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Syngeneic tumor cells can induce alloreactive T killer cells: A biological role for transplantation antigens

(tumor immunity/cell-mediated cytotoxicity/major histocompatibility antigens/regulation of gene expression/derepression)

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ABSTRACT **A chemically induced sarcoma of BALB/c (H-2^d) mice, MCG4, is shown to induce in BALB/c lymphocytes a primary anti-tumor cytolytic T lymphocyte (CTL) reaction *in vitro*. The anti-tumor CTL showed tumor specificity but reacted also with normal cells expressing distinct H-2 alloantigens. The CTL response could be shown to be induced by and directed against alloantigenic determinants expressed on two different molecules, one H-2K^k-like the other H-2D^k-like. The biological significance of these findings is discussed with regard to (*i*) possibility of derepression of normally silent *H-2* genes in tumor cells and normal cells, (*ii*) generation of alloreactivity in ontogeny, and (*iii*) role of alloreactive T cells in eliminating cells expressing wrong H-2 antigens.**

Several theories have been proposed to account for the high frequency of alloreactive T lymphocytes (1–4). None of these theories involves alloantigen itself as a possible stimulus because this is thought to be "forbidden" for genetic reasons. Tumor cells, on the other hand, have been reported to express alloantigens, or at least antigens very similar to normal transplantation antigens from other strains of the species (for reviews see refs. 5 and 6). This suggested that the genes for alloantigens of a species are not distributed within the cells of individual members as alleles but as genes (7), the majority of which would be normally not expressed and silent. A derepression mechanism could be postulated to activate such genes in tumor cells (8–10) or even in normal cells as a result of occasional error or disturbance in gene regulation. Derepressed H-2 transplantation antigens on normal cells would be difficult to detect because their frequency is expected to be low and also because they would be recognized by the immune system as "not self" and eliminated. Tumor cells that express H-2 like alloantigens may eventually escape immune destruction and grow out and thus are a powerful cell source for structural, functional, and genetic studies on these antigens.

We recently reported about a sarcoma of BALB/c (H-2^d) mice, MCG4, which expresses foreign H-2-like molecules (10–13). This tumor, which had been newly induced by methylcholanthrene and adapted to the ascites form, reacted with monoclonal BALB/c hybridoma-derived anti-H-2^k antibodies (12) and induced in BALB/c mice strong anti-H-2^k isoantibodies (13). The foreign H-2^k-like molecules on the tumor were recognized as targets by alloreactive cytolytic T lymphocytes (CTL) and as restricting elements by virus-specific CTL (14). Now we demonstrate that the H-2^k-like alloantigens on MCG4 are as immunogenic as normal H-2^k alloantigens in triggering syngeneic T cells to become alloreactive CTL. Anti-tumor CTL clones can be distinguished that recognize H-2^k determinants expressed on different H-2 molecules (K and D). From these data a theory for the biological significance of alloreactivity is proposed. It states (*i*) that H-2 alloantigens can occur in normal mice and be immunologically active and (*ii*) that the frequency of such an event is high enough to play a significant role in the generation during ontogeny of the extraordinarily high proportion of T cells specific for alloantigens.

MATERIAL AND METHODS

Mice. Mice, 3–6 weeks old, of the following strains were used: BALB/c/Han (H-2^d), DBA/2/Han (H-2^d), B10.D2/nSn (H-2^d), C3H/He Han (H-2^k), B10.BR/SgSn (H-2^k), C57BL/10Sn (B10;H-2^b), C57BL/6J (H-2^b), B10.S (H-2^s), A.SW/Sn (H-2^s), N.ZW (H-2^z), A.CA (H-2^f), S.WR/J (H-2^q), B10.A/SgSn (H-2^a), A.AL (H-2^{a1}), A/J (H-2^a), C3H.OH (H-2^{o2}), D2.GD (H-2^{g2}), B10.A(2R)/SgSn (H-2^{h2}), B10.A(4R) (H-2^{h4}), B10.A(3R) (H-2^{i3}), B10.A(5R)/SgSn (H-2^{i5}), A.TH (H-2^{t2}), A.TL (H-2^{t1}), B10.HTT (H-2^{t3}), and B10.WB (H-2^{ja}). The mice were bred in our animal facilities. Breeding pairs were obtained from Jackson Laboratory and Zentralinstitut für Versuchstierzucht (Hannover, West Germany), from Peter Demant (D2.GD; Vereniging Het Nederlands Krankerinstituut, Amsterdam, The Netherlands) and from Chella David (A.AL; Washington University School of Medicine, St. Louis, MO).

