, or increased Id expression and Ag-binding activity. Recent studies (4–11) have indicated that anti-Id may be useful in inducing protective immunity against a wide variety of infectious agents, and have suggested the potential of anti-Id as vaccine candidates (12, 13). Alternatively, anti-Id injection before exposure with another infectious agent resulted in an apparent suppression of the immune response, which manifested itself in an increase in pathogenicity of the agent for the host (14).

The use of anti-Id in the treatment of cancer has primarily focused on lymphoid tumors (reviewed in 15 and 16). In these studies, cell surface Ig present on neoplastic B lymphoid cells represents the Id, and suitable anti-Id could eliminate much or all of the tumor, with negligible effects on residual normal tissues. Injection of mice with anti-Id that appears to mimic the tumor Ag structure has been reported to induce antitumor immunity in human melanoma (17) and mammary adenocarcinomas (18). Alternatively, anti-Id generated against suppressor T lymphocyte receptors was also capable of reducing the in vivo tumor growth of a mastocytoma (19), whereas another report (20) indicated that anti-Id suppressed tumor rejection to fibrosarcomas when produced against T lymphocyte clones. A recent study (21) has implicated anti-Id, produced when humans were injected with mouse monoclonal antibodies (mAb) against gastroin-
testinal cancer cells, in the clinical improvement and longer remission of these patients with colorectal carcinoma. Together, these data indicate that Id networks may play some integral immunological role in the formation of tumors.

Simian virus 40 (SV40) is an oncogenic DNA virus that induces tumors in vivo when inoculated into newborn hamsters, and transforms cells in vitro from a variety of species, including rodents, primates, and humans. Rodent cells transformed by SV40 in vitro will frequently form tumors in hamsters and mice (reviewed in 22). Cells that have been transformed by SV40 express a virally encoded tumor antigen (T-Ag). T-Ag is predominantly localized in the nucleus of transformed cells; however, a small fraction of T-Ag is found on the cell surface (23–27). Mice immunized against T-Ag are protected from challenge by syngeneic SV40 tumor cells (28–31, reviewed in 32). Thus, the SV40 T-Ag is associated with the induction of tumor immunity.

In this report, we generate and characterize rabbit anti-Id reagents against mouse mAb to the carboxyl and amino terminal epitopes of SV40 T-Ag, and to a cellular protein, designated p53 (33–35), that is intimately associated with T-Ag on the surface of transformed cells (36, 37). These anti-Id reagents each recognized antibody-combining site–related, private Id determinants. Treatment of BALB/c mice with a pool of anti-Id generated against mAb to both the carboxyl and amino termini of SV40 T-Ag before challenge with a tumorgenic dose of SV40-transformed cells partially suppressed tumor growth. In some instances, this suppression was complete, since several of the pooled anti-Id–treated mice failed to develop any detectable tumors after challenge, whereas 100% of the control groups of mice developed tumors. The implications of these observations with regard to Id networks and SV40 tumor induction will be discussed.

Materials and Methods

Mice. Adult female BALB/c mice were purchased at 6–8 wk, from Charles River Breeding Laboratories, Inc., Wilmington, MA.

Virus and Cell Lines. The Baylor reference strain of wild-type SV40 (38) was grown and assayed as previously described (39). The transplantable SV40-transformed BALB/c mouse cell lines mKSA (40, 41) and VLM (41) were used for intraperitoneal and intradermal tumor cell challenges, respectively. The SV40-transformed hamster cell line, HaAsc (24), and the SV40-transformed mouse cell line, 3T3/SV-1/10 (42), were used for T-Ag and p53 purification. All SV40-transformed cell lines were cultured in Dulbecco's modified minimal essential medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco Laboratories), 0.3% sodium bicarbonate, and 25 μg/ml gentamicin sulfate (Schering Corp., Bloomfield, NJ).

Purification of SV40 T-Ag and p53 Cellular Protein. T-Ag and p53 were purified by immunoaffinity chromatography. mAb PAb419 was covalently crosslinked to protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) according to the method of Schneider and coworkers (43). The preparation containing only T-Ag was derived from extracts of the SV40-transformed hamster cell line, HaAsc (24), which contains very low levels of p53. The preparation containing T-Ag and p53 was obtained from the SV40-transformed mouse cell line, 3T3/SV-1/10 (33), which contains very high levels of p53. Thus, p53 was purified from the same cells, by virtue of its association with T-Ag. Briefly, T-Ag present in detergent extracts of tissue culture cells was bound to the affinity matrix during an overnight incubation, with shaking. A column was prepared from the T-Ag–containing affinity matrix, and T-Ag was eluted using 10 mM Tris-NaOH (pH 11.0) and 100 mM NaCl. Fractions were collected in an equal volume of 50 mM Tris-HCl (pH 7.0)
and 100 mM NaCl to rapidly neutralize the high-pH elution buffer. The peak fractions were pooled and concentrated in Centricon-30 tubes (Amicon Corp., Danvers, MA).

