Periodic loss of reactivity of a myeloma tumor with cytotoxic thymus-derived lymphocytes
(major histocompatibility complex/H-2 restriction/cell surface antigens/mouse plasmacytomas)

JOHN H. RUSSELL, ARTHUR H. HALE, LEO C. GINNS*, AND HERMAN N. EISEN

Department of Biology and the Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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ABSTRACT During each transplantation passage of a line of mouse myeloma tumor MOPC-315 through syngeneic (BALB/c) hosts, the tumor cells lose reactivity with cytotoxic thymus-derived lymphocytes directed against products of the BALB/c major histocompatibility complex (H-2d) and regain reactivity on transfer to fresh hosts. In contrast to this cyclical change, the tumor cells remain uniformly reactive with anti-H-2d alloantisera throughout the transplantation cycle.

The major histocompatibility complex (MHC) is a large cluster of linked genes coding for cell surface proteins that control a number of immune processes, including allograft rejection and interactions among syngeneic lymphoid cells. In a recently discovered effect, products of the MHC restrict the reactions of thymus-derived lymphocytes (T lymphocytes) with diverse surface antigens of syngeneic target cells. For example, in the mouse, in which the MHC is called H-2 and this restriction has been mostly clearly defined, lysis of virus-infected target cells by cytotoxic T lymphocytes (CTLs) has a dual requirement: the most effectively lysed cells are those with both the same virus infection and H-2 products as the stimulator cells that elicited development of the CTLs (1). By testing stimulator and target cells from various parental and recombinant mouse strains, differing only in restricted regions of the MHC, it has become apparent that the restrictive H-2 products are coded for by the H-2K and/or H-2D loci, located at either end of the H-2 complex (2).

H-2 restriction of CTL activity is also evident with target cells whose surface antigens are modified chemically with 2,4,6-trinitrophenyl or other groups (3–5) and with normal cells via their many minor histocompatibility antigens (i.e., antigens that elicit weak allograft rejection reactions and are controlled by genes outside the MHC) (6, 7). It is thus expected that syngeneic CTL responses to tumor-associated antigens on tumor cells are probably also subject to the same dual requirement for antigen plus H-2 products, and this expectation is supported by several observations (8–10). Accordingly, tumors that fail to react with syngeneic CTLs could be expected to lack either a tumor-specific antigen as has been described (11) or the appropriate H-2 products.

In accord with these considerations, we describe in this paper a transplantable mouse myeloma tumor of BALB/c origin (H-2d haplotype) that undergoes a periodic loss, during each transplantation cycle, of ability to interact with CTLs directed against products of the H-2d haplotype. Nevertheless, throughout the transplantation cycle the tumor retains undiminished ability to react with alloantibodies to H-2d products. This discrepancy suggests, in accord with some other observations (12–15), that anti-H-2 antibodies and T lymphocytes may be specific for different H-2 encoded determinants. Whether the myeloma cells' periodic loss of reactivity with anti-H-2 CTLs represents a mechanism whereby this tumor evades destruction by host cell-mediated immune responses or reflects a normal differentiation sequence in the maturation of B lymphocytes is not clear.

EXPERIMENTAL PROCEDURES

Mice. Congenic male and female mice of the following strains were used (H-2K and H-2D alleles in parentheses): BALB/c AnN (d,d), BALB.B (b,b), and BALB.Htg (d,b). They were produced in the Massachusetts Institute of Technology Center for Cancer Research colony from breeder kindly provided by Ralph Graff (Washington University), Edward A. Boyse (Sloan-Kettering Institute), and Frank Lilly (Albert Einstein College of Medicine). Jackson Laboratories, Bar Harbor, ME, provided C57BL/6 (b,b) and the following congenic C57BL/10 mice: B10 (b,b), B10.D2 (d,d), and B10.A(5R) (b,d).

Tumors. MOPC-167 and MOPC-315 [BALB/c (d,d) plasmacytomas] were obtained several years ago from M. Potter, National Cancer Institute, and maintained in ascites form by serial transfer of 1 × 10⁶ cells weekly in BALB/c AnN mice. To obtain adequate quantities of MOPC-315 cells at specified times after transfer, it was necessary to vary the inoculum. Unless stated otherwise, "early" MOPC-315 cells were collected 4 days after intraperitoneal injection of 1.5 × 10⁷ cells and "late" MOPC-315 cells were collected 11 days after injection of 5 × 10⁶ cells; the cells injected were obtained from a routine transfer generation. MOPC-167 cells were used 6–10 days after transfer of 1 × 10⁶ cells.