Tumors. MCG4 is a sarcoma induced in 1977 in a BALB/c (H-2^d) male mouse as a solid tumor by a subcutaneous injection of 0.2 mg of methylcholanthrene (11). The solid tumor was adapted to growth as an ascites tumor. The BALB/c origin of MCG4 has been confirmed by an isoenzyme analysis by H. H. Krog, Copenhagen. Upon transplantation in normal syngeneic animals the tumor induced a strong immune response (13) and was rejected after about 10–14 days (regressor mice). The tumor was routinely transplanted by inoculating 2×10^6 cells intraperitoneally into 400-roentgen (0.1 coulomb/kg)-irradiated BALB/c mice. Tumor cells were also maintained in suspension culture *in vitro*, using tissue culture medium (RPMI 1640 with 10% fetal calf serum, 7 mM glutamine, 50 μM 2-mercaptoethanol, penicillin, and streptomycin). The experiments were performed with the subline MCG4-A, which is the 1979 Heidelberg line of MCG4.

Generation of CTL. CTL against MCG4 were generated by culturing spleen cells from either normal BALB/c mice (primary response) or from BALB/c MCG4 regressor mice (secondary response) for 4–5 days with mitomycin C-treated MCG4 tumor cells. The cells were cultured in Falcon flasks at a density of 4×10^6 responder cells per ml and 2×10^5 mitomycin C-

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Abbreviations: CTL, cytotoxic T lymphocyte(s); Con A blast, concanavalin A-activated lymphoblast.

inactivated tumor cells per ml. CTL against alloantigens were generated in mixed lymphocyte cultures (14), using normal spleen cells (4×10^6 per ml) as responder cells and mitomycin C-treated allogeneic spleen cells (10^6 per ml) as stimulator cells. The effector cells were washed twice before they were tested in the cytotoxicity test.

^{51}Cr Release Cytotoxicity Test. This 4-hr assay was performed as described (15).

RESULTS

Primary Induction *in Vitro* of Anti-Tumor CTL Activity. When spleen cells from normal BALB/c mice were cocultured *in vitro* for 5 days with mitomycin C-treated BALB/c-derived MCG4 tumor cells, high anti-tumor cytolytic activity was generated. This could be detected in a 4-hr ^{51}Cr-release assay (Fig. 1). The cytolytic activity was sensitive to treatment with anti-Thy 1.2 serum and complement and was thus of T cell nature. This primary anti-tumor response *in vitro* was comparable both qualitatively and quantitatively to a primary response against normal allogeneic cells of H-2^k type. Thus, BALB/c anti-H-2^k CTL generated in a mixed lymphocyte culture reacted with MCG4 tumor cells and not with normal H-2^d target cells, just like the anti-tumor CTL. The results from Fig. 1 also demonstrate that MCG4 tumor cells differ from normal H-2^d type cells in the expression of H-2^d antigens. CBA anti-BALB/c and C57BL/6 anti-BALB/c CTL showed a strong cytolytic reaction with concanavalin A-activated lymphoblasts (Con A blasts) from BALB/c mice, whereas only C57BL/6 anti-BALB/c CTL reacted with the MCG4 target cells. These results thus show that MCG4 is immunogenic in a primary response with BALB/c responder cells *in vitro*, differs from normal H-2^d type cells, and can be recognized by anti-H-2^k CTL.