Antibodies and Their Purification. The following mAb recognize SV40 T-Ag: PAb 405, 416, 419, 423, and 430, and were the gifts of Dr. E. Harlow. mAb PAb 421 and HD 200 recognize p53, and were gifts from E. Harlow and A. DeLeo, respectively. These antibodies have been described in detail elsewhere (44, 45), and their specificities are given in Table I. mAb A-1, A-2, W-1, and W-2, which recognize hepatitis B surface antigen (HBsAg) were generated in this laboratory (46). All hybrid clones were passaged in ascites form by intraperitoneal injection of 1–5 × 10⁶ hybrid cells into pristane-primed BALB/c mice.

Mouse ascites fluid was delipidated at 4°C by the addition of manganese chloride to a final concentration of 2.5 mM. After centrifugation at 2,000 g to remove lipids, 18–20% (wt/vol) sodium sulfate was added, at room temperature. The resulting precipitate, containing the globulin fractions, was suspended in 0.16 M borate buffer (pH 8.2), and was further fractionated by molecular sieve chromatography on a Sephadex G-200 (Pharmacia Fine Chemicals) column equilibrated in borate buffer. The IgG-containing peak was concentrated, and the amount of protein was determined spectrophotometrically by using an extinction coefficient of 14 for a 1% preparation at 280 nm. IgG was also purified from BALB/c mouse serum using similar methods. To insure the purity of the IgG Id preparations, PAb 419 and 421 were recycled over Sephadex G-200 columns, while PAb 405 and HD 200 were further purified by preparative isoelectric focusing using an LKB electrofocusing apparatus in a sucrose ampholyte gradient (with a pH range of 3.0–9.5), according to the manufacturer’s specifications. The sucrose was removed by dialysis against borate buffer.

Immunoadsorbents for Id and Anti-Id Reagents. Individual IgG antibody preparations from normal BALB/c mouse sera, PAb 405, 419, 421, and HD 200 were covalently coupled to CNBr-activated Sepharose 4 B at a concentration of 3 mg/ml of Sepharose. Affinity-purified IgG rabbit anti-Id preparations to PAb 405, 419, 421, and HD 200 were individually coupled to CNBr-activated Sepharose 4 B at a concentration of 1 mg/ml of Sepharose.

Preparation of Anti-Id Antisera. The preparation of anti-Id in rabbits and its subsequent affinity purification have been described previously in detail (47–49). Briefly, 2 mg of IgG anti–SV40 T-Ag or anti-p53 from four separate clones (PAb 405, PAb 419, PAb 421, and HD 200) was emulsified in complete Freund’s adjuvant and injected into the footpads of at least two New Zealand White rabbits. Each rabbit received at least four biweekly injections before obtaining antiserum. The immune rabbit antisera were repeatedly adsorbed on a Sepharose 4 B column conjugated with normal BALB/c mouse IgG until all detectable reactivity with isotype and allotype determinants were removed. The presence of antiasotypic and antiiallootypic antibody was determined by a direct-binding

| Designation | Antibody class | Specificity | Reference |
|-------------|----------------|------------|-----------|
| PAb 405     | IgG1           | Anti-T-Ag  | 44        |
| PAb 416     | IgG2a          | Anti-T-Ag  | 44        |
| PAb 419     | IgG2a          | Anti-T-Ag  | 44        |
| PAb 421     | IgG2a          | Anti-p53   | 44        |
| PAb 423     | IgG1           | Anti-T-Ag  | 44        |
| PAb 430     | IgG3           | Anti-T-Ag  | 44        |
| HD 200      | IgG2a          | Anti-p53   | 45        |
| A-1         | IgG1           | Anti-HBsAg | 46        |
| A-2         | IgG1           | Anti-HBsAg | 46        |
| W-1         | IgG1           | Anti-HBsAg | 46        |
| W-2         | IgG1           | Anti-HBsAg | 46        |
radioimmunoassay (RIA) (see below). Potential shared determinants were removed by further adsorption of antisera over anti-T-Ag or anti-p53 mAb immunoadsorbent columns that were unrelated to the rabbit immunogen. Following the removal of all detectable antiisotype and antiallotype reactivity, the rabbit anti-Id preparations were affinity-purified on Sepharose 4 B columns containing the respective mAb Id preparations. The elution of affinity-purified rabbit anti-Id was performed with 1 M acetic acid, using methods previously described in detail (7, 49). The concentration of rabbit anti-Id was determined spectrophotometrically, using an extinction coefficient of 15 for a 1% preparation.

**Preparation of Affinity-purified Id.** Ascites fluid from the four Id preparations were delipidated and salt-fractioned as described above, and dialyzed overnight at 4 °C against borate buffer. The individual Id preparations were then adsorbed on affinity columns containing their respective rabbit anti-Id. The affinity columns were washed with excess borate buffer until no detectable protein was present in the eluent, and affinity-purified Id preparations were then eluted with 1 M acetic acid. The protein-containing fractions were pooled, neutralized, and dialyzed overnight at 4 °C in borate buffer. The affinity purification is similar to methods previously described (7, 49). These idotype preparations were concentrated, and the amount of protein was determined spectrophotometrically. Anti-SV40 T-Ag or p53 activity was determined by immunoprecipitation or indirect immunofluorescence, as described below. The affinity-purified Id preparations were used solely for the in vivo modulation experiments on SV40 tumor induction.