Production of CTLs. On day 0, spleen cells from mice of the appropriate strain were harvested and washed once. Nucleated cells were counted and their viability was determined with trypan blue. To 4 × 10⁶ viable cells in upright 25-ml plastic flasks (Falcon no. 3013, Oxnard, CA) were added 4 × 10⁶ irradiated [1000 R (SI, 0.26 C/kg)] spleen cells or 0.67–6 × 10⁶ irradiated (10,000 R) tumor cells in a total volume of 10 ml. Cells were γ-irradiated with a 137Cs source in a Gammacell 40 (Atomic Energy of Canada, Ltd.). After the cells were mixed, they were incubated at 37°C under 90% air/10% CO₂. On day 1, 2 ml of a nutritional supplement (see Media, below) was added and incubation was continued. The killing assay was performed on day 5.

Targets. Targets for cytotoxic assays were prepared by harvesting the appropriate spleen cells, eliminating erythrocytes

Abbreviations: MHC, major histocompatibility complex; T lymphocytes, thymus-derived lymphocytes; CTL, cytotoxic T lymphocyte; Con A, concanavalin A; P₃/NaCl phosphate-buffered saline (0.15 M NaCl/0.01 M potassium phosphate, pH 7.4).

* Present address: Pulmonary Division, Massachusetts General Hospital, Boston, MA 02114.
Table 1. Evidence that MOPC-315 tumor cells from ascites fluid of F1(BALB/c × C57BL/6) hosts are not contaminated by host cells

<table>
<thead>
<tr>
<th>Target cells</th>
<th>% lysis* by</th>
<th>B10 sera¹</th>
<th>B10 D2 sera²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Anti-B10</td>
<td>Normal</td>
</tr>
<tr>
<td>BALB/c spleen cells</td>
<td>4</td>
<td>93</td>
<td>6</td>
</tr>
<tr>
<td>F1(BALB/c × C57BL/6) spleen cells²</td>
<td>5</td>
<td>94</td>
<td>3</td>
</tr>
<tr>
<td>C57BL/6 spleen cells</td>
<td>6</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Early MOPC-315 cells</td>
<td>6</td>
<td>93</td>
<td>1</td>
</tr>
<tr>
<td>Late MOPC-315 cells</td>
<td>5</td>
<td>92</td>
<td>4</td>
</tr>
</tbody>
</table>

* % lysis measured by trypan blue exclusion, as in ref 16.

¹ B10 sera at 1:20 dil (1:60 final).
² B10.D2 sera at 1:10 dil (1:30 final).
³ 2-day Con A blasts.
⁴ Tumor cells grown in F1(BALB/c × C57BL/6); early and late ascites cells were collected at 4 and 11 days after transfer (see Fig. 6).

by suspending the spleen cell pellets in 5–10 times their volume of 0.75% NaCl/0.017 M sodium phosphate (final pH, 7.1) and incubating them for 2–5 min at room temperature and 2 min at 37°C. The cells were then washed twice, and 5 × 10⁹ viable cells were placed in a 60-mm plastic dish in 5 ml of medium containing 2 µg of concanavalin A (Con A; Miles, Kankakee, IL) per ml. After 2 days the cells were harvested and incubated with 0.05 M α-methyl mannose for 1–3 hr before use as targets. When tumor cells were targets, they were harvested from ascites fluid, diluted with phosphate-buffered saline (PBS), and centrifuged (1000 g, 5 min) to clear debris. Trypan blue exclusion (25% viability) was used to determine cell viability. Tumor cells at 1 × 10⁶/ml and spleen cells at 5 × 10⁶/ml were labeled by incubation at 37°C for 1–2 hr with 200 and 500 µCi of Na²¹CrO₄ (obtained in sterile saline at 1 mCi/ml from New England Nuclear, Boston, MA and diluted with medium) per ml, respectively.

Asays for Lysis by CTLs and for Lysis Inhibition. Various numbers of effector cells and 10⁶ target cells, in a final volume of 150 µl, were incubated in 12 × 75 mm plastic tubes (Falcon no. 2003, Oxnard, CA) for 4 hr at 37°C under 94% air/6% CO₂ at 100 rpm. The plates were centrifuged (450 × g, 5 min) at 37°C. The supernatant and all of the remaining material were assayed for radioactivity separately in a Packard γ-spectrometer. Inhibition by unlabeled target cells ('cold inhibitors') was performed by incubating effector cells with 50 µl of an inhibiting cell suspension at 37°C for 30 min before addition, in 50 µl of the 51Cr-labeled targets and proceeding as before. Specific lysis was calculated as [(ER – CR)/(T – CR)]100, in which ER is % release by stimulated effector cells, CR is % control release determined by incubating target cells with mock-stimulated effectors (e.g., BALB/c spleen cells incubated for 5 days with 1000 R irradiated BALB/c spleen cells), and T is total (100%) target cell radioactivity.

