PARTICIPATION OF THE H-2 ANTIGENS OF TUMOR CELLS IN THEIR LYSIS BY SYNGENEIC T CELLS*

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A feature of the allograft reaction is the generation of a subclass of T lymphocytes which are capable of lysing specifically target cells in vitro (1). The same class of cytotoxic T lymphocytes may be involved in the destruction in vivo of neoplastic (2) and virally infected syngeneic cells (3), suggesting that the physiological function of these cytotoxic T cells is related to the elimination of antigenically abnormal cells.

Many observations suggest that there is a special relationship between the activity of cytotoxic T lymphocytes and the serologically determined histocompatibility antigens. For example, when an immune reaction occurs against murine cells bearing a variety of foreign or chemically modified surface antigens, cytotoxic lymphocytes are not generated against all of the potential antigens, but only against the foreign (4, 5) or modified (6) H-2 antigens. On the other hand, cytotoxic activity may be undetectable in cultures containing a mixture of cells that are identical at the *H-2K* and *H-2D* loci but differ at other loci, despite the presence of a strong proliferative response (7, 8).

H-2 antigens also appear to be involved in the T-cell-mediated lysis of virally infected or neoplastic syngeneic cells in which the H-2 antigens would not be expected to be the targets of the cytotoxic cells. Cytotoxic lymphocytes generated against virally infected syngeneic cells kill virally infected target cells only if the target cells and the stimulating cells share the *H-2K* or *H-2D* region of the major histocompatibility complex (MHC)[1] (9). Furthermore, anti-H-2 antibodies inhibit the lysis of virally infected (10) or neoplastic cells (11, 12) by *H-2*-compatible cytotoxic T cells.

The exact nature of the role of the H-2 antigens in T-cell-mediated lysis is unknown. One key question is whether the H-2 antigens on both the cytotoxic lymphocyte and the target cells are important in the lytic event. Although several observations suggest that the H-2 antigens on the target cell are important in the lysis of syngeneic cells, modified either chemically (6) or by viral infection (9), there is no direct evidence that H-2 antigens on tumor cells are critical for their lysis by syngeneic cytotoxic cells. Even less is known about the possible role of H-2 antigens on the cytotoxic T cell, although one recent observation (11) suggests that they may not participate in the lysis of syngeneic tumor cells.

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[1] *Abbreviations used in this paper:* H-RPMI, HEPES-buffered RPMI medium with 10% fetal calf serum; MHC, major histocompatibility complex; TASA, tumor-associated surface antigen.

In the present study, we have, therefore, investigated more closely the role of H-2 antigens in the killing of tumor cells by cytotoxic lymphocytes of the same H-2 type. We have found that although H-2 antigens are present on these cytotoxic lymphocytes, they do not participate in the lytic event. Our data indicate, rather, that it is the H-2 antigens on the tumor cells that are crucial to the cytotoxic interaction.

Materials and Methods

Animals. BALB/c mice were bred in our own colony and were used at 2–8 mo of age. DBA/2J, C57BL/6J, and F_1-hybrid (C57BL/6 × DBA/2) mice were obtained from The Jackson Laboratories (Bar Harbor, Maine) and were used at 2–6 mo of age.

Antisera. Anti-H-2^d serum was prepared by a schedule of repeated intraperitoneal injections of C57BL/10J mice with spleen, thymus, and lymph node cells from B10.D2/OSn mice. The anti-H-2^b antiserum was prepared by a similar method in which B10.D2/OSn mice were immunized with lymphoid cells from B10/ScSn mice. In both cases, the antisera used were prepared from the fourth to eighth bleedings. The Anti-Thy-1.2 serum (AKR anti-C3H) was obtained from Litton Bionetics (Kensington, Md.).

Tumor Cells. The lymphomas EL4.BU *(H-2^b)*, originally derived from a C57BL/6 mouse, and P388 *(H-2^d)*, originally derived from a DBA/2 mouse, were obtained from the Salk Institute. They were passaged in vitro and in some cases for short periods in ascitic form in vivo.

Immunization of Mice with Tumor Cells. BALB/c mice were immunized by the subcutaneous injection of 3×10^6 untreated P388 cells. In the case of DBA/2 mice, it was necessary to use P388 cells which had been irradiated with 4,000 rads to prevent proliferation. C57BL/6 mice were immunized with EL4 cells either by subcutaneous injection of irradiated cells or by injection in one hind leg with living cells, followed after 7–14 days by surgical amputation of the limb. Mice were used as spleen cell donors 1–2 mo after immunization.

