Hybrid Resistance to BALB/c Plasmacytomas: F₁, Hybrid Anti-MPC-11 Immunological Responses Correlated with Resistance to Tumor Challenge

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ABSTRACT

BALB/cJ × C57BL/10SnF₁ hybrids resist challenge with the BALB/c plasmacytoma, MPC-11, by a radiation-sensitive, silica-insensitive mechanism, whereas BALB/cJ × BALB.B F₁ hybrids are as susceptible to MPC-11 as are homozygous BALB/c mice themselves. To investigate the mechanism of resistance, we have compared anti-MPC-11 immune responses by these F₁ hybrids both before and at various times after tumor challenge. Resistance is not determined by natural killer cell reactivity inasmuch as neither hybrid harbors splenic natural killer cells with lytic activity directed against MPC-11. Nor is it determined by antibody-dependent cell-mediated cytotoxicity since neither hybrid produces an appropriate anti-MPC-11 antibody. Spleen cells and lymph node cells from both hybrids are capable of generating high levels of anti-MPC-11 cytotoxic T-lymphocyte activity in both primary and secondary mixed-lymphocyte tumor cell cultures. Such cytotoxic T-lymphocytes protect susceptible hybrids from tumor growth in Winn assays. The susceptible but not the resistant hybrids lose the ability to generate high levels of cytotoxic T-lymphocytes activity in spleen mixed-lymphocyte tumor cell cultures by 28 days, and in lymph node mixed-lymphocyte tumor cell cultures by 14 days postchallenge. The reduction in spleen cell reactivity is due to suppression mainly by adherent cells and can be abrogated by pretreatment of the susceptible hybrids with a low dose of Cytoxan 2 days before challenge. This pretreatment does not, however, protect the mice. They develop tumor at the same rate and die at the same time as do controls. Both the late appearance of suppression and the lack of effect on survival of its ablation suggest it to be a concomitant of tumor growth rather than its cause. Resistance to tumor growth in this model system may reflect an enhanced ability of the resistant hybrid to deliver effector cells to the site of tumor implantation.

INTRODUCTION

F₁ hybrids between inbred strains of mice often resist challenge with parental tumors to which the syngeneic parent is highly susceptible (1–8). This phenomenon, which is called hybrid resistance, may provide a model system in which the importance of genes controlling different mechanisms of resistance can be assessed; e.g., comparisons of the antitumor responses of resistant or susceptible F₁ hybrids might point up an immunodeficiency associated with susceptibility. Ideally the resistance phenomenon should be attributable to the action of only one or a small number of genes rather than to some nebulous effect of hybrid vigor.

The system with which we work fulfills this requirement. Hybrid resistance to the BALB/c plasmacytoma, MPC-11, is expressed by F₁ hybrids between BALB/c and mice of various H-2 types with C57BL/10 (hereafter called B10) strain background genes (9) and is not expressed by F₁ hybrids between BALB/c and mice of the same H-2 types and BALB background genes. In hybrids of BALB/c and B10 mice, resistance is due to a single, dominant, autosomal gene of B10 origin which is not linked to H-2 and which is uninfluenced by sex or maternal strain (9). In the presence of this gene, inheritance of certain H-2 haplotypes, including the H-2° haplotype of B10 itself, increases resistance. In its absence, heterozygosity within H-2 has no demonstrable effect on susceptibility.

Resistance is radiation sensitive, is i.v. silica insensitive (10), and is manifested by marked reduction and delay in tumor take, slow tumor growth, and some late tumor regression. At appropriately low tumor doses, resistant hybrid groups may come to contain up to 75% mice that are tumor free at a time when 100% of BALB/c mice have progressively growing tumors, from which they will eventually die.

In this paper, we describe our efforts to establish whether there are any differences in anti-MPC-11 responses of H-2-matched resistant B10F₁ and susceptible BALB/cJ × BALB.B F₁ (hereafter called BALB.BF₁) mice which could account for their difference in susceptibility. Our results lead us to the conclusion that most of the major differences that we observe in the spleens of these mice are probably a consequence rather than a cause of tumor growth in the susceptible hybrids. Susceptibility in this model system may depend on a shutdown of the regional lymph node response, perhaps coupled with a failure to deliver effector cells to the site of tumor implantation.

MATERIALS AND METHODS

Mice. C57BL/10SnJ and CBA/J mice were purchased from The Jackson Laboratory, Bar Harbor, ME; AKR/Cum mice were from Cumberland View Farms, Clinton, TN; and BALB/cCrI mice, used for tumor passage, were from Charles River Breeding Laboratories, Inc., Wilmington, MA. The line of BALB/cJ mice used in breeding hybrids was derived by strict sib mating from pedigreed pairs supplied to us by Dr. Dan Menuelo, New York University Medical Center, New York, NY. Our line of BALB.B mice was bred from pairs originally given to us by Dr. Frank Lilly, Albert Einstein College of Medicine, New York, NY. Age- and sex-matched hybrids of both sexes (maternal strain always BALB/cJ), between 8 and 30 weeks old, were used both in vitro studies and for
tumor resistance assays. No significant age- or sex-related effects on either resistance or susceptibility to MPC-11 have been noted in these hybrids within this age range. Because H-2<sup>dm</sup>F<sub>i</sub> hybrids carrying the B10 background gene for resistance to MPC-11 are more resistant than homozygous H-2<sup>dm</sup> hybrids with the same background gene (9), we performed our studies on in vitro correlates of resistance in B10.F<sub>i</sub> and BALB.BF<sub>i</sub>, hybrids, rather than in the more obvious BALB/c x B10.D2 F<sub>i</sub>, hybrid and BALB/c pair. This accentuated differences due to the essential B10 background gene and was also more valid in the sense that both strains were F<sub>i</sub> hybrids, bred in our facility.

