Myeloma Proteins as Tumor-Specific Transplantation Antigens
(BALB/c mice/plasmacytomas/tumor variants/idiotypes/anti-2,4-dinitrophenyl)

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ABSTRACT BALB/c mice immunized with myeloma proteins 315 or 460 made antibodies to the individually specific (“idiotype”) determinants of these proteins and suppressed growth of the corresponding transplanted tumor cells (MOPC-315 or MOPC-460). Stable, variant MOPC-315 tumors that produce only the light chain of protein 315 grew in several of the mice immunized with this protein, probably because the anti-idiotypic immune response selects against those myeloma cells that form the intact myeloma protein.

Antibodies made by BALB/c mice to myeloma proteins from mouse plasmacytomas of BALB/c origin were previously found to be specific for the individually distinctive (“idiotype”) determinants that seem to be localized in the respective ligand-binding sites of these proteins (1). The tumor cells that produce a myeloma protein should have altered growth properties (enhancement or suppression) in mice that produce such an immune response if the protein were localized in the cell membrane. In this study, we show that BALB/c mice immunized with myeloma proteins 315 or 460 can specifically suppress the growth of the corresponding tumor, MOPC-315 or MOPC-460. Selection by the host immune response was probably responsible for the additional finding that variant MOPC-315 tumors that produce only the light chain of protein 315, grew in several of the mice immunized with whole molecules of protein 315 (light plus heavy chains).

MATERIALS AND METHODS

Immunogens and Immunization Schedules. 10- to 12-week old female BALB/cAnN mice were injected with purified myeloma-proteins 315 or 460, which were isolated by subjection of the proteins to mild reduction and alkylation (in this work with iodoacetic acid) (2, 3). Proteins 315 and 460 purified in this manner (s10, w = 6.5) have about eight carboxymethyl groups per molecule (150,000 daltons), one on the C-terminal or penultimate cysteine of the light chain and 3–4 on the heavy chain (4). Both purified proteins had intact ligand-binding sites, as shown by specific quenching of their trypto-

Abbreviation: TD50, number of cells required to produce tumors in 50% of control (untreated) animals; Dnp, 2,4-dinitrophenyl.

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Phen fluorescence: at 4° in phosphate buffered saline [0.15 M NaCl–0.01 M potassium phosphate (pH 7.4)], protein 315 was quenched 62% by Dnp-L-lysine and protein 460 was quenched 40% by 2,4-dinitroanaphthol (2, 5). The proteins were injected according to immunization schedules given in detail in Table 2.

Tumors and Challenge with Tumor Cells. 10 Days after the last injection with myeloma proteins 315 or 460, mice were inoculated subcutaneously with specified numbers of myeloma cells. 10 Days later and frequently thereafter (daily for about 50 days), mice were examined for palpable tumors. The inoculated cells were obtained from mice carrying MOPC-315 and MOPC-460 in ascites form. The ascites tumors had been initiated about 4 months earlier by intraperitoneal injection of dispersed cells from the corresponding “solid” subcutaneous tumor; they were maintained by successive intraperitoneal transfer at about 10-day intervals with 0.2–0.3 ml of ascites fluid per mouse. (10 Days after intraperitoneal inoculation, a BALB/c mouse generally yielded 2-4 ml of ascites fluid with about 1 × 107 tumor cells/ml; usually, about 90% of the cells were viable according to the trypan blue dye-exclusion test (15). For challenge of immunized and control mice, the 10-day ascites fluid was diluted with Hank’s balanced salt solution to contain an appropriate number of tumor cells.

Myeloma tumors were generously provided by Dr. Michael Potter, National Institutes of Health. MOPC-315 and MOPC-460 have been maintained for 3–4 years in this laboratory by subcutaneous passage in various BALB/c mice.

Table 1. Hapten inhibition of reactions between BALB/c anti-idiotype antibodies and 125I-labeled myeloma proteins with anti-Dnp activity

<table>
<thead>
<tr>
<th>Antigen: BALB/c anti-idiotype antiserum</th>
<th>2,4-Dinitroanaphthene sulfonic acid (2.3 mM)</th>
<th>125I-labeled myeloma proteins (1.9 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>315: Anti-315</td>
<td>60</td>
<td>15</td>
</tr>
<tr>
<td>460: Anti-460</td>
<td>90</td>
<td>&gt;95</td>
</tr>
<tr>
<td>EA: Anti-EA</td>
<td>&lt;5</td>
<td>&lt;5</td>
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</table>

* 5 μl of antiserum, 0.1 μg of 125I-labeled protein as antigen, 100 μl of goat antiserum to mouse immunoglobulins (adsorbed with proteins 315 and 460), in a total volume of 500 μl, containing 50 μg of bovine serum albumin and the Dnp ligands indicated (see Methods). EA, egg albumin.
Table 2. Induced specific resistance to plasmacytomas and immune selection of tumor variants