**Assays for Antibody Activity Against SV40 T-Ag and p53.** Sera from mice immunized with rabbit anti-SV40 T-Ag and p53 Id were assayed for anti-T-Ag and anti-p53 activity by immunofluorescence and immunoprecipitation. Immunofluorescence assays for nuclear T-Ag or p53 were performed on acetone-fixed monolayers of VLM cells, as previously described (50), using serial twofold dilutions of the mouse serum, followed by fluorescent-conjugated rabbit anti-mouse IgG (Cappel Laboratories, Cochranville, PA). Detergent extracts of VLM cells labeled with [35S]methionine were immunoprecipitated with the different mouse sera and analyzed for T-Ag and p53 on sodium dodecyl sulfate (SDS)-polyacrylamide gels as previously described (24).

**Assays for Detecting Anti-Id Activity:** Binding Assays. A solid-phase RIA was used for the binding analysis of the anti-Id antisera (47–49). Briefly, 50 μl containing 200 ng of mouse IgG prepared from mAb-containing ascites fluid or BALB/c mouse serum were coated onto the wells of flat-bottomed microtiter polyvinyl plates for 8 h at 4 °C. After blocking nonspecific sites with 1% bovine serum albumin, 50 μl of various dilutions of rabbit anti-Id antisera or concentrations of affinity-purified anti-Id were added, and incubated for 1 h at 25 °C. The microtiter plates were washed with a 0.01% Tween 20 phosphate buffered saline and 50 μl of a reagent containing 100,000 cpm of a 125I-labeled goat anti-rabbit gamma globulin (GARGG) were added. The preparation and chloramine-T iodination of GARGG are described elsewhere (47). After incubation for 1 h at 25 °C, the residual radioactivity was removed, and the plates were washed. The wells of the microtiter plates were cut out, and bound radioactivity was counted in an automatic gamma counter. The binding curves generated were expressed as cpm 125I-labeled GARGG bound vs. concentration of anti-Id.

**Inhibition Assays.** A solid-phase RIA with IgG mAb used as inhibitors was performed according to the previously described methods (45). Briefly, equal volumes of various inhibitors and anti-Id, used at a concentration corresponding to the linear portion of the binding curve, were preincubated for 4 h at 4 °C before the addition of 50 μl of the inhibitor–anti-Id complex to the Id-coated wells. This assay was then performed similarly to that described above for direct binding. The percent inhibition was calculated from the formula: Percent inhibition = 1 − [(cpm bound with inhibitor)/(cpm bound without inhibitor)] × 100. The percent inhibition was determined from the mean of triplicate values.

To ascertain whether purified SV40 T-Ag and p53 would inhibit the Id–anti-Id binding, a modification was made in the inhibition assay. The microtiter wells were coated with 50 μl of a concentration of anti-Id corresponding to the linear portion of the binding curve.
After adding 1% bovine serum albumin to block nonspecific sites, we added 50 μl of a solution containing equal volumes of various concentrations of T-Ag or p53 and 50,000 cpm of 125I-labeled Id that had been previously preincubated for 4 h at 4°C. After incubating for 1 h at 25°C, removing residual radioactivity, washing the plates, and counting were performed as described. The percent inhibition of binding was calculated from the formula described above.

**Assay for Detecting Anti-anti-Id (Ab3) Activity.** This assay is similar to that described above for the inhibition of the Id-anti-Id reaction, except that mouse sera obtained from anti-Id-treated mice were used to inhibit the solid-phase RIA. Radioactively labeled GARGG was used to detect the inhibition of the anti-Id binding to its respective Id.

**In Vivo Administration of Id and Anti-Id Preparations.** BALB/c mice were given intraperitoneal injections of either affinity-purified Id or anti-Id, as an alum precipitate at pH 7.0. Control antibodies were affinity-purified rabbit anti-Id that recognized a common human Id on antibodies against HBsAg. This preparation was generated by adsorptions similar to those described for the anti-SV40 T-Ag Id preparations. The specificity of this reagent has been described elsewhere (7, 47). Mice received three injections of 50 μg alum-precipitated anti-Id, every 10 d, and serum was obtained 7 d after the third immunization. Serum obtained from each mouse before any immunization served as a negative control. In addition, groups of mice received three injections of 50 μg of either anti-Id or Id preparations every 10 d, and were challenged with a tumorigenic dose of SV40-transformed cells (VLM or mKSA) 7 d after the final immunization. The tumor cell inoculum corresponded to a dose where 100% of untreated animals would develop tumors. The tumorigenic immunization was given either intradermally, behind the neck of the animals (VLM cells), or intraperitoneally (mKSA cells). The VLM tumors that developed from the intradermal injection were measured by calipers, and expressed as the size of the tumor diameter. Tumor growth in mice receiving mKSA cells intraperitoneally was determined by the survival time of the animals. Numerous pilot experiments were performed to establish the optimal parameters with regard to concentrations of the antibody preparations, dose of the SV40 tumor cells, and time interval between injections of antibodies and tumor cells.

**Statistical Methods.** The levels of significance (P values) were obtained using the two-tailed student’s t test on the mean survival time of each treatment group of mice.