**Tumor Cells.** MPC-11, an IgG2b x-secreting plasmacytoma, was maintained by s.c. passage in BALB/cCrI mice and F<sub>i</sub>, hybrid blood chimeras were challenged by s.c. injection of 3 x 10<sup>5</sup> viable MPC-11 cells prepared from solid tumor as described previously (9). This number of viable MPC-11 cells regularly kills virtually 100% of BALB/cCrI, BALB/cJ, and BALB/cChallenged by s.c. injection of 3 x 10<sup>5</sup> viable MPC-11 cells prepared from A/HeJ origin, was obtained from Dr. Dan Meruelo, New York University Medical Center. The culture supernatant from 30-H12(R4) hybridoma cells (Dr. N. L. Warner, University of New Mexico School of Medicine, Albuquerque, NM) AKR/Cum splenic stimulator cells were added. Cr-labeled BW5147 cells were used as targets in assays of these responses.

**Generation of CTL in Vitro by MLTC:** Evaluation of Suppressor Cell Activity. Anti-MPC-11 CTL were generated in MLTC at various R:S ratios by a modification of the technique of Burton et al. (13). One-mi volumes of complete medium containing 7.5 x 10<sup>5</sup> responding cells were placed in 24-well tissue culture plates (Costar, Cambridge, MA), and 1-mi volumes of medium containing various numbers of MPC-11-plasmyoma stimulator cells, irradiated with 8000 rads from a cesium source (Gaemtor; Radiation Machinery Corp., Parsippany, NJ) or medium alone, were added. After 5 days culture at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere, the cells were harvested and washed, and viable cell numbers were determined by vital dye exclusion. The anti-MPC-11 lytic activity of the effector cells was assessed at various E:T ratios against a standard number (10<sup>4</sup>) of Cr-labeled MPC-11 ascites target cells in 4-h 51Cr release assays. We considered the remote possibility that irradiated BALB/c T-cells contaminating the MPC-11 asces stimulator-stimulating populations might institute a mixed lymphocyte reaction against the F<sub>i</sub> responding cells, thus confusing the results, but found that anti-Thy-1.2 plus complement treatment of the stimulator populations had no effect on the level of anti-MPC-11 CTL activity generated at any R:S ratio tested.

Anti-H-2<sup>dm</sup> responses were generated in similar cultures except that the number of responding cells was reduced to 2.5 x 10<sup>4</sup>/well and equal numbers of mitomycin C-inactivated (25 µg/ml; Sigma Chemical Co., St. Louis, MO) AKR/Cum splenic stimulator cells were added. Cr-labeled BW5147 cells were used as targets in assays of these responses.

**Preparation of Stimulator, Target, and Effector Cells.** The culture medium was RPMI 1640 (Grand Island Biological Co., Grand Island, NY) containing 10% fetal bovine serum, streptomycin (100 µg/ml), penicillin (100 units/ml), 15 µm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (Research Chemical Co., Sun Valley, CA) and, for effector cell induction, 5 x 10<sup>-4</sup> M 2-mercaptoethanol (Fischer Scientific, Fairlawn, NJ).

As previously (9), we routinely used cells dissociated from solid tumors for in vitro culture by using ascites cells as both stimulators and targets in vitro. These were collected 6 to 10 days after passage. Spleen and lymph node cells were prepared from pooled spleens or peripheral (inguinal, axillary, brachial, and superficial cervical) lymph nodes of normal or tumor-bearing mice by repeated teasing and flushing with cold complete medium or by pressing through a nylon sieve. Erythrocytes were lysed with distilled water and clumps were removed by sedimentation at 4 °C. After two washes with complete medium, cells were counted, their viability was checked (erythrosin B dye exclusion), and they were suspended in complete medium at appropriate viable cell concentrations. Ascites cell suspensions were always virtually 100% viable. Suspensions prepared from solid tumors could not be used as labeled target cells because of the high rate of spontaneous 51Cr release. They were, however, as effective as stimulators as ascites cells.

**Treatment of Effector Cells and Their Progenitors with Anti-Thy-1.2 Plus Complement.** Lymphoid cells, suspended at 1 x 10<sup>7</sup>/ml with two the predetermined minimal concentration of anti-Thy-1.2-containing culture supernatant from 30-H12(R4) hybridoma cells (Dr. N. L. Warner, University of New Mexico School of Medicine, Albuquerque, NM) required for maximal cytolyis (1:12), were incubated for 30 min at 4 °C. Unbound antibody was removed by a single wash with culture medium, and freshly thawed "low-tox" rabbit complement, prepared from rabbits irradiated with 300 rads 3 days prior to serum harvest, was added at a final concentration of 1:40. After 30 min incubation at 37 °C, the cells were washed three times in culture medium. Control cells were treated with the same concentrations of complement or anti-Thy-1.2 alone.