Tissue Culture. Suspensions of spleen cells were prepared, and 2.5×10^7 viable nucleated cells were cultured together with 10^6 tumor cells previously irradiated with 4,800 rads. In some experiments, the spleen cells were cultured with 10^7 allogeneic spleen cells which had been pretreated for 30 min at 37°C with mitomycin C (40 μg/ml). Each culture contained 10 ml of medium in plastic flasks (No. 3012, Falcon Plastics, Oxnard, Calif.). The medium used was RPMI-1640 (Associated Biomedic Systems, Buffalo, N. Y.) supplemented with glutamine (0.292 mg/ml), β-mercaptoethanol (5×10^{-5} M), and fetal calf serum (5%) (Associated Biomedic Systems). Cultures were incubated in humidified air with 5% CO_2 for 5–6 days. Cells from these cultures were termed "effector cells" and after harvest were washed and resuspended in 0.5 ml of RPMI-1640 medium, containing 10% fetal calf serum and HEPES (20 mM), (H-RPMI).

Assay of Cytotoxic Activity. A modified ^{51}Cr-release assay (13) was used. Assays were performed in triplicate in round-bottomed microtitre trays (Limbro, IS-MRC-96, Ace Scientific Supplies, Linden, N. J.). To assay cytotoxicity, 100 μl of serial dilutions of the effector cell suspension were incubated with 100 μl of H-RPMI containing 2.5×10^4 ^{51}Cr-labeled target cells. In experiments to test the effect of antisera on cytotoxicity, 50 μl of either H-RPMI or of serial dilutions of the antiserum under test were included in each well together with 100 μl of H-RPMI containing 2.5×10^4 ^{51}Cr-labeled target cells and 50 μl of H-RPMI containing the cultured spleen cells. Control assays in which the spleen cells were omitted were performed for each dilution of each antiserum, as the presence of some mouse sera tended to reduce the spontaneous release of ^{51}Cr by these target cells. The total counts releasable from the target cells were determined from wells in which the target cells were incubated in the presence of 1.25% NP-40. The trays were incubated from 1–3 h at 37°C in an atmosphere of 5% CO_2 and were then stood in a water bath at 45°C for 60 min (13). A 0.1-ml aliquot of supernate was then carefully removed from each well and counted in a scintillation counter. Specific lysis was calculated from the formula:

$$\text{Specific lysis} = \frac{\text{Experimental counts released} - \text{control counts released}}{\text{total releasable counts} - \text{control counts released}}$$

Results are presented as the mean specific lysis of triplicate assays ± the standard error of the mean and were evaluated using Students *t* test.

Treatment of Spleen Cells with Antisera and Complement. A two-stage procedure with reconstituted lyophilized guinea pig serum (Grand Island Biological Co., Grand Island, N. Y.) absorbed with agarose as a source of complement was used as described elsewhere (14). The necessary concentrations of anti-H-2 or anti-Thy-1.2 sera were determined in preliminary experiments.

Results

The Generation of Cytotoxic T Lymphocytes Directed against Syngeneic or Nonsyngeneic H-2-Compatible Tumors. Cytotoxic lymphocytes were generated in vitro by culturing spleen cells with syngeneic or nonsyngeneic, but H-2-compatible, irradiated tumor cells (Fig. 1). The two syngeneic combinations used were DBA/2 *(H-2^d)* spleen cells with P388 tumor cells and C57BL/6 *(H-2^b)* spleen cells with EL4 tumor cells. The nonsyngeneic, *H-2*-compatible combination was BALB/c *(H-2^d)* spleen cells together with P388 *(H-2^d)* tumor cells. Spleen cells were taken from donors that were previously immunized in vivo with the appropriate tumor because preliminary experiments showed that this resulted in higher cytotoxic activity. Cytotoxic activity was abolished when the cultured cells were treated with anti-Thy-1.2 serum and complement before the cytotoxic assay was performed (Fig. 1). This indicated that the cytotoxic cells were T cells, as previously described in similar systems (1–3).

The antigens against which these cytotoxic lymphocytes were directed have not been fully defined, but they cannot be simply H-2 antigens (or other products of the MHC) inasmuch as both the tumor and the responding population possess the same MHC. In the syngeneic systems, the antigens involved in the cell-mediated lysis can be described as tumor-associated surface antigens (TASA). The cytotoxic response, however, was much greater in the *H-2*-compatible system than in the syngeneic systems, and therefore, many of the experiments were performed using cytotoxic lymphocytes generated by culturing BALB/c spleen cells with the P388 tumor cells. In this case, because the BALB/c and DBA/2 strains differ at loci other than the *H-2* locus, it is possible that the BALB/c cytotoxic lymphocytes could be directed not only against TASA on P388 cells, but also against antigens on normal DBA lymphoid cells. In fact, we have observed some lysis of normal DBA spleen cells by BALB/c cells which had been cultured with P388 cells, although the specific lysis was much less than that observed when P388 cells were used as targets (unpublished observations).