**Results and Discussion**

**Rabbit Antisera to SV40 mAb Recognize Private Id Determinants.** The anti-Id nature of four different rabbit antisera was demonstrated by the binding curves shown in Fig. 1. Two rabbit antisera, anti-419 Id and anti-405 Id were generated against mAb that recognized SV40 T-Ag. These antisera bound only their respective monoclonal Id preparation, and failed to significantly bind to IgG from other SV40 T-Ag mAb, and also IgG prepared from normal BALB/c mouse serum, mAb against p53, and mAb against HBsAg (anti-HBs) (data not shown). In this regard, at a concentration of 400 ng, anti-419 Id bound 30,100 cpm with the 419 Id, while only 150–300 cpm were bound with all other mouse IgG preparations (Fig. 1A). Similar results are illustrated by the binding curves generated with anti-405 Id (Fig. 1B). Two other antisera were produced by immunizing rabbits with IgG obtained from two p53 mAb. Again, anti-421 Id and anti-HD 200 Id demonstrated the ability to bind only their respective Id preparations (Fig. 1, C and D).

In the next series of experiments, the individual Id-anti-Id reactions were examined by inhibition analysis to further determine whether the rabbit anti-Id recognized determinants associated only with the immunizing IgG mAb prepa-
Table I

Inhibition of Binding of Rabbit Anti-Id to Their Respective Id by Various IgG Inhibitors

| Inhibitor  | 405 Id-anti-405 | 419 Id-anti-419 | 421 Id-anti-421 | HD 200 Id-anti-HD 200 |
|------------|----------------|----------------|----------------|----------------------|
| PAb 405    | 0              | 0              | 0              | 0                    |
| PAb 416    | 0              | 0              | 0              | 0                    |
| PAb 419    | 0              | 0              | 0              | 0                    |
| PAb 421    | 0              | 0              | 0              | 0                    |
| PAb 423    | 0              | 0              | 0              | 0                    |
| PAb 430    | 0              | 0              | 0              | 0                    |
| HD 200     | 4              | 4              | 4              | 4                    |
| A-1        | 1              | 1              | 1              | 1                    |
| A-2        | 1              | 1              | 1              | 1                    |
| W-1        | 0              | 0              | 0              | 0                    |
| W-2        | 0              | 0              | 0              | 0                    |

* All inhibitors were used at a final concentration of 5 μg.

* Represents the mean percent inhibition of binding obtained from triplicate values.

A summary of the characteristics and specificity of all the mAb used in the inhibition analysis is given in Table I. Based on the ability to block the binding of the anti-Id to the respective Id, only the specific IgG preparation used as the immunogen was capable of significantly (>95%) inhibiting the reaction (Table II). At a concentration of 5 μg, normal mouse IgG and IgG purified from various non-Id mAb did not significantly inhibit any of the four Id-anti-Id reactions. Inhibition values from 11 different IgG preparations ranged from 0
to 9%. The inhibition curves obtained using the homologous Id to inhibit the various Id-anti-Id reactions are depicted in Fig. 2. The inhibitor concentration that gave 50% inhibition (I50) of the 419 Id-anti-419 Id reaction was 10 ng (Fig. 2A). The I50 values for 405 Id-anti-405 Id, 421 Id-anti-421 Id, and HD 200 Id-anti-HD 200 Id were 12 ng, 9.6 ng, and 5 ng, respectively (Fig. 2, B, C, and D). Based on these data, it was apparent that each of the four rabbit antisera detected a unique determinant present on only the immunizing IgG mAb preparation.

Inhibition of Id-binding Anti-Id by Purified SV40 T-Ag and p53 Protein Preparations. To ascertain whether the determinants recognized by the various Id-anti-Id reactions were associated with the antibody-combining site of the Id, purified preparations containing either T-Ag or both T-Ag and p53 as a complex were used as inhibitors. The reactions of 421 Id-anti-421 Id and HD200 Id-anti-HD 200 Id were inhibited only by the T-Ag-p53 complex preparation and not by purified T-Ag alone (Table III). At a total protein concentration of 2 μg, the T-Ag-p53 preparation inhibited 421 Id-anti-421 Id and HD 200 Id-anti-HD 200 Id reactions by 55% and 36%, respectively. These data indicate that the two anti-Id preparations recognized determinants associated with the antibody-combining site of the Id.

Table III

| Inhibitor                  | Concentration (μg) | 421 Id-anti-421 | HD 200 Id-anti-HD 200 | 419 Id-anti-419 | 405 Id-anti-405 |
|---------------------------|--------------------|----------------|----------------------|----------------|----------------|
| BSA                       | 1,000              | 0*             | 0                    | 0              | 0              |
| Ovalbumin                 | 10,000             | 0              | 0                    | 0              | 0              |
| SV40 T-ag/p53 complex     | 2                  | 55             | 36                   | 32             | 31             |
|                           | 0.4                | 43             | 19                   | 7              | 20             |
| SV40 T-ag                 | 2                  | 0              | 2                    | 32             | 93             |
|                           | 0.4                | 1              | 0                    | 26             | 88             |