Alloantiserum to H-2d. This was prepared by multiple intraperitoneal injections of 2 × 10⁶ B10.D2 spleen cells into B10 mice.

Absorption of Antibodies to H-2d. Serial dilutions of cells that had been washed three times by repeated cycles of centrifugation were distributed into microtest tubes (Sarstedt, Princeton, NJ) and centrifuged (450 × g, 5 min); the supernatants were removed, and the packed cells were resuspended in 60 µl of antiserum to H-2d at dilutions that would lyse approximately 75% of the target cells in a standard cytotoxic assay with complement (see below). After 30 min on ice, during which the tubes were periodically agitated by gentle vortex mixing, the cells were packed by centrifugation (8000 × g, 5 min, Brinkman centrifuge, model 3200) and samples of the supernatant were diluted and distributed into microtiter plates (linbro Scientific) for storage at −70°C until assayed for cytotoxic antibodies (below).

Cell Lysis by Anti-H-2d Antibodies Plus Complement. To 25-µl dilutions of unadsorbed or adsorbed sera in microtiter plates (above) was added 25 µl containing 5 × 10⁶ 51Cr-labeled Con A blasts of spleen cells from mice of the appropriate strains. After incubation for 30 min at room temperature, the plates were centrifuged (450 × g, 5 min), the supernatants were discarded, and the cells were washed once. Complement was then added (50 µl) as a 1:4 dilution of normal rabbit serum (previously selected for minimal cytotoxicity and also adsorbed with a mixture of mouse tumor and normal cells). After 1 hr at 37°C, the cells were washed once and their radioactivity (51Cr) was determined. In one experiment, cytotoxicity was evaluated by trypan blue exclusion rather than by 51Cr release (see Table 1).

Media. RPMI 1640 was supplemented with various additions for different procedures. For eliciting CTLs by 5-day incubation in culture, 90 ml of RPMI 1640 was supplemented with 1.0 ml each of minimal Eagle's medium nonessential amino acids (100X), sodium pyruvate (Gibco, 100X), L-glutamine (5%) and with 1.5 ml of 1 M N-2-hydroxyethyl-N-2-ethansulfonic acid, pH 7.4 (Sigma, St. Louis, MO), penicillin, streptomycin, 2-mercaptoethanol, and heat-inactivated fetal calf serum (56°C, 45 min, Flow Laboratories, Cockeysville, MD) were then added to final concentrations of 2.5 × 10⁵ units/liter, 25 mg/liter, 50 µM, and 5% (vol/vol), respectively. For the 4-hr killing assay, the medium was identical except that it contained 10% (vol/vol) heat-inactivated fetal calf serum. The 5-day cultures were supplemented on day 1 with 2.0 ml of a modification of the Mishell–Dutton supplement (17).

For all the reactions involving adsorption of antibodies or lysis of cells by antiserum and complement, unsupplemented RPMI 1640 containing 0.1% gelatin (Difco) was used to wash and suspend cells and to dilute antiserum and complement.

**RESULTS**

In another study it was noticed that CTLs elicited in BALB/c mice by MOPC-167 lysed different preparations of MOPC-315 cells to different extents (J. H. Russell, C. Terres, L. C. Ginz, and H. N. Eisen, unpublished data). To analyze these fluctuations, MOPC-167 cells were compared with MOPC-315 cells taken at different times in the transplantation cycle in regard to susceptibility to lysis by allogeneic anti-H-2d CTLs (e.g., BALB.B anti-BALB/c). As shown in Fig. 1A, MOPC-315 cells harvested 9 days after transfer were less sensitive to lysis than those harvested 5 days after transfer; at neither time were they as susceptible as MOPC-167 cells.

The difference in sensitivity of "early" and "late" MOPC-315 cells was verified with cells harvested 4, 7, and 11 days after transfer (Fig. 1B). The same cells were also tested for their ability to inhibit the lysis of standard H-2d target cells (Con A blasts of BALB/c spleen) by alloreactive anti-H-2d CTLs. As shown in Fig. 2, the progressively decreasing susceptibility to lysis during the transplantation cycle was matched by a corresponding decrease in ability to inhibit the anti-H-2d CTLs. Inhibition of lysis at inhibitor target ratios > 100:1 is probably not significant because nonspecific controls (EL-4) were also somewhat inhibitory at this level. The cyclical change in reactivity with CTLs was evident when cells taken at 11 days were
inoculated into fresh recipients: cells collected 4 days later had regained reactivity with CTLs.