Adherent and Nonadherent Effector Cells. Effector cells were separated into adherent and nonadherent subpopulations by a modification of the technique of Kumagai et al. (11) as described by Robinson and Wheelock (12).
in PBS ranged from 92 to 98% of the total cell-associated cpm. The percentage effector-associated lysis was calculated as

\[
\frac{\text{cpm released by effector cell interaction}}{\text{cpm released spontaneously}} = \frac{\text{cpm released by detergent lysis}}{\text{cpm released spontaneously}}
\]

Antibody-dependent complement-mediated cytolysis assays were performed as described by Wienhold et al. (15). ADCC assays were performed as described by Kiessling et al. (16). Cr-labeled MPC-11 cells were incubated at 4°C for 30 min with dilutions ranging from 1:4 to 1:128 of heat-inactivated pools of serum collected from groups of F1 hybrid mice bled before or 7, 14, 21 or 28 DPC with 3 x 10^5 viable MPC-11 cells or, as a positive control, with B10 anti-BALB/c anti-H-2\* anti-serum (a gift of Dr. Ross Basch, New York University Medical Center) at dilutions ranging from 1:32 to 1:128. They were then cultured at 37°C with normal SPC from resistant or susceptible hybrids at an E:T ratio of 50:1 for 4 h. Release of 51Cr was measured as above.

Winn Assays. SPC, harvested either directly from mice or from MLTC, were admixed with 3 x 10^5 viable MPC-11 plasmacytoma cells at various effector cell:tumor cell ratios in a total volume of 0.2 ml in PBS. The cell mixtures were immediately injected s.c. into the right ventral flank of normal BALB.BF1 hybrid mice. Mice were palpated for appearance of tumor every other day. Caliper measurements in three dimensions of established tumors were recorded three times per week until all the controls had died.

Cytoxan Pretreatment. Mice received a single i.p. injection of Cytoxan (25 mg/kg body weight; Mead-Johnson, Evansville, IN), in 0.2 ml sterile PBS, 48 h before s.c. tumor challenge. Control mice received 0.2 ml sterile PBS alone.

Statistical Methods. Differences in 51Cr released by effector cells from the different hybrids were compared by Student’s t test for paired observations. Differences in time of tumor appearance or survival were assessed by Wilcoxon’s nonparametric ranking test.

RESULTS

Growth of Tumor in BALB/c, BALB.BF1, Hybrid, and B10F1, Hybrid Mice. Following s.c. injection of 3 x 10^5 viable MPC-11 cells in PBS alone. No protective effect of the SPC was observed even at SPC:MPC-11 ratios of 100:1, the extent by the highly active NK cells present in CBA spleen. Control mice received 3 x 10^5 MPC-11 cells alone. No protective effect of the SPC was observed even at SPC:MPC-11 ratios of 100:1, the tumors becoming palpable at the same time and growing at the same rate in the recipients of SPC-tumor cell mixtures and of MPC-11 alone (data not shown).

NK Cell Activity Is Present in Both B10F1 and BALB.BF1, Mice but Does Not Account for Hybrid Resistance to MPC-11. High NK cell-mediated cytolytic activity is associated with hybrid resistance to several transplantable tumors (17–20). To determine whether it plays any role in resistance to MPC-11, the splenic NK cell activity of normal and of MPC-11-challenged B10F1 and BALB.BF1 hybrids was measured against both YAC-1 and MPC-11 target cells (Chart 1). Unactivated NK cells contained in normal SPC populations from both resistant and susceptible hybrids, at the highest E:T ratio tested (200:1), within 4 h released 5 to 12% 51Cr from highly NK-sensitive YAC-1 targets but had no effect on MPC-11 target cells. Seven days after s.c. challenge, SPC taken from resistant and susceptible hybrids had significantly higher (10 to 19%) lytic activity against YAC-1 at the 200:1 E:T ratio, indicating either an influx of NK cells into the spleen or activation and/or proliferation of resident NK cells in response to tumor, but again had no effect on MPC-11 target cells. In contrast, SPC from CBA mice, a strain with high splenic NK cell activity (21) and capable of lysing an even higher proportion of YAC-1 target cells than activated B10F1, SPC (52 versus 19%), lysed a significant number of MPC-11 cells (15%). These data indicate that although MPC-11 is much less susceptible to NK cell-mediated lysis than is YAC-1, it can be lysed to some extent by the highly active NK cells present in CBA spleen.