* Mean of triplicate values.
bining site of anti-p53 protein mAb. Similarly, the two anti-Id generated to SV40 T-Ag mAb also appeared to recognize the antibody-combining site. Inhibition of 419 Id-anti-419 Id and 405 Id-anti-405 Id reaction was obtained with both the T-Ag-p53 complex--containing preparation and with purified T-Ag (Table III). In the 405 Id-anti-405 Id reaction, the purified T-Ag was a much more efficient inhibitor (2 μg inhibited 93%) compared with the T-Ag-p53--containing preparation (2 μg inhibited 31%). Conversely, both preparations were equally efficient as inhibitors of the 419 Id-anti-419 Id reaction. The reason for the difference in inhibitor capacity of the antigen preparations in the two SV40 T-Ag Id-anti-Id reactions is not known. However, the possibility exists that during affinity purification and elution with high pH from an affinity column containing IgG purified from 419, antigenic determinants associated with the amino terminal portion of T-Ag, which are bound by 419, were more labile. Thus, denaturation of epitopes associated with the 419, SV40 T-Ag--binding site might explain the apparent differences in inhibitor capacity of the purified T-Ag preparation to inhibit the two Id-anti-Id reactions. Alternatively, 419 IgG may have leached off the affinity column and contaminated the T-ag preparation. In this instance, some of determinants recognized by 419 Id may have been bound by contaminating 419 and diminished the capacity of the T-Ag preparation to inhibit the 419 Id-anti-419 Id. This possibility seems less likely, since no Ig heavy or light chain contamination was seen in silver-stained SDS-polyacrylamide gels of the purified T-Ag (data not shown). Thus, any contamination of 419 from the affinity column would be minimal.

Based on the binding and inhibition studies, each Id-anti-Id reaction recognized unique Id determinants associated, at least in part, with the antibody-combining site of the immunized Id preparation. These data indicate that these anti-Id reagents do indeed detect Id determinants.

Numerous studies had previously reported the use of anti-Id in modulating the immune response to both infectious agents (4-11) and tumors (17-21). We decided to use the anti-Id reagents against SV40 T-Ag and p53 in an attempt to modulate the immune response to SV40 tumor induction in vivo. We selected BALB/c mice as the animal of choice for these studies because tumorigenic, SV40-transformed BALB/c cells lines were available, and because the Id preparation was of BALB/c origin. In our initial studies, we selected a dose of SV40-transformed cells that would induce visible tumors in 100% of the inoculated mice after 21 d. The route of inoculating 10^4 SV40 tumor cells (VLM cells) was intradermal, behind the neck. All anti-Id immunizations were given intraperitoneally as an alum precipitate, since we had previously demonstrated that alum adsorption of anti-Id against HBSAg induced high titers of antibodies in mice (7).

Effect of Anti-Id Immunization on SV40 Tumor Induction. In our first set of experiments, nine groups of eight mice each were given three injections of 50 μg of anti-Id, 10 d apart, then challenged with 10^4 VLM tumor cells 7 d after the final injections. Control groups included mice immunized with saline, rabbit IgG obtained before Id injection (pre-IgG), and anti-Id directed against human anti-HBSAg, which had been raised by methods similar to those described for production of anti-SV40 Id. One group of eight mice was also given cyclophos-
phamide (50 mg/kg body weight) 2 d before the tumor inoculum. Cyclophosphamide treatment at this dose selectively destroys T suppressor cells (51), and the role of this subset of T cells in tumor immunity has been documented for several systems (19, 20, 52–55). Studies have also indicated that both the amino and carboxyl portions of T-Ag are exposed on the surface of transformed cells (37, 56–59). For this reason, we thought that a pool of the two anti-T-Ag Id, recognizing both the amino and carboxyl determinants of T-Ag might be more effective in inducing tumor immunity. Of the nine groups of mice injected with anti-Id or control antibodies and challenged with SV40-transformed cells, only the group receiving a pool of anti-419 and anti-405 Id showed any reduction of tumor size (Table IV). All tumors induced in each mouse were measured with calipers 27 d after the SV40 tumor cell challenge, and the diameter was recorded. The mean tumor diameter of eight mice treated with pooled anti-419 and anti-405 Id was 12.9 mm. This value was compared with a range of mean tumor diameters of 20–30 mm for the other eight treated groups of mice. The range of individual tumor diameters for the 64 mice was 20–32 mm. In the pooled anti-SV40 T-Ag Id–treated mice, two out of eight failed to develop any visible signs of tumors, and three others showed a reduction in tumor size (9–17 mm) compared with the other treatment groups. To ensure that the two mice that failed to develop any tumors had indeed received SV40-transformed cells, the mice were bled 40 d after tumor challenge, and the sera were examined for antibody activity against SV40 T-Ag. Both sera were positive for anti-T-Ag by immunofluorescence, indicating that these two mice had received the tumor challenge. Cyclophosphamide had no effect on tumor growth, since no difference in the mean tumor diameter was observed between this treatment group of mice and controls. Assuming that cyclophosphamide treatment abolished T suppressor cell activity, these data suggest that T suppressor cells may have no detectable

| Treatment | Mean tumor diameter (mm) |
|-----------|--------------------------|
| Anti-405 Id | 22.4 ± 0.51 |
| Anti-419 Id | 24.2 ± 0.76 |
| Anti-T-Ag Id (pool)* | 12.9 ± 2.33 |
| Anti-421 Id | 27.2 ± 1.41 |
| Anti-HD 200 Id | 24.4 ± 2.12 |
| Cyclophosphamide* | 21.6 ± 0.46 |
| Saline | 20.4 ± 0.79 |
| Pre IgG | 23.0 ± 2.10 |
| Anti-Id (HBsAg) | 29.6 ± 1.71 |

Each group of eight mice received three intraperitoneal injections 10 d apart, and were challenged with SV40-transformed cells, subcutaneously, 7 d after the final injections. Tumors were measured using calipers, 27 d after tumor inoculation. Data are mean diameters, in millimeters ±SEM.