<table>
<thead>
<tr>
<th>Exp. Pro. inj.</th>
<th>Tumor cells injected</th>
<th>No. per mouse (×10³)</th>
<th>No. with tumors</th>
<th>No. producing myeloma protein</th>
<th>No. not producing myeloma protein</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A None</td>
<td>MOPC-315</td>
<td>5</td>
<td>10/10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>460 B</td>
<td>MOPC-315</td>
<td>5</td>
<td>9/10</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>315 A</td>
<td>MOPC-315</td>
<td>5</td>
<td>1/9</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
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<td>MOPC-315</td>
<td>5</td>
<td>1/9</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>315 C</td>
<td>MOPC-315</td>
<td>5</td>
<td>1/9</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
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<td>MOPC-315</td>
<td>10</td>
<td>10/10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
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<td>8/8</td>
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<td>0</td>
</tr>
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<td>4/10</td>
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<td>3</td>
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<tr>
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<td>13/13</td>
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<td>0</td>
</tr>
<tr>
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<td>3/9</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>E None</td>
<td>MOPC-460</td>
<td>50</td>
<td>5/6†</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
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<td>MOPC-460</td>
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<td>14/16</td>
<td>14</td>
<td>0</td>
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<tr>
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<td>0</td>
</tr>
<tr>
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<td>MOPC-460</td>
<td>50</td>
<td>5/18§</td>
<td>4</td>
<td>1**</td>
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</table>

* Designations to experiments shown in Fig. 2.
† In immunization schedule A each mouse received a total of 1.0 mg of protein in five biweekly injections. Schedule B differed from A only in that the injections were weekly. In schedule C, each mouse received 600 μg of protein (total) in three weekly injections. Schedule D was the same as B, except that the total immunogen per mouse was 1.4 mg of protein given in seven injections. The first injections were in complete Freund's adjuvant, the second were in incomplete adjuvant, and all subsequent ones were in phosphate-buffered saline; at each injection, the antigen was distributed in rear footpads and the same four subcutaneous sites. Four additional mice immunized with Donpe-bovine gammaglobulin and four injected with complete Freund's adjuvant that lacked added immunogen were also tested with 5,000-10,000 MOPC-315 cells; all of these controls also developed tumors.
‡ 2 of 3 tumors resumed production of protein 315 when transferred to nonimmunized mice.
§ BALB/cJ mice; all others were BALB/cAnN.
¶ 77% of 48 unimmunized mice developed tumors in additional controls (see Fig. 1).
‖ Two additional mice developed tumors that regressed completely.
** Resumed production of protein 460 when transferred to nonimmunized mice.

mice. For 2 years the mice were obtained from a commercial source (Cumberland View Farms, Tenn.); in the past year we used BALB/cAnN and BALB/cJ mice from a colony maintained by one of us (RJG).
MOPC-460 arose in a pedigreed BALB/cAnN mouse (6). MOPC-315 arose in a mouse ("BALB/c-2") that was in the seventh generation of successive backcrosses to BALB/cAnN, starting with the offspring of a BALB/cAnN x C57B1/Ka cross; the progeny selected for each backcross had both BALB/c and C57B1 allotypic markers on heavy immunoglobulin chains (7). However, the α chain of protein 315 has the BALB/c allotype (7); the light chain of this protein has been sequenced and designated λ-2 type (8).

BALB/c Antibodies to Myeloma Proteins. The day before mice were challenged with tumor cells, they were bled under ether anesthesia from the orbital venous plexus. Sera were examined for antibodies as follows (1): 5 μl of serum, 0.1 μg of 125I-labeled myeloma protein (1.4 and 2.2 × 10⁶ cpm/μg for proteins 315 and 460, respectively) and various amounts of unlabeled myeloma protein were mixed in a total volume of 400 μl phosphate-buffered saline with 10 mg/ml bovine serum albumin. In some assays the BSA was reduced to 50 μg/ml; see Table 1). After 15 min at 37°C, 100 μl of goat antiserum to mouse immunoglobulins was added; this antiserum was previously adsorbed on Sepharose columns with covalently attached proteins 315 and 460. After 60 min at 37°C and overnight at 4°C, the precipitates were washed and 125I was counted. The values obtained were corrected for blanks prepared in the same way with serum from BALB/cAnN mice immunized with chicken egg albumin. Counts in the blank were 1-2% of the counts found in assays with 0.1 μg of myeloma protein. When large excesses of unlabeled myeloma proteins were added to insure saturation of antibodies (about 55 μg of protein 315 and 220 μg of protein 460 were required for 5 μl of a randomly prepared pool of anti-315 and of a pool of anti-460 sera, respectively) a corresponding small proportion of added cpm were bound; the counts obtained were nonetheless at least 3-fold greater than blanks.
125I-labeled protein 15 was tested as an additional control; this IgA (α) myeloma protein, produced by BALB/c plasmacytoma TEPC-15, was a gift from Drs. B. Chesebro and H. Metzger, NIH. All assays were in duplicate (±10%). Proteins were iodinated as described (9).