Lysis of Allogeneic Normal Cells by Anti-Tumor CTL. BALB/c anti-MCG4 CTL did not react with several other BALB/c-derived tumor target cells, be they induced by chemicals (Meth.A, ULMC), tumor viruses (LSTRA, YC8), or irradiation (RLδ1) (Table 1A). Allogeneic normal cells, however, such as lymphoblasts from H-2^k strain mice, were lysed by these H-2^d-derived anti-tumor CTL (see Table 1). More detailed information about the allospecificity of the anti-tumor CTL was obtained by testing primary and secondary CTL against a large panel of Con A blasts from distinct H-2 congenic and recombinant mouse strains. Thus we could distinguish in primary effector cells CTL reactive with H-2K^k (see reactivity with A.AL vs. A.TL) and H-2D^k (see reactivity with C3H.OH vs. B10.D2). Secondary effector cells showed generally a higher cytolytic activity and reacted with most of the allogeneic cells tested but not with H-2^d type cells. They could, however, distinguish between cells of the *H-2* genotype *bbbddd* [e.g., B10.A(3R), B10.A(5R)] and *dddbbb* (e.g., D2.GD).

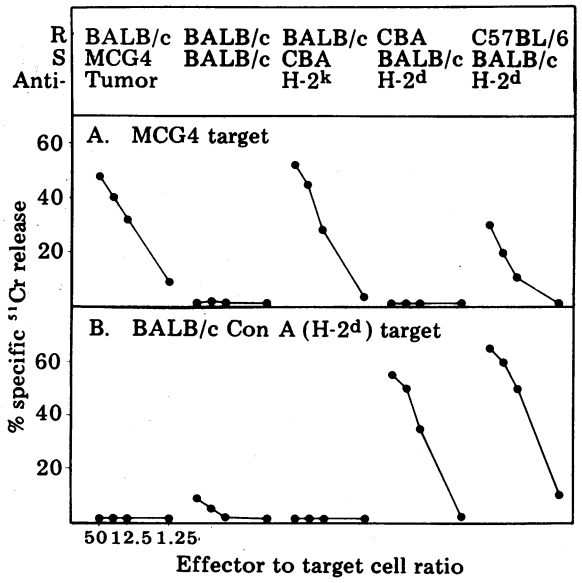

FIG. 1. Induction of primary anti-tumor cytotoxicity *in vitro* by tumor cells or by normal allogeneic cells. Spleen cells from the indicated normal mice as responder cells (R) were cocultured for 5 days with mitomycin C-treated MCG4 tumor cells or with the indicated mitomycin C-treated spleen cells as stimulator cells (S). The cells were then washed and tested for cytotoxic activity against ^{51}Cr-labeled target cells, which were either MCG4 tumor cells (*A*) or BALB/c-derived Con A lymphoblasts (*B*).

"Cold" Target Competition Analysis. In order to test whether the MCG4-induced CTL that reacted with allogeneic cells were the same as those that reacted with the tumor, "cold" target competition experiments were performed (see Fig. 2). Secondary BALB/c anti-MCG4 CTL were tested against ^{51}Cr-labeled ("hot") MCG4 cells in the absence or presence of different numbers of nonlabeled ("cold") MCG4 tumor cells or allogeneic Con A blasts. Strong inhibition was observed with either MCG4 tumor cells or B10.BR (H-2^k) Con A blasts. Lymphoblasts expressing H-2^d or H-2^b antigens did not lead to competitive inhibition (Fig. 2*A*). Within the anti-MCG4 CTL two populations with different target cell specificity could be distinguished when either B10.A (expressing H-2K^k) or C3H.OH (expressing H-2D^k) Con A blasts were used as "hot" target cells (Fig. 2*B*). In both instances "cold" MCG4 tumor cells caused strong inhibition, but each of the targets was inhibited only by the corresponding "cold" target. When Con A blasts from B10.A(5R) (expressing K^b molecules) were used as "hot" targets, not only MCG4 and the corresponding "cold" targets [B10.A(5R)] but also Con A blasts expressing H-2K^k and H-2D^k molecules caused inhibition (Fig. 2*D*). Similar results were obtained when anti-MCG4 CTL were tested against "hot" H-2^b (B10), H-2^s (B10.S), or H-2^f (A.CA) target cells (data not shown). Taken together, these data indicate that BALB/c anti-MCG4 CTL contain predominantly anti-H-2^k (anti-K^k and anti-D^k) reactive cells. Reactivity with normal cells from other haplotypes was not seen in "cold" target competition assays with MCG4 as "hot" target (Fig. 2*A*) but could be detected when selectively tested for with the respective "hot" target cell type.