* Consists of a pool of 25 μg each of affinity-purified anti-405 Id and anti-419 Id.

* 50 mg/kg body weight was given intraperitoneally 2 d before tumor inoculation.
effect on in vivo SV40 tumor immunity. This is in contrast to a previous study
(60) where cyclophosphamide treatment was reported to abolish suppressor T
cell activity, thus augmenting the cell-mediated cytotoxicity against SV40 tumors.
Our study differs in that we measured in vivo SV40 tumor induction, whereas
the previous report measured the in vitro response to SV40-induced tumors
using a $^{51}$Cr-release assay to determine cytotoxicity. In other systems (19, 20),
selective elimination of T suppressor cells has resulted in either an inhibition of
in vivo tumor formation or suppression of tumor rejection. In addition, it
appeared that anti-Id directed against Id with specificity for both the carboxyl
and amino determinants of SV40 T-Ag together could partially suppress in vivo
tumor formation. However, anti-Id directed to either the carboxyl or amino
portions of T-Ag by themselves had no effect. Also, anti-Id directed against p53
did not appear to suppress in vivo tumor formation. At that point we did not
know whether a pool of the two anti-Id for p53 would have an effect on tumor
immunity.

In the next set of experiments, we decided to repeat the in vivo tumor
immunity induced by anti-SV40 T-Ag Id using intraperitoneal injections of an
SV40-transformed mouse cell line (mKSA) that grows in the ascites form, using
survival time as a parameter of immunity. The SV40-transformed tumor cell
line was titrated in order to give a dose of transformed cells that resulted in no
survivors in untreated mice 21 d after injection. In addition, we also decided to
examine what effects p53 and T-Ag Id injection had on the induction of SV40
tumor immunity. Previous investigators (21) had implicated in vivo anti-Id
production induced by Id immunization for protection or reduction of tumor
growth. 16 groups of 10 mice each were injected with Id, anti-Id, control
antibodies, cyclophosphamide, saline, or SV40 before tumor challenge. Injection
of mice with SV40 does not result in tumor formation, but provides immunity
to subsequent challenge with SV40-transformed cells. The mean survival time
for each treatment group of mice was determined, and the results of this
experiment are shown in Table V. The mean survival time for all treatment
groups except for mice receiving the anti-T-Ag Id pool, SV40, or the SV40 T-
Ag Id (pool) ranged from 18.6 to 21.7 d. Mice treated with the anti-T-Ag Id
pool or SV40 had mean survival times of 27.4 and 34.6 d, respectively. Based
on the two-tailed student's t test, these two groups of mice had significantly
higher mean survival times, compared with 13 other treatment groups ($P <0.05$,
anti-T-Ag Id pool; $P <0.001$ SV40). These results were in agreement with the
above observations, where tumor induction was determined by the size of the
tumor. The anti-T-Ag Id pool suppressed the in vivo formation of tumors in
mice, whereas cyclophosphamide used at a dose to selectively abolish suppressor
T cell activity, or individual anti-SV40 T-Ag and p53 Id had no effect. In
addition, Id preparations purified on anti-Id-containing affinity columns admin-
istered both individually and as a pool, and an anti-p53 Id pool had no statistically
significant effect on SV40 tumor induction. However, the T-Ag Id pool had a
longer mean survival time compared with other treatment groups (23.2 vs. 21.7
and 10 d).

It is noteworthy that three out of the 10 anti-T-Ag Id pool-treated mice
failed to develop tumors, and three others survived longer than any of the 140
**TABLE V**

*Effects of Various Id and Anti-Id Treatments on In Vivo SV40 Tumor Formation*

| Treatment | Number of survivors in groups of ten mice each | Mean survival time\(d\) |
|-----------|-----------------------------------------------|--------------------------|
| Anti-Id (405) | 0 | 18.6 ± 0.65 |
| Anti-Id (419) | 0 | 20.1 ± 1.38 |
| Anti-T-Ag Id (pool) | 3 | 27.4 ± 2.55* |
| Anti-Id (421) | 0 | 19.0 ± 0.56 |
| Anti-Id (HD200) | 0 | 19.4 ± 0.59 |
| Anti-p53 Id (pool) | 0 | 19.1 ± 0.81 |
| 405 Id | 0 | 19.5 ± 1.17 |
| 419 Id | 0 | 21.7 ± 0.73 |
| T-Ag Id (pool) | 0 | 23.2 ± 0.78 |
| 421 Id | 0 | 20.4 ± 0.80 |
| HD 200 Id | 0 | 19.4 ± 0.59 |
| p53 Id (pool) | 0 | 19.2 ± 0.72 |
| SV40‡ | 7 | 34.6 ± 1.54‡ |
| Cyclophosphamide | 0 | 18.9 ± 0.52 |
| Saline | 0 | 21.4 ± 0.86 |
| Anti-Id (HBsAg) | 0 | 18.7 ± 0.54 |

Each group of 10 mice received three intraperitoneal injections, each containing 50 μg of alum-precipitated antibodies 10 d apart. The mice were then challenged with 10⁶ SV40-transformed cells (mKSA) intraperitoneally 7 d after the final injections. 50 mg/kg body weight of cyclophosphamide was given intraperitoneally 2 d before tumor inoculation. The day of death was determined for each mouse.