Activated NK cells from resistant F1 hybrids might conceivably differ from those of susceptible hybrids in their ability to kill MPC-11 over a longer period, but longer in vitro assays with 51Cr-labeled MPC-11 target cells were not feasible due to high spontaneous release of isotope from the cells. Instead we tested the protective effect of normal SPC or of activated SPC, from B10F1, or BALB.BF1 hybrid mice that had been challenged s.c. with MPC-11 7 days earlier, in in vivo Winn assays. Various numbers of SPC were admixed with 3 x 10^5 viable MPC-11 cells and injected s.c. into groups of six BALB.BF1 hybrids. Control mice received 3 x 10^6 MPC-11 cells alone. No protective effect of the SPC was observed even at SPC:MPC-11 ratios of 100:1, the tumors becoming palpable at the same time and growing at the same rate in the recipients of SPC-tumor cell mixtures and of MPC-11 alone (data not shown).

Taken together, these results suggest that splenic NK activity is not of major importance in hybrid resistance to MPC-11.

Lack of Antibody Capable of Mediating ADCC against MPC-11 in the Sera of MPC-11-challenged Resistant and Susceptible Hybrids; Presence of ADCC Effector Cells in Both Kinds of Hybrid. Hybrid resistance to MPC-11 might depend on production of anti-MPC-11 antibody which could theoretically pro-
in vivo. Measurement of antibody-dependent complement-mediated cytotoxicity on MPC-11 target cells proved, however, impossible due to spontaneous release of 51Cr and uptake of vital dye by MPC-11 cells in the presence of either guinea pig or rabbit "low-tox" complement, extensively absorbed in the cold with lymphoid or MPC-11 cells. Since, in any case, MPC-11 cells grow and form tumors in BALB/c mice, which are not complement deficient, the progenitor cells of the solid tumor must presumably be protected from complement activity whether it is activated by moieties intrinsic to the tumor or by a hypothetical anti-MPC-11 antibody.

ADCC effector cells, on the other hand, might be able to infiltrate the tumor and promote its destruction; resistant hybrids might either contain a natural antibody or respond to MPC-11 challenge by elaborating an antibody capable of mediating ADCC. To test this hypothesis, ADCC effector function in SPC from both hybrids was tested in the presence of serum taken from the two hybrids at various times before and after tumor challenge. None of these sera mediated ADCC against MPC-11. Significant 51Cr release from MPC-11 target cells was, however, detected in the presence of control anti-H-2d antibody (57.8% at the highest concentration) and was then observed whether the effector cells were obtained from resistant or susceptible F1 hybrids. These results indicate that neither resistant nor susceptible hybrids produce antibody capable of mediating ADCC against MPC-11, but both kinds of hybrid have the necessary effector cells.

Anti-MPC 11 CTL Responses Are Generated In Vitro by SPC from Both Resistant and Susceptible Hybrids but the Responses of the Latter Decline with Time after Tumor Challenge. Antitumor CTL responses are readily generated during coculture of SPC from normal BALB/c mice with certain BALB/c plasmacytomas, including MPC-11 (22-24). As BALB/c mice are, however, highly susceptible to MPC-11, it initially seemed unlikely that such CTL activity could be responsible for hybrid resistance. The absence of any apparent association between either NK cell or antibody-mediated activity and hybrid resistance nevertheless prompted us to explore the possibility that CTL activity might, after all, be important; e.g., a more intense response might be generated by resistant than by susceptible hybrids.

SPC from both normal and tumor-challenged (3 x 106 viable MPC-11 cells) hybrids of both susceptible and resistant types generated anti-MPC-11 cytotoxic activity which was abolished by treatment of the responding SPC populations with anti-Thy-1.2 antibody plus complement, either before or after culture; i.e., T cells were involved both in the generation and in the effector phase of the cytotoxic activity (data not shown). The amount of CTL activity generated by both kinds of hybrid was dependent on the time after tumor challenge and on the number of stimulating tumor cells. 10:1 or 30:1 being optimal R:S ratios in different experiments. High concentrations of tumor cells (R:S ratio, 3:1) were very inhibitory especially at the later time points after challenge. Low but detectable responses ranging from 10 to 20% 51Cr release at an E:T ratio of 200:1 were always generated in the absence of added tumor cells. However, because the viable cell recoveries were generally lower than in cultures containing stimulator cells, the yield of effector cells/106 cells initially cultured was much lower than in stimulated cultures.

No statistically significant differences between the hybrids were observed in comparisons of CTL generated from either unprimed or 7 or 14 DPC SPC preparations (Table 1; Chart 2). The spleens of both hybrids doubled or tripled in size during the first week after tumor implantation, viable cell yields per spleen increasing from 50.2 ± 38.7 (SD, six experiments) to 173.4 ± 41.5 million cells (three experiments) in B10F1, hybrids and from 71.2 ± 46.2 (six experiments) to 181.0 ± 77.1 million cells (three experiments) in BALB.BF1 hybrids. Thus the secondary response measured at days 7 or 14 postchallenge was magnified considerably over the primary when the yield of potential LU per spleen was taken into account (data not shown). By 21 DPC, the CTL responses of the resistant and susceptible hybrids began to diverge. Those of the resistant hybrids remained high, while those of the susceptible hybrids declined and were no longer detectable by 28 DPC.