In addition to the failure of the late MOPC-315 cells to interact with anti-H-2d CTLs, either as targets or as inhibitors, these cells were also less effective than the early tumor cells as stimulators of the development of anti-H-2d CTLs in BALB.B (H-2^b) spleen cells (Fig. 3A). A mixing experiment (Fig. 3B) demonstrated that late cells did not interfere with the response to MOPC-167. Thus, the failure of the late cells to stimulate a response is related to antigen recognition rather than to inhibition of proliferation and maturation of the CTLs.

To determine whether the presence of the serologically defined cell surface antigens coded for by the H-2 complex were also intermitting diminished, tumor cells were tested both for their susceptibility to lysis by anti-H-2^d alloantibodies and for their ability to bind these antibodies. In contrast to the progressive loss of reactivity with CTLs, MOPC-315 cells collected 4, 7, and 11 days after transfer were identical as targets for the anti-H-2^d antisera (Fig. 4) and as adsorients of cytotoxic antibodies from these sera (Fig. 5). By using, as targets, spleen cells from various congenic and recombinant mouse strains, it was possible to show more specifically that the early and late MOPC-315 cells had indistinguishable amounts of cell surface products of both the K and D loci of the H-2 complex.

The main finding in this study is that, late in each transplantation cycle, cells of MOPC-315, a BALB/c myeloma tumor, undergo a periodic loss in ability to elicit or react with anti-H-2d CTLs. Nevertheless, throughout the entire cycle the tumor cells retain undiminished their ability to react with anti-H-2d alloantibodies. These findings are in accord with studies of mutant H-2K^b alleles, which suggest that anti-H-2 CTLs and anti-H-2 alloantibodies react with different determinants on the cell surface molecules encoded by the H-2K and H-2D loci (14, 15). According to this view, the determinants recognized by CTLs are expressed on the surface of early MOPC-315 cells but are not expressed, or are somehow not accessible to CTLs, on the late tumor cells.

Our results, however, could also be accounted for by other possibilities. For instance, the CTLs and antibodies might react
Immunology: Russell et al.

Figure 3. Ability of various tumor cells to elicit anti-H-2\(^{d}\) CTLs in culture. \(^{51}\)Cr-Labeled Con A blasts of BALB/c spleen cells served as target cells in all assays (control release was 22%). (A) BALB.B spleen cells were cultured for 5 days with 1 x 10\(^6\) cells of the following types: MOPC-167 (\(\bigcirc\)), MOPC-315 taken 4 (\(\triangle\)) or 11 (\(\Delta\)) days after transfer, and H-2\(^{b}\) control EL-4 (\(\triangledown\)). (B) In this mixing experiment, BALB.B spleen cells were cultured with the following cells: 1 x 10\(^6\) MOPC-167 (\(\bigcirc\)), 2 x 10\(^6\) MOPC-167 (\(\bigtriangleup\)), 1 x 10\(^6\) MOPC-315 plus 1 x 10\(^6\) MOPC-315 taken at 4 days (\(\triangle\)), and 1 x 10\(^6\) MOPC-167 plus 1 x 10\(^6\) MOPC-315 taken at 11 days (\(\Delta\)).

Figure 4. Detection of cell surface products of the H-2\(^{d}\) locus on various cells by sensitivity to alloreactive antisera plus complement. Serum from B10 mice immunized with B10.D2 spleen cells, plus complement provided by normal rabbit serum (1:4), lysed the following \(^{51}\)Cr-labeled target cells: BALB/c spleen Con A blasts (\(\bigcirc\)) and MOPC-315 taken at 4 (\(\triangle\)), 7 (\(\bigtriangledown\)), or 11 (\(\Delta\)) days. EL-4 cells (\(\bigtriangledown\)) served as specificity control.

Figure 5. Detection of cell surface products of the H-2\(^{d}\) locus on various cells by absorption of anti-H-2\(^{d}\) serum (B10 anti-B10.D2). The absorbed and unabsorbed serum samples were tested on \(^{51}\)Cr-labeled Con A blasts of spleen cells from the following mouse strains, with the indicated H-2\(^{K}\) and H-2\(^{D}\) alleles (in parentheses): (A) BALB/c (d,d); (B) BALB.HTG (d,b); and (C) B10.A (5R) (b,d). The cells used for adsorption were BALB/c spleen Con A blasts (\(\bigcirc\)) and MOPC-315 taken at 4 (\(\triangle\)), 7 (\(\bigtriangledown\)), or 11 (\(\Delta\)) days. EL-4 cells (\(\bigtriangledown\)) served as controls. Lysis by unadsorbed serum and complement was 65, 60, and 62% in A, B, and C, respectively; lysis by normal mouse (B10) serum and complement was 21, 19, and 27% in A, B, and C, respectively.