* To aid calculations, the last death observed in the anti-T-Ag Id pool and SV40 treatment groups was 37 d after tumor inoculation. Mice that survived tumor challenge were arbitrarily given a survival time of 37 d.

Mean survival time ±SEM.

* Based on the two-tailed student’s t test; \(P < 0.05\).

‡ Mice received 10⁵ mKSA SV40-transformed cells 7 d before tumor challenge.

Based on the two-tailed student’s t test; \(P < 0.001\).

mice receiving the 14 different treatments that resulted in no significant reduction in tumor growth. Thus, 4 out of 10 of the anti-T-Ag Id pool–treated mice showed no apparent reduction of SV40 tumor growth. Similar results were obtained when tumor size was measured, with three out of the eight mice receiving anti-T-Ag Id pool showing no reduction in tumor size. These data suggest that anti-Id preparations that recognize both the amino and carboxyl determinants of SV40 T-Ag have some role in suppressing tumor formation. However, these putative Id networks induced by anti-Id injection do not appear to be universally active in suppression of tumor formation in all mice. The injection of SV40 should confer protection against challenge with a tumorigenic dose of SV40-transformed cells. Indeed, 7 of 10 mice given SV40 before challenge survived with no apparent tumor formation. The reasons for tumor development resulting in death in the other three mice from this group are not
known. Note that, in the calculations of mean survival time, mice that survived tumor challenge in the anti-Id (pool) and SV40 treatment groups were arbitrarily given 37 d as their survival time to aid in the calculations. This represented the last day of death observed for mice in both treatment groups. This arbitrary assignment of 37 d for survival time resulted in a smaller value for mean survival time; however, the values obtained were still significantly different from other treatment groups.

**Induction of an Ab-3 (Anti-anti-Id) that Lacks Anti-T-Ag Activity.** Previous studies (5–9, 11) have demonstrated that anti-Id may mimic or represent the internal image of the antigen and induce an in vivo antiantigen response. In this light, the ability of the anti-Id pool to mimic T-Ag and induce an anti-T-Ag response capable of suppressing tumor formation was examined. Mouse serum was obtained from anti-Id–injected mice 7 d after the third immunization, and assayed for anti-T-Ag activity by both immunofluorescence and radioimmunoprecipitation. No anti-T-Ag activity was found in any of these sera (data not shown). In addition, the ability of these sera to inhibit the various Id-anti-Id reactions was also analyzed. The data presented in Table VI demonstrate the ability of sera obtained from anti-Id–treated mice to inhibit the different Id-anti-Id reactions. Sera from four anti-405 Id–treated mice inhibited the 405 Id–anti–405 Id reaction 36–47%, whereas the same sera inhibited the other three Id-anti-Id reactions by <8%. Only sera from anti-421 Id–injected mice failed to inhibit (6–11%) its respective Id-anti-Id reaction significantly when compared with the other three Id-anti-Id reactions. It was interesting that serum from mice immunized with the anti-T-Ag Id pool inhibited both the 405 Id– and 419 Id–anti-Id reactions, yet no anti-T-Ag activity could be demonstrated. Based on the 405 and 419 Id inhibition curves shown in Fig. 2, and the levels of inhibition obtained with the mouse sera diluted 1:10, the approximate amounts of 405 and 419 Id present in these mouse sera were 120 and 80 ng, respectively. This quantity of 405 and 419 antibody is well within the range of the limits of detecting anti-T-Ag activity by both immunofluorescence and immunoprecipitation. Therefore, the antibody induced by anti-Id injection that is capable of

| Table VI
| Induction of an Anti-anti-Id (Ab-3) Response in Mice Receiving Anti-Id |
| Treatment | 405 Id-anti-405 | 419 Id-anti-419 | 421 Id-anti-421 | HD 200 Id-anti-HD 200 |
|-----------|----------------|----------------|----------------|----------------------|
| Anti-405 Id | 36–47* | 0–7 | 0–4 | 0–3 |
| Anti-419 Id | 4–8 | 29–44 | 2–6 | 1–5 |
| Anti-T-ag Id (pool) | 34–45 | 31–46 | 0–4 | 1–5 |
| Anti-421 Id | 4–7 | 1–6 | 6–11 | 2–7 |
| Anti-HD 200 Id | 1–4 | 2–7 | 3–5 | 28–46 |
| Each group of four mice received three intraperitoneal injections 10 d apart. Serum was obtained 7 d after the final injections. |
| * The range of percent inhibition values determined using a 1:10 dilution of serum as an inhibitor of the various Id-anti-Id reactions. |
| ‡ Consists of a pool of 25 μg each of affinity-purified anti-405 Id and anti-419 Id. |
inhibiting the Id-anti-Id reactions must be an anti-anti-Id (Ab-3) response that does not represent a true internal image and lacks anti-T-Ag activity.