Presence of H-2 Antigens on Cytotoxic Lymphocytes. A first step in the evaluation of the role of H-2 antigens in the T-cell-mediated lysis of *H-2*-compatible cells was to determine whether H-2 antigens were present on the surface of the cytotoxic lymphocytes. Spleen cells which had been cultured with irradiated *H-2*-compatible tumor cells were first treated with specific anti-H-2 serum and complement and then assayed for cytotoxic activity. This pretreatment of cell populations with anti-H-2 serum and complement completely abrogated cytotoxic activity (Fig. 2), indicating that H-2 antigens were present on the surface of these cytotoxic lymphocytes.

H-2 Antigens on Cytotoxic Cells are not Involved in the Lysis of Allogeneic Target Cells. We next investigated the question of whether the H-2 antigens on the cytotoxic lymphocytes are involved in the lysis of target cells. One test of the importance of these H-2 antigens in cell lysis is whether an antiserum directed against the H-2 antigens of cytotoxic lymphocytes will inhibit the lysis

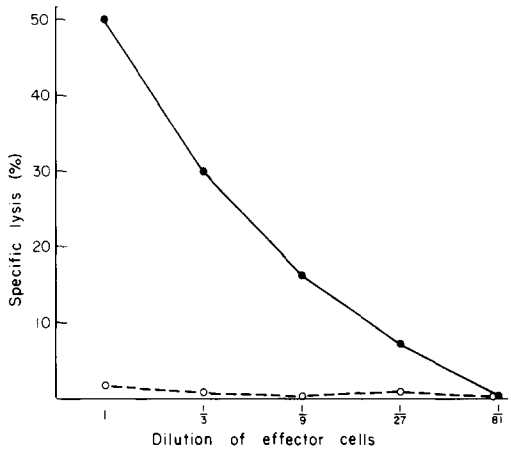

FIG. 1. Lysis of *H-2*-compatible tumor cells by cytotoxic T lymphocytes. BALB/c spleen cells were cultured for 6 days with irradiated P388 cells and assayed for cytotoxic activity against P388 target cells. Before assay two portions of the culture, each containing approximately 10^6 BALB/c cells, were treated with either normal AKR serum (—●—) or anti-Thy-1.2 serum (--○--) and complement. Assay time was 150 min.

of target cells of a different *H-2* type. Several reports of experiments using cytotoxic lymphocytes generated against allogeneic cells indicate that the presence of anti-H-2 sera specific for the *H-2* type of the cytotoxic lymphocytes does not inhibit the killing of H-2-incompatible allogeneic targets (1, 7). In these studies, however, the disparity between the number of the effector and target cells in the assay was not taken into account. Because the ratio of effector to target cells is usually large, e.g. 100:1 (1) and 40:1 (7), it is likely that much more specific anti-H-2 antibody would be needed to block the H-2 antigens on the effector population than would be required to block the H-2 antigens on the target cells. For this reason, we carried out similar experiments using smaller ratios of effector to target cells.

Fig. 3 shows the results of such an experiment. Anti-H-2^d serum directed against the cytotoxic lymphocytes did not inhibit lysis, in contrast to the inhibitory effect of the anti-H-2^b serum directed against the *H-2* type of the target cells. When the ratio of effector cells to target cells was reduced from 10:1 to 0.4:1, the antiserum directed against the *H-2* type of the cytotoxic lymphocyte still did not inhibit cell-mediated lysis (Fig. 3). The reciprocal experiment using *H-2*^{d} target cells and a series of dilutions of *H-2*^{b} effector cells was also carried out. In this experiment, the anti-H-2^d serum blocked lysis, and even when the ratio of effector to target cells was less than one, the anti-H-2^b serum had no effect.

The results of these experiments indicate that H-2 antigens on the cytotoxic lymphocytes are unlikely to play a role in the T-cell-mediated lysis of allogeneic cells. Furthermore, these experiments suggested that anti-H-2 sera inhibited the lysis of tumor cells by *H-2*-compatible lymphocytes (11, 12) because they blocked the H-2 antigens of the tumor cells. Therefore, we turned our attention to the effect of anti-H-2 sera on the activity of cytotoxic lymphocytes generated against *H-2*-compatible tumor cells.