Suppression Is Responsible for Reduction of CTL Responses in Susceptible Hybrids. To determine whether this loss of responsiveness in susceptible hybrids late after tumor challenge was due to suppression, 14 DPC "responder" and 28 DPC "suppressor" SPC populations from both hybrids were cultured with MPC-11 stimulators either separately or admixed in equal cell numbers. Marked suppression of generation of anti-MPC-11 CTL activity occurred in the cultures containing BALB.BF1, 28 DPC suppressor SPC whether the responding cells were of BALB.BF1, (Chart 3) or B10F1, origin (data not shown). No suppression occurred (data not shown) in cultures containing B10F1, 28 DPC SPC, which were themselves highly responsive.

To establish what cell type was responsible for suppression, pooled BALB.BF1, suppressor 28 DPC SPC were separated into T-cell-depleted (anti-Thy-1.2 plus complement), adherent, and nonadherent fractions and the CTL activity generated by each subpopulation was tested against MPC-11. Significant suppression of activity was observed with adherent cells and this was preventable by treatment of the responding SPC populations with anti-Thy-1.2 antibody plus complement (data not shown). Thus the suppression late after tumor challenge was due to the presence of suppressor T cells in the responding SPC populations.

SPC donors

<table>
<thead>
<tr>
<th>BALB.BF1</th>
<th>B10F1</th>
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<tbody>
<tr>
<td>Normal (12)</td>
<td>39.1 ± 15.0</td>
</tr>
<tr>
<td>7 DPC (5)</td>
<td>63.5 ± 10.8</td>
</tr>
<tr>
<td>14 DPC (7)</td>
<td>41.5 ± 19.6</td>
</tr>
<tr>
<td>21 DPC</td>
<td>26.6</td>
</tr>
<tr>
<td>28 DPC (5)</td>
<td>5.8 ± 7.2</td>
</tr>
</tbody>
</table>

* Number of experiments; includes data from experiments shown in Chart 2 and in Tables 2 and 3 as well as other data.

* Mean ± SD at an E:T ratio of 100:1 in all the experiments. The effectors were generated at the optimal 10:1 or 30:1 E:S ratio for each experiment (always the same for both hybrids in a given experiment).

* Significance determined from Student's t test for paired observations.

Table 1: CTL responses generated in vitro by SPC taken from BALB.BF1 and B10F1, normal donors or from donors challenged with MPC-11 at various times before sacrifice

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or control cells.

Cells or T-depleted responder cells together with 7.5 x 10^5 irradiated MPC-11 cells of 3.75 x 10^6 responder and 3.75 x 10^6 fractionated or nonfractionated suppressor BALB.BF, SPC served as a dilution control in this experiment. Cultures contained 7.5 x 10^5 lymphoid cells (3.75 x 10^6 responder cells plus 3.75 x 10^6 suppressor or control cells).

**Table 2**

<table>
<thead>
<tr>
<th>Population cultured with irradiated MPC-11 cells</th>
<th>% 51Cr release</th>
<th>% suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Responder&quot; cells (normal SPC)</td>
<td>53.0</td>
<td></td>
</tr>
<tr>
<td>T-depleted responder cells</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>Responders + T-depleted responders</td>
<td>50.5</td>
<td>4.7</td>
</tr>
<tr>
<td>28 DPC &quot;suppressor&quot; cells</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>Nonadherent suppressor cells</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Responders + suppressors</td>
<td>27.7</td>
<td>47.7</td>
</tr>
<tr>
<td>Responders + T-depleted suppressors</td>
<td>32.8</td>
<td>38.1</td>
</tr>
<tr>
<td>Responders + nonadherent suppressors</td>
<td>29.3</td>
<td>44.7</td>
</tr>
<tr>
<td>Responders + adherent suppressors</td>
<td>11.3</td>
<td>78.7</td>
</tr>
</tbody>
</table>

*Cultures contained 7.5 x 10^6 responder or suppressor cells alone or mixtures of 3.75 x 10^6 responder and 3.75 x 10^6 fractionated or nonfractionated suppressor cells or T-depleted responder cells together with 7.5 x 10^6 irradiated MPC-11 cells per 2-ml culture well (10:1 R:S). Data shown were obtained at the 100:1 E:T ratio.

When They Develop Large Tumors. In all the preceding experiments, nonadherent fractions. Responder BALB.BF, normal SPC and the suppressor population fractions were then cultured with irradiated MPC-11 stimulator cells either separately or admixed in equal cell numbers. Cultures of normal SPC with either intact, T-depleted, nonadherent or adherent suppressor cells produced markedly diminished CTL responses (Table 2). Only a very small (8.4%) diminution in response was detectable in control cultures containing T-depleted normal spleen cells, indicating that the reduction in response in the presence of suppressors is not simply an effect of dilution. The T-depleted suppressor population was somewhat less suppressive than the intact suppressor population (38.1% versus 47.7% suppression), the nonadherent population was more like the intact population (44.7 versus 47.7% suppression) and the adherent population was considerably more suppressive (78.7% versus 47.7% suppression). Nonadherent cells cultured alone gave no appreciable response indicating either that removal of adherent cells is insufficient to release them from suppression, i.e., they are themselves tolerant or suppressed by nonadherent cells, or that they need adherent cells for CTL generation.