Anti-MCG4 CTL Reactivity in (H-2^d × H-2^k) F_1 or (H-2^d × H-2^b) F_1 Mice. If all of the anti-MCG4 CTL activity was directed against determinants similar to or identical with allodeterminants of K^k or D^k molecules, no anti-MCG4 reactivity should be generated in spleen cells from (H-2^d × H-2^k) F_1 mice. To test this, we investigated the anti-tumor response of BALB/c × B10.BR mice in comparison to that of the two *H-2* congenic strains BALB/c × B10.D2 and BALB/c × B10. Spleen cells from MCG4-immunized mice were cultured for 4 days with or without mitomycin-treated MCG4 tumor cells and then tested for cytotoxic activity against MCG4 or Con A blasts from B10.D2, B10.BR, or B10 mice. As illustrated in Fig. 3, this genetic approach allowed the distinction of three different anti-MCG4 CTL populations, those from BALB/c × B10.BR reacting with MCG4 only (Fig. 3*B*), those from BALB/c × B10 reacting with MCG4 and H-2^k but not H-2^b targets (Fig. 3*C*), and those from BALB/c × B10.D2 reacting with MCG4, H-2^k, and H-2^b target cells (Fig. 3*A*).

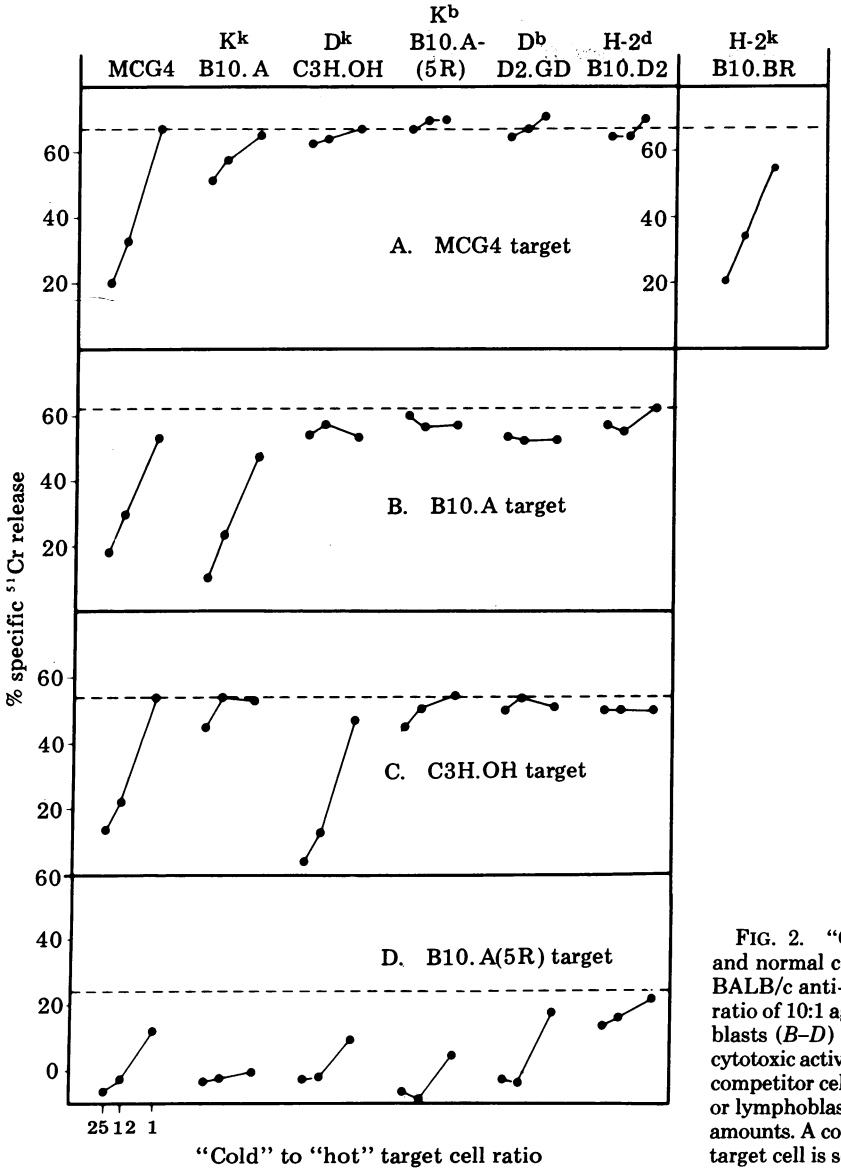

FIG. 2. "Cold" target competition between MCG4 tumor cells and normal cells expressing defined H-2 alloantigens. Secondary BALB/c anti-MCG4 CTL were tested at an effector to target cell ratio of 10:1 against either MCG4 tumor cells (*A*) or various Con A blasts (*B–D*) as "hot" target cells. The broken line indicates the cytotoxic activity obtained in these assays in absence of "cold" target competitor cells. In other test groups nonlabeled MCG4 tumor cells or lymphoblasts from the indicated strains were added in different amounts. A competition between a "cold" target cell and the labeled target cell is seen by a reduction in the % specific ^{51}Cr release.

DISCUSSION

The major findings of this report are the following: A chemically induced tumor from BALB/c (H-2^d) mice, MCG4, is shown to induce in BALB/c lymphocytes a strong primary CTL response. These anti-tumor CTL could be induced as in the allogeneic mixed lymphocyte reaction (17) by culturing normal lymphocytes with the tumor for 5 days. The killer cells reacted specifically with the tumor and with normal allogeneic cells expressing distinct H-2 alloantigens. A detailed analysis of the tumor cells revealed that they express H-2K and H-2D type molecules that are antigenically not of the host type H-2^d but rather of the apparently foreign haplotype H-2^k. The evidence for this and the biological implications of these findings will be the subject of this discussion.