The excessive counts in the blanks found previously with 125I-labeled protein 460 were not encountered (1); they were probably due in the earlier study to inadequate absorption of the goat anti-mouse immunoglobulin with protein 460. Over 95% of freshly prepared 125I-labeled proteins 315 and 460 were specifically precipitated with a large excess of the cor-
estimated by the method of Reed and Muench (14) to be 1600 cells for MOPC-315 and 25,000 cells for MOPC-460. Once a tumor appeared in a control (BALB/cAnN) mouse, it was progressive and lethal.

responding BALB/c antisera (0.1 μg of 125I-labeled protein and 25 μl of antisera); after 4 or 5 months storage at 4°C the proteins were only about 50-60% precipitable.

Serum IgA and Anti-Dnp Activity. Sera obtained from mice near death with massive tumors were tested for proteins 315 and 460 by radial immunodiffusion precipitin assays (16; see ref. 15) in which agar contained either monospecific rabbit antisera to mouse α chains (to measure IgA levels) or Dnp-bovine gammaglobulin at a concentration of 0.3 mg/ml (to measure anti-Dnp activity)

Antiserum to the Light Chain of Protein 315. Rabbits were injected with 0.7 mg of the Fab fragment of protein 315 (4) in complete Freund's adjuvant, distributed among the four foot pads. 2 and 8 months later, the rabbits were boosted with 0.9 and 0.3 mg, respectively, of the light chain of protein 315 (in incomplete adjuvant), and they were bled 2 weeks after the final injection.

RESULTS

Anti-Idiotype Antibodies. With saturating amounts of myeloma proteins (see Methods), the amount of protein 315 bound per ml of serum from 10 mice immunized with this protein (Schedule B) was equivalent to 250 ± 30 μg (average ±SEM; the range was 136-422 μg/ml). The amount of protein 460 bound per ml of serum from nine mice immunized with this protein (Schedule D) was equivalent to 307 ± 265 μg (range: 84-882 μg/ml). In an experiment where the antiserum to protein 315 bound 80% of protein 315 (25 μl serum plus about 1.0 μg of 125I-labeled myeloma protein), about 2% of protein 460 and <1% of protein 15 were bound. Antiserum to protein 460 was similarly reactive only with protein 460. In accord with this and extensive earlier evidence that these BALB/c antisera are anti-idiotype (1), their reactions with the respective immunogens were blocked by Dnp ligands. Since protein 315 has higher affinity for Dnp-lysine than for dinitroanphthalene sulfonate and protein 460 binds dinitroanphthalene sulfonate better than Dnp-lysine (10), the results (Table 1) indicate that Dnp-ligands and anti-idiotype compete for the same sites on the myeloma proteins (11).

However, protein 315 has about 100-fold greater affinity than protein 460 for Dnp-lysine (2, 6), and yet this hapten inhibited the 460 more than the 315 reaction (Table 1). This suggests that the anti-protein 460 molecules had relatively low affinity for protein 460, in agreement with the finding with two randomly prepared pools of antisera that the concentration of protein 460 needed to saturate anti-460 was about 4-fold greater than the concentration of protein 315 required to saturate anti-315 (see Methods). However, sera from individual mice varied considerably in concentrations of antibody (especially anti-460; see range, above); they might also vary widely in affinity for the immunogen, and hence in susceptibility to inhibition by Dnp ligands (see ref. 1).

Tumor immunity

Immunized mice were challenged with 5,000-10,000 MOPC-315 cells or with 50,000-100,000 MOPC-460 cells. These quantities were two to six times the numbers required to produce tumors in 50% of control mice (TD50) (Fig. 1).

Mice immunized with protein 315 were resistant to MOPC-315: tumors developed in all control mice, but in only 11% of immunized mice challenged with 5000 cells and in 40-60%
of those mice tested with 10,000 cells (Fig. 2, Table 2). The MOPC-315 tumors that grew in mice immunized with protein 315 appeared later than in controls, and most (12/18) were variants that no longer produced protein 315 (Table 2). After two transfers in nonimmunized hosts, two of the MOPC-315 variants were found to produce protein 315, as indicated by elevated serum concentrations of IgA and precipitin reactions of serum with Dnp-proteins. The remaining variants (10/12) continued not to produce protein 315 through 9 transfers over 6 months in unimmunized mice. Instead, these stable variants produced the light chain of protein 315: urine samples from mice carrying the stable variants and authentic light chain isolated from purified protein 315 gave reactions of identity in double diffusion gel precipitin tests with a rabbit antisera specific for the light chain (see Methods). (This observation, by Dr. Kristian Hannestad, will be described in detail in a later report.) Urine from mice carrying the standard ("wild-type") MOPC-315 did not form a precipitate with this rabbit antisera.