Since excess adherent cells are known to inhibit generation of CTLs in MLTC, we compared the effects of adding normal BALB.BF, versus 28 DPC BALB.BF, adherent SPC to normal BALB.BF, and B10F; responder cell cultures (Table 3). Normal adherent SPC were much less suppressive than 28 DPC adherent SPC with respect to responder cells from both F1 hybrids. Responder SPC from the BALB.BF, hybrid appeared somewhat more suppressible than those from the B10F, hybrid in terms of percentage suppression (56.9 versus 41.2%), but the reduction in percentage of 51Cr released was actually approximately the same (15.3 versus 14.0%) in the two populations, possibly indicating that the suppressors interact on a numerical basis with effector cells or their precursors.

We were concerned that suppression might have been due simply to the presence of large numbers of tumor cells, which are known to be suppressive, in the SPC populations. In additional experiments, however, in which complement controls were included for anti-Thy-1.2 plus complement depletion of suppressors, treatment of suppressor populations with complement alone failed to alleviate their diminished responsiveness (data not shown). Since MPC-11 is highly sensitive to complement, the major suppressive elements in suppressive SPC populations are probably not MPC-11 cells themselves although, if these are present, they may well contribute to suppression.

**Cytotoxicity Pretreatment of Susceptible Hybrids Ablates Suppression in 28 DPC SPC Preparations but Has No Effect on Susceptibility.** The possibility that suppression might account for the susceptibility of BALB.BF, hybrids was investigated with the aid of Cytoxan. Pretreatment of mice i.p. with Cytoxan (25 mg/kg body weight) 48 h before s.c. tumor injection increased the in vitro CTL responses of both B10F, and BALB.BF, hybrids, essentially reversing the suppression usually seen in the latter hybrid at 28 DPC (Chart 4). The same dose of Cytoxan, however, had no significant effect on the frequency or time of tumor take or the percentage survival or MST of groups of 15 B10F, and BALB.BF, hybrids (data not shown). Thus the splenic suppression phenomena do not appear to be of major importance in susceptibility.

**Reduced CTL Responses Are Seen Even in B10F, Mice When They Develop Large Tumors.** In all the preceding experiments,
ments, the B10F, SPC were prepared from unselected mice, set up in groups at the beginning of the experiment; i.e., they contained cells from both tumor-bearing mice and mice that were apparently tumor free at the time of sacrifice. When B10F, hybrid mice were deliberately separated into those with no tumor and those bearing tumor greater than 10 mm in diameter, there was at 21 DPC a significantly lower CTL response in the SPC preparations from the tumor-bearing mice (27.8 versus 56.6% 51Cr release at an E:T ratio of 200:1). Conversely when SPC from one unusual BALB.BF, mouse that regressed its tumor were compared with those of tumor-bearing BALB.BF, mice at 28 DPC, the former developed a higher CTL response than the latter (15.8 versus 5.3% 51Cr release at an E:T ratio of 200:1). These results strongly suggest that suppression in the spleen is a concomitant of tumor growth rather than its cause and that even the resistant hybrid is subject to this suppression.

**Efficacy of CTLs in Vivo.** Since CTLs were potent in the in vitro destruction of MPC-11 cells, we tested their ability to prevent tumor growth in in vivo Winn assays. SPC taken from B10F, mice 14 DPC were stimulated in MLTC with MPC-11 (R:S 30:1) for 5 days and then admixed with 3 x 10⁶ MPC-11 cells and injected s.c. into groups of five BALB.BF, hybrid mice. Tumor appeared in and killed five of five control mice, not receiving effector cells, but did not appear in any of four groups of mice receiving effector cells: tumor cell ratios ranging from 100:1 down to as few as 1:1. This result indicates that antitumor effector cells, presumably CTLs, are highly potent in protecting against MPC-11 growth. Any factor which prevents their generation or their ability to reach the tumor may well be decisive in determining susceptibility to MPC-11 challenge.

**CTL Activity Generated by Cells from Lymph Nodes Regional to Tumor in B10F, and BALB.BF, Hybrids.** Because we were unable to account for resistance versus susceptibility by comparing early splenic effector cell responses, we turned our attention to the LN draining the site of tumor implantation. The CTL activities generated by the pooled axillary, brachial, and inguinal LN on the same side as the tumor were compared in three experiments. In the fourth experiment, the tumor was injected in the middorsal line in the cervical region and the superficial cervical nodes were included in the pool. The difference in the injection site and the lymph nodes used may account for the generally lower responses seen in this experiment. The results of these four experiments and the calculated LU per MLTC and potential LU per LN are given in Table 4. In both F₁ hybrids, there was considerable enlargement of draining LN by 7 days after challenge. CTL responses by LN cells from unprimed B10F, mice were slightly higher than those of BALB.BF, hybrids, but there were more cells per LN in the latter; therefore we conclude that the unprimed hybrids have approximately the same reactivity on a per LN basis (Experiment 1). The major difference between the hybrids observed in this limited number of experiments was a rather striking reduction in the ability of the BALB.BF, hybrids to generate CTL 14 days after challenge. This is most easily seen when the potential LU per LN are compared within and between the BALB.BF, hybrid groups of Experiment 2 (123.6 at day 7 and 0.2 at day 14) or Experiment 4 (0.4 at day 7 and 0.01 at day 14). In Experiment 3, responses were lower in the BALB.BF, groups than in the B10F, groups at both day 7 and day 14 after challenge. We do not attach a great deal of significance to the exact numbers of potential LU, given the inaccuracies involved in calculating them and our lack of knowledge regarding the cell types increased in the lymph nodes draining the site of tumor inoculation, but the trend is obvious.