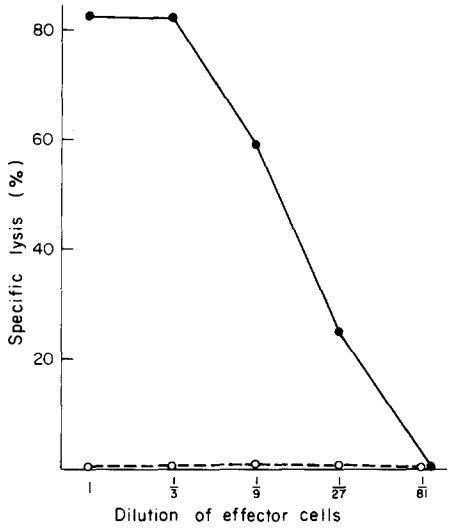

FIG. 2. H-2 antigens are present on the cytotoxic T lymphocytes which lyse *H-2*-identical tumor cells. BALB/c spleen cells were cultured with irradiated P388 cells for 6 days, and before assay two portions of the culture, each containing approximately 3×10^5 BALB/c spleen cells, were treated with either anti-H-2^d antiserum (--○--) or normal mouse serum (—●—) and complement. Assay time was 210 min.

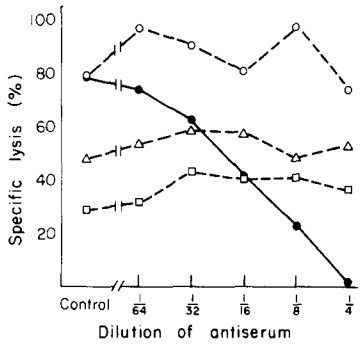

FIG. 3. The failure of antiserum directed against the H-2 antigens of the cytotoxic lymphocytes to inhibit cell lysis. BALB/c *(H-2^d)* spleen cells were cultured with mitomycin-C-treated C57BL/10 *(H-2^b)* spleen cells for 5 days. Cytotoxic activity was assayed using ^{51}Cr-labeled EL4 cells *(H-2^b)* in the presence of the indicated final dilution of anti-H-2^b serum (solid circles, -●-) or anti-H-2^d serum (open symbols, --○--, --△--, --□--). The cytotoxic activity was tested at three dilutions of the cultured BALB/c spleen cells so that the BALB/c cells present in each well of the assay-tray were: 2.5×10^5 (--○--); 5×10^4 (--△--); or 10^4 (--□--). The antiserum and spleen cells were maintained at 37°C for 30 min before the EL4 cells were added. The assays were then incubated for 150 min.

Target Specific anti-H-2 Sera Inhibit Lysis by Cytotoxic Lymphocytes Generated Against Antigens other than H-2 Antigens. First, we examined whether the ability of anti-H-2 sera to inhibit the lysis of tumor cells by *H-2*-compatible lymphocytes was correlated with their specificity for the H-2 antigens on the tumor cells. As shown in Table I, anti-H-2^d serum inhibited the killing of P388 cells by cytotoxic lymphocytes of *H-2*-compatible (BALB/c) origin, confirming

our previous observations (12). Similarly, anti-H-2^d serum inhibited the lysis of P388 cells by syngeneic, cytotoxic lymphocytes of DBA/2 origin (Table I). In contrast to anti-H-2^d serum, an anti-H-2^b serum, produced by a reciprocal immunization, did not inhibit the lysis of P388 *(H-2^d)* target cells (Table I). The same anti-H-2^b serum did, however, block cell lysis in a system in which C57BL/6 *(H-2^b)* spleen cells were activated against the syngeneic tumor, EL4. In this case, the anti-H-2^d antiserum had no effect.

It should be noted that the *H-2*-compatible BALB/c spleen cells produced about 15% specific lysis of P388 target cells in 60 min, whereas a comparable level of lysis by the syngeneic DBA/2 spleen cells was achieved only after 180 min (Table I). This stronger response to P388 cells by BALB/c spleen cells, as compared with DBA/2 spleen cells, was reproducible and was seen whether the spleen cells were taken from normal or immunized mice. Also shown in Table I are the results of an experiment in which immune BALB/c cells were stimulated twice in vitro with P388 cells. This tertiary response resulted in 50% specific lysis of P388 cells in a 60-min assay.

To investigate further the role of the H-2 antigens in lysis by cytotoxic cells generated against antigens other than H-2, we used (C57BL/6 × DBA/2)F_1 effector cells which carried both the *H-2^b* and *H-2^d* haplotypes. These effector cells were generated and assayed against the *H-2^b* tumor, EL4. Lysis of EL4 cells was not inhibited by antiserum directed against the *H-2^d* haplotype, carried only by the effector cells, but was inhibited by antiserum against the *H-2^b* haplotype carried by both effector and target cells (Table II). When (C57BL/6 × DBA/2)F_1 cytotoxic lymphocytes were generated and assayed against P388 *(H-2^d)* cells, lysis was inhibited by the anti-H-2^d serum, but not the anti-H-2^b serum. These results suggest that anti-H-2 sera inhibit tumor-specific lysis when they are directed against H-2 antigens present on the target cells, but not when directed against H-2 antigens present only on the effector-cell population.