This report characterizes four private or unique Id associated with mAb directed against SV40 T-Ag and p53, using anti-Id produced in rabbits. These Id determinants were associated, in part, with the antibody-combining site, since either purified T-Ag or T-Ag-p53 complexes specifically inhibited the four Id-anti-Id reactions. The specificity of these reactions was demonstrated by the ability of both T-Ag and T-Ag-p53 complexes to inhibit the 405 Id-anti-405 Id and 419 Id-anti-419 Id reactions, whereas only the preparations containing T-Ag-p53 complexes inhibited the p53 monoclonal Id-anti-Id reactions. These various anti-Id were then used in vivo in an attempt to modulate tumor formation in BALB/c mice subsequently challenged with SV40-transformed cells.

Previous studies (28–31, reviewed in 32) have demonstrated that either a subtumorigenic dose of SV40-transformed cells, SV40 alone, or purified T-Ag given before tumor cell challenge could completely suppress in vivo tumor formation. Alternatively, studies with viral Ag systems have shown that anti-Id can behave as the internal image, and induce an antiviral response (5–9, 11, 61, 62). The first of such reports, by Urbain et al. (61), characterized the induction of an anti-tobacco mosaic virus capsid protein response in mice injected with anti-Id alone. The original postulate of this report was that anti-Id directed against mAb to SV40 T-Ag may represent the internal image of T-Ag and induce in vivo tumor immunity. Thus, we used the anti-Id characterized herein to modulate the immune response to SV40-transformed cells.

In our initial experiments, anti-Id to either SV40 T-Ag or p53 individually had no effect on in vivo SV40 tumor formation. Other studies (37, 56–59) had demonstrated that both the amino and carboxyl termini of SV40 T-Ag are exposed on the surface of transformed cells, and both may play a role in tumor immunity. Because mAb 405 and 419 bind to the carboxyl and amino portions of SV40 T-Ag, respectively, we examined what effects a pool of these two anti-Id had on in vivo tumor formation. In two different sets of experiments, only this pool of the two anti-Id directed against T-Ag was capable of suppressing the formation of tumors by SV40-transformed cells. Not all mice in the anti-Id pool–treated group showed suppression of tumor growth. In the first experiment, two of a group of eight mice failed to develop any tumors, and three other mice showed a reduction in tumor size compared with groups of mice treated with control antibodies. In the second experiment, of 10 mice treated with anti-Id (pool), 3 failed to demonstrate any tumor formation, and 5 others showed increased survival times compared with control groups. Therefore, in two different experiments, anti-Id pool treatment before SV40 tumor challenge suppressed or partially suppressed tumor formation in 62.5% and 60% of the immunized mice. Why anti-Id treatment affected only certain mice within a given strain is not known. However, protection was not reproducibly observed for each mouse. In addition, serum from each mouse that did not develop tumors was assayed for anti-T-Ag activity by indirect immunofluorescence to ascertain whether these mice had been successfully challenged with SV40-transformed cells. All five mice developed an anti-T-Ag response.

The in vivo suppression of tumor growth by the injection of anti-Id or Id
generated to a given tumor Ag has been shown in other systems (17–19, 21, 63–70). The mechanisms of this suppression represented the production of either anti–anti-Id or anti-Id that was capable of stimulating or suppressing effector or inducer cells bearing anti-Id or Id receptors (17–19, 21, 63–70). We attempted to determine and postulate the potential mechanism of anti-Id–induced suppression in the SV40 tumor system.

The role of T suppressor cells has been demonstrated in the induction or suppression of tumor immunity in several systems (19, 20, 52–55, 63–65). We used cyclophosphamide (50 mg/kg body weight) to selectively destroy T suppressor cells (51) before SV40 tumor challenge, and found that this treatment had no effect on in vivo tumor growth. Thus, T suppressor cells did not appear to have a major role in the mechanism of SV40 tumor immunity. However, this does not rule out the possibility that T suppressor cells bearing Id or anti-Id receptors may have a role in the anti-Id–induced suppression of tumor growth.

Next, we studied whether the anti-Id represented the internal image of SV40 T-Ag and induced an anti–T-Ag response. No detectable anti–T-Ag activity was found in the serum of anti-Id–treated mice. However, it is possible that the polyclonal anti-Id preparations have a very minor internal image component that cannot be detected in our assay systems. Together, these data indicate that traditional immunization with SV40 T-Ag was more effective than anti-Id in producing tumor immunity. The failure of Id to provide protection is consistent with earlier evidence (71, 72), and that effector T cells were not analyzed in this study but may well play a central role in the anti-Id–induced immunity via the induction of regulatory idiotopes.

The studies reported herein demonstrate that Id networks may have some role in SV40 tumor immunity. An interesting mechanism that supports the data on tumor suppression reported in this manuscript involves the induction of an immune response to regulatory idiotopes. Further characterization of the potential Id networks produced during the course of SV40 tumor growth will be required before the mechanisms that occur during this complex immunological event will be unraveled.

Summary

This study characterizes four private idiotypes (Id) associated with monoclonal antibodies (mAb) to simian virus 40 (SV40) tumor antigen (T-Ag), and to a cellular protein, p53. Anti-Id recognized Id determinants associated with the antibody-combining site. BALB/c mice receiving a pool of anti-Id directed against mAb recognizing distinct amino and carboxyl terminal epitopes of T-Ag before receiving a tumorigenic dose of SV40-transformed cells showed suppression of tumor formation. Serum obtained from these mice before tumor challenge contained anti–anti-Id that failed to bind T-Ag. These data support the potential role of regulatory idiotopes in tumor immunity.

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