In Experiment 3, BALB.BF, 14 DPC LN cells admixed in equal cell numbers with B10F, 7 DPC LN cells, and, in Experiment 4, BALB.BF, 14 DPC LN cells admixed in equal cell numbers with BALB.BF, 7 DPC LN cells were also stimulated in MLTC. The 51Cr release in both these mixing experiments was intermediate between that in the responder 7 DPC LN preparation and that in the 14 DPC LN preparation (data not shown). This result may indicate that the majority of any suppressors that are involved in the 14 DPC reduction in the response are no longer present in the 14 DPC LN. In Experiment 4 and in another experiment not shown, the allogeneic (anti-H-2³) responses of BALB.BF, LN cells were also measured. There was no significant reduction in this response at day 14 suggesting that the reduction in the anti-MPC-11 CTL response may be antigen specific.

**DISCUSSION**

Our results strongly suggest that the events responsible for hybrid resistance are set in motion within the first 2 weeks of tumor implantation. Previous work (10) showed that resistance was sensitive to sublethal irradiation implying that the mechanism probably required an intact immune system. The present data are compatible with the idea that the main effectors of resistance are T-cells, most likely the extremely potent CTL evoked by the plasmacytoma. This does not exclude the possibility that macrophages, activated by T-cells in the presence of tumor, can also participate in resistance although the silica insensitivity of the phenomenon (10) suggests that they are probably not the effectors of major importance.

The idea that T-cells are responsible for resistance is supported by several findings. (a) The primary CTL response in vitro takes only 5 days and is high. Secondary responsiveness is detectable in populations taken from lymphoid organs only 7 days after tumor challenge. Thus the CTL response occurs well within the tumor latency period and could be induced in draining lymph nodes in good time for the effector cells to migrate to the tumor and kill tumor cells in situ. (b) Populations of cells known to contain large numbers of CTL are capable of preventing tumor growth in vivo Winn assays at an E:T ratio as low as 1:1; i.e., the effector cells in these populations are very potent. Furthermore the progenitor cells of the tumor, which may form a
relatively minor fraction of the tumor challenge, evidently bear a relatively
relevant group of determinants for effector cell-mediated killing. (c) NK cells, the most likely alternative effectors that we considered,
do not seem to be responsible for resistance; i.e., there is no
great difference in the in vitro NK activity of resistant and susceptible hybrids as measured against YAC-1 cells. Our tumor,
MPC-11, is in any case relatively insensitive to NK cell activity.
Furthermore neither normal nor 7 DPC “activated” splenic NK
cells could prevent or in any way alter the rate of tumor growth in in vivo Winn assays. (d) Antibody-dependent mechanisms seem unlikely to be involved because no antibody capable of participating in ADCC against MPC-11 is present in either hybrid before or after tumor challenge. The tumor is highly sensitive to complement in vitro even in the absence of antibody. Evidently
in vivo, the progenitor cells of the tumor can evade complement-
mediated killing, which makes it unlikely that antibody-dependent complement-mediated mechanisms are responsible for protection. Resistance is radiation sensitive which tends to rule out hypotheses involving preexisting “natural” antibodies to determinants such as those of murine leukemia viruses expressed on the tumor. Such antibodies and complement would not be expected to be radiosensitive.

Available evidence suggests that MPC-11 is actually eliminated from the resistant B10F, hybrids. The hypothesis that in resistant hosts the plasmacytoma might survive but might become dormant (25), perhaps by being regulated in a manner analogous to that for normal B- and plasma cells, was not supported by the results of experiments in which large numbers of pooled spleen and draining lymph node cells from B10F, long-term survivors of MPC-11 challenge were injected into BALB/c mice. Under these circumstances, any H-2\textsuperscript{K} hybrid regulatory cells should have been eliminated by the H-2\textsuperscript{K}BALB/c hosts thus freeing surviving tumor cells to grow out and form a tumor. No such antibodies and complement would not be expected to be radiosensitive.

If CTL are in fact responsible for resistance, why are BALB.BF, hybrids, which early after tumor challenge make CTL responses just as high as B10F, hybrids, susceptible? One possibility is that CTL generated by the susceptible hybrid are not delivered to the site of tumor implantation. Perhaps the tumor evokes a local change in the macrophages or endothelium of the resistant but not the susceptible hybrid such that effectors are stimulated to extravasate from blood and infiltrate the tumor at that site. Alternatively the effectors of the two hybrids could have different abilities to extravasate at sites of tumor implantation or have different abilities to leave the lymphoid organs where they are stimulated. Yet other possibilities are that CTLs are not maintained or that their activity is suppressed in susceptible but not in resistant hybrids.