These experiments (Tables I and II) confirm that H-2 sera block the lysis of tumor cells by *H-2*-compatible cytotoxic lymphocytes. Furthermore, they support the conclusion that the sera are blocking because of their anti-H-2 activity. The anti-H-2^b serum inhibited the lysis of *H-2^b* tumor cells but not the lysis of *H-2^d* cells, and reciprocally, the anti-H-2^d serum inhibited the lysis of *H-2^d* cells but not *H-2^b* cells. In addition, the experiments described suggest that while the H-2 antigens on cytotoxic lymphocytes are not important in lysis (Fig. 3, Table II), the H-2 antigens on the tumor cells are.

The Effect of Pretreatment of the Target Cells with anti-H-2 Serum. To test this hypothesis, H-2 antigens on target cells were blocked by treatment with anti-H-2 serum before exposure to the cytotoxic lymphocytes. During labeling with ^{51}Cr, P388 cells were incubated with anti-H-2^d serum and washed. To insure maximal masking of H-2 antigens (15), the cells were then incubated for an additional 30 min with rabbit antibodies against mouse Ig and washed. These pretreated target cells were partially protected from lysis by *H-2*-compatible cytotoxic lymphocytes (Fig. 4).

The incompleteness of the inhibition could not be accounted for by the dissociation of antibody from the P388 cells, because staining with fluorescein-

TABLE I

Specificity of the Inhibition by Anti-H-2 Sera of the Killing of P388 Cells by H-2-Identical or Syngeneic Cytotoxic Lymphocytes

Cytotoxic cells	Assay time	Final dilution of mouse serum	Specific lysis			
			No mouse serum	NMS	Anti-H-2^d	Anti-H-2^b
	min		%	%	%	%
BALB/c*	60	1/16	–	13 ± 1.4	18 ± 1.3	14 ± 0.6
		1/8	–	16 ± 1.5	13 ± 2.0	18 ± 0.7
		1/4	–	16 ± 0.7	7 ± 1.1‡	16 ± 0.7‡
BALB/c*	70	–	19 ± 2.0	–	–	–
		1/8	–	–	20 ± 2.5	21 ± 1.0
		1/4	–	–	10.6 ± 1.5§	25 ± 3.5§
DBA/2*	180	1/16	–	–	16 ± 0.3	18 ± 0.7
		1/8	–	–	17 ± 0.1	18 ± 3.0
		1/4	–	–	9 ± 1.0‖	17 ± 1.0‖
BALB/c¶	60	–	51 ± 1.3	–	–	–
		1/16	–	–	47 ± 4.5	50 ± 4.0
		1/8	–	–	50 ± 1.2	53 ± 1.2
		1/4	–	–	33 ± 5.5**	50 ± 4.5**

Comparable results were obtained in eight similar experiments.

* Spleen cells from immunized mice were cultured with irradiated P388 cells for 5 days.

‡ $P < 0.005$.

‖ $P < 0.005$.

§ $0.01 < P < 0.025$.

¶ Spleen cells from immunized BALB/c mice were cultured for 7 days with irradiated P388 cells. Another 10^6 irradiated P388 cells were then added and the cultures were assayed after a further 5 days.

** $0.025 < P < 0.05$.

TABLE II

The Effect of Anti-H-2^b and Anti-H-2^d Sera on the Cell-Mediated Lysis of EL4 Cells by (C57BL/6 × DBA/2)F_1 Lymphocytes

Dilution of antisera	Specific lysis	
	Anti-H-2^d	Anti-H-2^b
	%	
1/16	49 ± 3.4	42 ± 2.2
1/8	56 ± 2.3	39 ± 7.4
1/4	46 ± 11.0*	22 ± 1.6*

Spleen cells from (C57BL/6 × DBA/2)F_1 mice, previously immunized with irradiated EL4 cells, were cultured for 6 days with irradiated EL4 cells. Cytotoxic activity against EL4 cells was measured in a 3 h assay in the presence of the indicated dilutions of either the anti-H-2^d or anti-H-2^b serum.