As in the 315 system, BALB/cAn mice immunized with protein 460 became resistant to MOPC-460, but the effect was more difficult to discern. In one experiment resistance was suggested only by delayed onset of tumors and prolonged survival of tumor-bearing mice (not shown), rather than by a decreased incidence of tumors (Fig. 2C and D). However, in a second experiment, in which the immunization schedule (D) was prolonged, a decreased incidence of tumors was observed (Table 2, Fig. 2E). Moreover, MOPC-460 tumors appeared and then regressed completely in two mice immunized with protein 460, whereas tumor regression was not observed in any of the control BALB/cAn mice. In one mouse immunized with protein 460 a massive (fatal) tumor developed but the serum contained no detectable protein 460. However, when this tumor was transferred to a nonimmunized host an IgA myeloma protein with anti-Dnp activity (probably protein 460) was detectable in serum about after 3 weeks.

Skin Grafts

Because MOPC-315 arose in a heterozygous BALB/c mouse with some chromosomal segments of the C57Bl/Ka strain (see Methods), it was of interest to determine if this tumor carries normal transplantation antigens of C57Bl/Ka. BALB/c mice were therefore immunized with \(10^6\) lyophilized MOPC-315 cells and challenged 10–20 days later with 5,000 or 10,000 viable MOPC-315 cells. 70 Days later the approximately 20% survivors were grafted with skin from Ka mice. The same median survival time was found for the immunized mice and for unimmunized controls (11.1 ± 0.5 and 10.8 ± 0.5 days, respectively).

**DISCUSSION**

These experiments show that myeloma proteins 315 and 460 can function as tumor-specific transplantation antigens: each protein can induce an immune response that leads to specific rejection of the corresponding tumor. As was shown (1), and confirmed here, antibodies made by BALB/c mice to proteins 315 and 460 are specific for their respective idiotypic determinants. The resistance to myeloma cells induced by these proteins is probably also specific for idiotypic determinants, because mice rendered resistant to MOPC-315 were not significantly resistant to MOPC-460, and vice versa. Moreover, stable variants of MOPC-315, arising only in 315-immunized mice, produced just the light chain of protein 315; this chain does not react with BALB/c anti-idiotypic antibodies to the intact protein (light plus heavy chains) (1). It is not clear, however, if suppression of the myeloma cells is due to the anti-idiotypic serum antibodies, or to cell-mediated immunity with the same specificity, or to both acting cooperatively or antagonistically (i.e., serum antibodies tending to protect the tumor cells against cell-mediated immunity).

The anti-idiotypic response to protein 315 (antibodies or cell-mediated immunity) doubtless selects against tumor cells that produce this protein, allowing preferential growth of the light chain-producing variants. In accord with the interesting work of Coffino and Scharff (12), most of the MOPC-315 variants (10/12) are probably stable mutants: they did not revert in unimmunized (unselective) hosts. Immune selection of variants that are deficient in a distinctive tumor-associated antigen seems not to have been previously demonstrated unequivocally (13).

However, immune selection of tumor variants that lack normal transplantation antigens has been observed with heterozygous tumors growing in hosts of one parental type (13). Variation of this type is probably also represented in tumor MOPC-315, whose apparent lack of C57Bl transplantation antigens could be a consequence of prolonged maintenance of the tumor in BALB/c mice (see Methods). The loss of a tumor-associated antigen (myeloma protein) and the probable loss of some normal transplantation antigens in this one tumor emphasize the adaptability of tumor cell populations to the selective pressure of host immune responses.

Protein 460 induced less intense resistance to tumor MOPC-460 than protein 315 did to MOPC-315; even with a relatively small challenge dose of 460 cells (twice the TD<sub>50</sub> as compared with three- to six-times the TD<sub>50</sub> for the 315 reaction) the resistance was less pronounced, and only one MOPC-460 variant (an unstable one) was recognized. The affinity of the anti-idiotypic antibodies for protein 460 was probably lower than that of the corresponding antibody for protein 315 (Table 1). It is possible that protein 315 is a particularly effective immunogen (in BALB/c mice) because of the unusual amino-acid sequence in the C-terminal half of this protein's light chain (8). It is also possible that tumor MOPC-460 is less susceptible than MOPC-315 to immune attack. This could come about if, for instance, these tumors differed in the number or disposition of myeloma proteins in their respective cell membranes. That these proteins are localized in the myeloma cell plasma membrane has all along been a tacit assumption underlying these experiments and their interpretation. In a later report the existence and some properties of the membrane-bound myeloma proteins will be considered (Hannestad, K., Kao, M. S. & Eisen, H. N., in preparation).

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