The reduction in the splenic CTL response of BALB.BF, hybrids late after tumor implantation is attributable to the activity of at least two kinds of suppressor; one is Thy-1 positive and probably nonadherent, the other, much more strongly suppressive, is plastic adherent. Whether these cells are dependent on one another or are independently triggered by MPC-11 cells arriving in the spleen from the large s.c. tumors carried by the mice is not known. Excess irradiated MPC-11 cells are, themselves, capable of inhibiting CTL generation in primary or secondary MLTC of either B10F, or BALB.BF, hybrids both in our hands and as reported by others (26). It is not clear whether the MPC-11 cells are directly suppressive or induce suppression by T-cells and/or macrophages in the cultures. Plasmacytoma cells are well known to induce suppression of PFC responses in culture (27, 28) by releasing a factor which acts on macrophages (29, 30) causing them to release a substance (31, 32) that apparently prevents B-cell proliferation (33). They are known not to influence T-cell responses such as mixed lymphocyte reactions or phytohemagglutinin responses (34) but could conceivably exert their effect on CTL generation at the level of effector cell proliferation or maturation. In two experiments conducted in this laboratory with the generous help of Dr. Margaret Amy, we found that primary in vitro IgM PFC responses to sheep erythrocytes of SPC from both B10F, and BALB.BF, mice were over 70% suppressed when MPC-11 cells were included in Mini Marbrook cultures at a SPC:MPC-11 cell ratio of 25:1. Thus both hybrids clearly have the potential to be suppressed when their SPC are exposed to excess MPC-11 cells whether this is measured in terms of CTL generation or of PFC generation in vitro.

The reduction in the LN CTL response of susceptible hybrids at 14 DPC seems not to depend on a suppressor still present at this time. A suppressor that appeared and then emigrated before 14 DPC is probably nonadherent, the other, much more strongly suppressive, is plastic adherent. Whether these cells are dependent on one another or are independently triggered by MPC-11 cells arriving in the spleen from the large s.c. tumors carried by the mice is not known. Excess irradiated MPC-11 cells are, themselves, capable of inhibiting CTL generation in primary or secondary MLTC of either B10F, or BALB.BF, hybrids both in our hands and as reported by others (26). It is not clear whether the MPC-11 cells are directly suppressive or induce suppression by T-cells and/or macrophages in the cultures. Plasmacytoma cells are well known to induce suppression of PFC responses in culture (27, 28) by releasing a factor which acts on macrophages (29, 30) causing them to release a substance (31, 32) that apparently prevents B-cell proliferation (33). They are known not to influence T-cell responses such as mixed lymphocyte reactions or phytohemagglutinin responses (34) but could conceivably exert their effect on CTL generation at the level of effector cell proliferation or maturation. In two experiments conducted in this laboratory with the generous help of Dr. Margaret Amy, we found that primary in vitro IgM PFC responses to sheep erythrocytes of SPC from both B10F, and BALB.BF, mice were over 70% suppressed when MPC-11 cells were included in Mini Marbrook cultures at a SPC:MPC-11 cell ratio of 25:1. Thus both hybrids clearly have the potential to be suppressed when their SPC are exposed to excess MPC-11 cells whether this is measured in terms of CTL generation or of PFC generation in vitro.

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the explanation for the reduction in responsiveness since early tumors in susceptible hybrids contain very few T-cells. It would be satisfying to be able to conclude that the reduction in regional lymph node response in BALB/c x B10.D2 F hybrids, which also carry the B10 background gene for resistance but which are H-2d (9), is due to suppression of the background gene for resistance but which are H-2d (9). We have also accumulated a large, although less complete, body of information on BALB/c x B10.D2 F hybrids and BALB/c, their homozygous H-2d partner. Broadly speaking, the results are similar to those obtained with the H-2d hybrids although, in simultaneous experiments, the CTL responses in both the H-2d strains are generally lower than those of the H-2d hybrids. It is obvious that when experiments are done with plasmacytomas to which BALB/c mice are poorly or completely unresponsive, the major gene(s) controlling hybrid resistance is (are) likely to appear to be H-2 linked (36). If, as in our experiments, H-2 is eliminated as a source of variation in the intensity of response, the role of background genes can be investigated. We think it is likely that the same B10 background gene influences the outcome of many studies of hybrid resistance to BALB/c plasmacytomas (36, 37). The tumor-associated antigens peculiar to each plasmacytoma may well be associated with different H-2-linked immune response and/or immunosuppression effects on the CTLs generated. The background gene may be responsible for delivery of the effectors to the tumor site.

ACKNOWLEDGMENTS

We thank David Akeman for his excellent technical assistance and Jacquelyn Green for patiently typing the manuscript.

REFERENCES


Hybrid Resistance to BALB/c Plasmacytomomas: F₁ Hybrid Anti-MPC-11 Immunological Responses Correlated with Resistance to Tumor Challenge

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