* $0.05 < P < 0.1$.

conjugated antibodies against rabbit-Ig indicated that bright caps persisted for at least 4.5 h. The difficulty of completely inhibiting cell-mediated lysis by capping of specific antigens has been reported previously (16) and probably is due to the thermodynamic properties of the T-cell–target cell interaction (17). Nevertheless, this experiment indicates that a procedure that blocks the H-2 antigens on the tumor cell alone can inhibit lysis by *H-2*-compatible cytotoxic cells.

The Blocking of Cytotoxicity by anti-H-2 Serum Directed Against the Target Cell Alone. We have observed that cytotoxic lymphocytes generated against *H-2*-compatible tumor cells are able to lyse *H-2*-incompatible tumor cells. This finding allowed us to test the effects of anti-H-2 sera directed only against the target cell. When BALB/c *(H-2^d)* spleen cells from normal mice were cultured together with *H-2^d* P388 cells, cytotoxic cells were generated which were able to lyse the *H-2^b* lymphoma, EL4 (Table III). Lysis was inhibited (Table III) by anti-H-2^b serum, which was directed against H-2 antigens present only on the target cells. The anti-H-2^b serum was raised in *H-2^d* (B10.D2) mice and, therefore, should not bind to the H-2^d antigens of the BALB/c cytotoxic lymphocytes. Similar concentrations of anti-H-2^d antiserum had no effect in this system. This experiment indicates that antiserum directed towards the H-2 antigens of the tumor cell alone inhibits lysis by cytotoxic lymphocytes which have been generated against antigens on *H-2*-compatible cells.

Discussion

The demonstration that specific anti-H-2 sera inhibit the lysis of virally infected (10) or neoplastic (11, 12) cells by syngeneic or *H-2* identical cytotoxic lymphocytes provides direct evidence for a special role for the H-2 antigens (or a closely-linked product of the MHC) in immune surveillance. The major conclusion that can be drawn from the present experiments is that the H-2 antigens on the target cell, but not those on the cytotoxic lymphocyte, are critical in the lysis of tumor cells by *H-2*-compatible T cells.

Our results showed that H-2 antigens were present on the cytotoxic lymphocytes active against *H-2*-compatible tumor cells (Fig. 2). Two types of experiment, however, indicated that the H-2 antigens on cytotoxic cells were not involved in the lysis of target cells. First, cytotoxic lymphocytes were generated and assayed against allogeneic target cells. Antiserum specific for the H-2 type of the cytotoxic lymphocytes failed to block cytotoxicity even when very small numbers of cells of this *H-2* type were present (see Fig. 3). Furthermore, these same antisera were able to block cytotoxicity in reciprocal experiments when they were directed against the *H-2* type of the target cell. Second, the lysis of *(H-2^b)* EL4 cells by (C57BL/6 × DBA/2)F$_1$ lymphocytes, which carry both the *H-2^b* and *H-2^d* haplotypes, was not inhibited by anti-H-2^d serum, but was inhibited by anti-H-2^b serum (Table II). The same anti-H-2^d serum could, however, block the lysis of *H-2^d* target cells (Table I, Fig. 4). Similarly, it has been observed recently that neither an anti-H-2K^k or an anti-H-2D^k antiserum inhibited the lysis of *H-2^d* tumor cells by cytotoxic lymphocytes from F$_1$ hybrids carrying the *H-2^k* and *H-2^d* haplotypes (11).

The results of these two types of studies suggest that it is unlikely that H-2

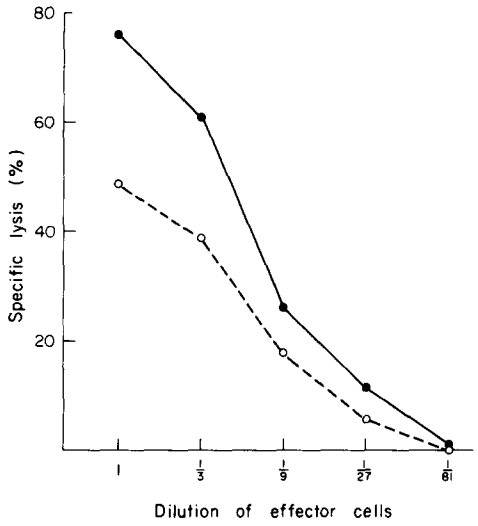

FIG. 4. Coating tumor cells with anti-H-2 antibody inhibits their lysis by *H-2*-identical cytotoxic lymphocytes. P388 cells (2.5×10^6) were incubated for 30 min at 37°C with either anti-H-2^d serum (100 μl) or medium alone together with Na-(^{51}Cr) chromate (50 μC). After washing, the P388 cells were incubated for an additional 30 min at 37°C with a gamma globulin fraction (200 μg) of a rabbit antiserum against mouse Ig. These P388 cells were then used as targets for cytotoxic BALB/c cells which had been generated against irradiated P388 cells. Lysis obtained using the P388 cells treated with anti-H-2^d serum is shown by the open circles (--○--); lysis obtained using as targets the P388 cells treated with medium alone is shown by the closed circles (-●-).