Fig. 3. Ability of various tumor cells to elicit anti-H-2\(^{d}\) CTLs in culture. \(^{51}\)Cr-Labeled Con A blasts of BALB/c spleen cells served as target cells in all assays (control release was 22%). (A) BALB.B spleen cells were cultured for 5 days with 1 x 10\(^6\) cells of the following types: MOPC-167 (\(\bigcirc\)), MOPC-315 taken 4 (\(\triangle\)) or 11 (\(\Delta\)) days after transfer, and H-2\(^{b}\) control EL-4 (\(\triangledown\)). (B) In this mixing experiment, BALB.B spleen cells were cultured with the following cells: 1 x 10\(^6\) MOPC-167 (\(\bigcirc\)), 2 x 10\(^6\) MOPC-167 (\(\bigtriangleup\)), 1 x 10\(^6\) MOPC-315 plus 1 x 10\(^6\) MOPC-315 taken at 4 days (\(\triangle\)), and 1 x 10\(^6\) MOPC-167 plus 1 x 10\(^6\) MOPC-315 taken at 11 days (\(\Delta\)).

with the same determinants on the same surface molecules, but in cells that are late in the transplantation cycle the surface microenvironment around the H-2 products might be somehow altered so that the reactions with CTLs, but not with antibodies, are hindered. Another possibility is that H-2 products and cell surface structures are uniform at all times, but that late in the transplantation cycle the tumor cells develop an internal change (e.g., in a cytoskeletal structure or function) that interferes with target cell interaction with CTLs. Systematic comparison of H-2 encoded and other cell surface molecules from early and late MOPC-315 cells may distinguish among these possibilities.

The late MOPC-315 cells are less reactive than early cells not only with allogeneic anti-H-2\(^{d}\) CTLs but also with CTLs that have the same H-2\(^{d}\) haplotype as MOPC-315 (H-2\(^{d}\)) and are directed against minor transplantation antigens on the tumor cell surface—i.e., CTLs elicited with BALB/c spleen cells in DBA-2 mice (A. H. Hale and J. H. Russell, unpublished data). Hence, the defect in the late tumor cells doubtless applies to syngeneic as well as to allogeneic CTLs. Accordingly, the loss could represent a mechanism that enables tumor cells to escape destruction by host CTLs that would otherwise react with the tumor-associated antigens.

Fig. 4. Detection of cell surface products of the H-2\(^{d}\) locus on various cells by sensitivity to alloreactive antisera plus complement. Serum from B10 mice immunized with B10.D2 spleen cells, plus complement provided by normal rabbit serum (1:4), lysed the following \(^{51}\)Cr-labeled target cells: BALB/c spleen Con A blasts (\(\bigcirc\)) and MOPC-315 taken at 4 (\(\triangle\)), 7 (\(\bigtriangledown\)), or 11 (\(\Delta\)) days. EL-4 cells (\(\bigtriangledown\)) served as specificity control.

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The recurrent loss of reactivity with anti-H-2\textsuperscript{d} CTLs in late MOPC-315 cells means that these tumor cells undergo periodic alteration in certain cell surface properties during each transplantation cycle. Another MOPC-315 line has been observed by Rohrer et al. (18) to undergo periodic changes in cytology and in production of myeloma protein in each transplantation cycle. A similar phenomenon was suggested for another myeloma tumor in tissue culture (19). It is possible that the loss in reactivity with CTLs by MOPC-315 reflects a normal differentiation step in the maturation of B lymphocytes. Such a loss could offer an interesting biological advantage, preventing emerging immunoglobulin idiotypes on maturing B lymphocytes from eliciting or reacting with potentially lethal anti-idiotypic syngeneic CTLs.

Note Added in Proof. The line of MOPC-315 used by Rohrer et al. (18) was kindly provided by Richard Lynch and was found not to show the same loss of reactivity with CTLs as the MOPC-315 line used in the present study. Several other MOPC-315 lines in this laboratory also do not show the loss (E. Cells, unpublished data). The particular line of MOPC-315 described in this study is now designated MOPC-315-EL (for early, late).

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