TABLE III
Inhibition of Cell-Mediated Lysis by Serum Directed Against H-2 Antigens Only on the Tumor Cell

Dilution of mouse serum	Specific lysis		
	No serum	Anti-H-2^b	NMS
–	22 ± 0.4	–	–
1/8	–	12 ± 1.5	18 ± 4.0
1/4	–	0	23 ± 0.5

BALB/c (*H-2*^{d}) spleen cells were cultured with the *H-2*^{d} tumor P388 and were assayed against the *H-2*^{b} tumor EL4. Assays were performed in medium alone or in the presence of either anti-H-2^b serum or normal mouse serum. Assay time was 150 min.

antigens function either as a part of the T-cell receptor or through binding by complementarity to H-2 antigens on the target cells. Furthermore, they imply that anti-H-2 sera inhibit the lysis of syngeneic tumor cells by blocking H-2 antigens on these cells. Two lines of evidence support this contention. First, coating of the H-2 antigens on the target cell by pretreatment of the target cells with anti-H-2 serum followed by rabbit antibodies against mouse Ig, clearly reduced lysis of tumor cells by *H-2*-compatible cytotoxic lymphocytes (Fig. 4). Second, when cytotoxic lymphocytes generated against *H-2*-compatible tumor cells were assayed against cross-reacting *H-2*-incompatible target cells, lysis

was inhibited by anti-H-2 sera that could bind only to the target cell (Table III). It seems unlikely that the binding of anti-H-2 sera to target cells blocks lysis for nonspecific reasons. For example, there is evidence that the presence of anti-H-2 serum directed against specificities carried by only some of the *H-2* molecules on a target cell does not prevent lysis of the target cell by cytotoxic lymphocytes directed against other *H-2* specificities carried by the target cell (1, 7). We conclude, therefore, that the H-2 antigens of tumor cells play a crucial role in lysis by *H-2*-compatible cytotoxic lymphocytes.

The fact that the anti-H-2 sera used inhibit the lysis of tumors of only the corresponding H-2 type (Tables I and II) suggests that this inhibition is not due to the antiviral antibodies which are found in most alloantisera (18), (R. J. Milner and G. M. Edelman, unpublished observations). Such antibodies may possibly be provoked by the production of virus, which has been reported to occur in allograft reactions (10).

Some critical experimental details related to the inhibition of cell-mediated cytotoxicity of antisera in this system are worthy of mention. The sigmoidal character of the plot of the extent of lysis against the number of effector cells added (e.g., Fig. 2) means that at very high levels of lysis, inhibition may be inherently difficult to detect. Moreover, longer incubation times were found to be associated with reduced degrees of inhibition, even when the concentrations of spleen cells were reduced to give comparable final levels of lysis. This increasing resistance of cell-mediated cytotoxicity to inhibition by anti-H-2 serum with longer incubation times has been described in an allogeneic system (20) and was predicted on thermodynamic grounds (17). Optimal conditions for the demonstration of inhibition by antiserum therefore involve short incubation periods and a more moderate degree of lysis (15–50%). The presence of effector cell populations with high cytotoxic activity allows the use of short incubation times and lower numbers of effector spleen cells, the latter being important in reducing competition with the target cells for the anti-H-2 antibody.

It was striking that there was a larger cytotoxic response against the DBA/2 tumor P388 by BALB/c spleen cells than by DBA/2 spleen cells (Table I). The mixed lymphocyte reaction generated by cells differing at the *M* locus (21), which is among the non-*H-2* loci at which BALB/c and DBA/2 mice differ, has been reported to enhance the production of cytotoxic lymphocytes directed against H-2 antigens on third party cells (22). The present observation, therefore, may represent an example of a similar enhancement of a response to a tumor-associated antigen on *H-2*-identical cells.

Previous studies indicate that T-cell-mediated lysis is restricted to target cells sharing private *H-2* specificities with the stimulating cells (9, 23). The killing of EL4 cells ($H\text{-}2^b$) by BALB/c cytotoxic lymphocytes which have been activated against P388 cells ($H\text{-}2^d$), (Table III), appears to contradict this hypothesis. Moreover, we and others (6) have found that cytotoxic lymphocytes generated against chemically-modified syngeneic spleen cells will kill chemically-modified tumor cells which differ at the *H-2* locus. The fact that the lysis of EL4 cells by BALB/c cytotoxic cells was inhibited by anti-$H\text{-}2^b$ serum (Table III) suggests, however, that the cytotoxic lymphocyte must recognize H-2 antigens on the target cell, even when the target cell does not share any private specificities

with the stimulating cells. Other data suggest that some cytotoxic lymphocytes recognize regions of the *H-2* molecule other than those determining the private specificities (7). Furthermore, cross-reactions between modified H-2 antigens of different private specificities might be expected because such antigens share at least some chemical similarities (24).

H-2 molecules on the target cell could participate in the cytotoxic events described here in at least two ways: they could be involved in determining the nature of the target antigen recognized by a single receptor on the cytotoxic T cell or they could be the target of a second set of T-cell receptors which are specific for H-2 antigens. Dual recognition of tumor-associated antigens and H-2 antigens by two separate receptor systems on the T cell seems unlikely for a variety of reasons (6, 9, 23). The first hypothesis is, therefore, more attractive, but requires an explanation of how *H-2* molecules affect the specificity of a single target antigen recognized by the cytotoxic T cell.

One explanation may be that the MHC specifies a glycosyl transferase which modifies other cell surface components in a manner characteristic of each *H-2* haplotype (25). However, because anti-H-2 sera precipitate only one major cell surface component (26) and the carbohydrate moieties of H-2 antigens do not contribute to their serological specificity (discussed in ref. 15, p. 372), experiments demonstrating that anti-H-2 antisera inhibit the lysis of target cells by *H-2*-compatible cytotoxic lymphocytes (10–12, and the present data) suggest that the H-2 antigens are more directly involved in the lytic event. Another possibility is that the H-2 antigens themselves are antigenically altered as a result of viral infection (6, 27).

One possible mechanism is that both H-2 and, for example, tumor viral antigens contribute specificity to a hybrid antigenic determinant (27, 23, 12). We have recently found that H-2 and viral antigens on tumor cells can be cocapped and copatched, suggesting that these antigens can become physically associated on the cell surface (12). The observation that both antiviral (28) and anti-H-2 sera (10) can block lysis of virally-infected cells is consistent with the view that the T-cell receptor binds to such a physical complex of viral and H-2 antigens. Furthermore, cytotoxic lymphocytes are specific for particular viral or tumor-associated antigens (29, 30), suggesting that the target for cytotoxic lymphocytes cannot result from perturbations of the *H-2* molecule that are independent of the nature of those antigens. A complex of H-2 and viral antigens would form a hybrid antigen in which the determinants of the junction region would have elements of both the self (H-2) and viral components. The receptor repertoire of the population of cytotoxic T-cell precursors (31) would then be directed against such hybrid antigens formed from H-2 antigens and viral or other foreign cell surface antigens.

Finally, it may be of value to consider the relevance of the present findings and those of other workers to the possible functions of H-2 antigens. First, H-2 antigens may serve as a substrate for the production of a new antigenic determinant (27, 6, 12). The polymorphism of H-2 antigens could provide a variety of new antigens and thus increase potential immunogenicity (32, 6, 33). Second, *H-2* molecules may associate in some way with foreign cell surface antigens (12), and the mechanism for the association may involve the polymorphism of

H-2 antigens (e.g., 33). Third, the *H-2* molecule may be a specialized participant in the lytic event (23, 5). Such a function could reflect a structural and evolutionary relationship with complement components.

Summary

Cytotoxic T lymphocytes were generated in vitro against *H-2*-compatible or syngeneic tumor cells. In vitro cytotoxic activity was inhibited by specific anti-H-2 sera, suggesting that H-2 antigens are involved in cell lysis. Two observations directly demonstrated the participation of the H-2 antigens on the tumor cells in their lysis by H-2-compatible T cells. First, coating of the H-2 antigens on the target tumor cell reduced the number of cells lysed on subsequent exposure to cytotoxic T cells. Second, when cytotoxic T cells were activated against an *H-2*-compatible tumor and assayed against an *H-2*-incompatible tumor, anti-H-2 serum that could bind to the target cell, but not to the cytotoxic lymphocyte, inhibited lysis.

H-2 antigens were also shown to be present on the cytotoxic lymphocytes. Specific antisera reacting with these H-2 antigens, but not those of the target cell, failed to inhibit lysis when small numbers of effector cells were assayed against *H-2*-incompatible target cells or when effector cells of F_1-hybrid origin and bearing two *H-2* haplotypes were assayed against a tumor cell of one of the parental strains. These findings suggest that it is the H-2 antigens on the tumor cell and not those on the cytotoxic lymphocytes that are important in cell-mediated lysis of *H-2*-compatible tumor cells.

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