Anti-MCG4 CTL contained different alloreactive T cell clones, mainly anti-K^k and anti-D^k reactive ones. These could be distinguished by "cold" target competition analysis using MCG4 tumor cells and Con A blasts of different H-2 types as radioactively labeled targets and as "cold" target competitor cells (Fig. 2). The expression of K^k-like and D^k-like alloantigens on MCG4 was confirmed by a typing analysis with alloreactive CTL (14). In addition, MCG4 expresses antigens that are not crossreactive with normal H-2^k or H-2^b alloantigens and that can induce CTL in H-2^d × H-2^k hybrid mice (Fig. 3). Whether these antigens represent tumor-specific antigens distinct from H-2 alloantigens is not known at present. BALB/c anti-MCG4 CTL also reacted with cells of H-2^b (Figs. 2*D* and 3*A*), H-2^s, or H-2^f haplotypes (Table 1). A recent analysis of cloned MCG4 tumor lines revealed that the original tumor cell population was heterogenous with regard to expression of H-2 antigens, which could explain the above results (unpublished data). With regard to expression of H-2^k-like antigens, the results presented could be reproduced with the MCG4-A clone 52. This cell line induced in BALB/c T cells anti-K^k and anti-D^k CTL reactivity, reacted with anti-H-2 sera directed against the private specificities of H-$2K^k$ and H-$2D^k$, and expressed K and D end H-2 molecules that could be precipitated with BALB/c anti-MCG4 isoantiserum (unpublished results). Concomitant with the appearance of apparently foreign H-2 molecules was the loss or the reduced expression of H-2^d type (self) molecules as revealed by immunochemical and cellular immunological analysis (see for instance the reaction with CBA anti-BALB/c CTL in Fig. 1; the reactivity with C57BL/6 anti-BALB/c CTL could be due to public determinants shared with H-2^k).

The alloantigens on MCG4-A appeared antigenically and functionally so similar to normal H-$2K^k$ and H-$2D^k$ antigens

Table 1. BALB/c anti-MCG4 CTL tested on BALB/c tumor cells and on allogeneic Con A blasts

Target	H-2 K	H-2 I-A	H-2 I-B	H-2 I-C	H-2 S	H-2 D	% specific cytotoxicity* Primary stimulation 50:1	Primary stimulation 12.5:1	Primary stimulation 1:1	Secondary stimulation 50:1	Secondary stimulation 12.5:1	Secondary stimulation 1:1
A. BALB/c-derived tumor cells†												
MCG4-A							67.50‡	56.39	24.18	85.65	82.59	58.38
Meth.A							0	0	0		ND	
ULMC							0	0	0		ND	
LSTRA							0	0	0		ND	
YC8							0	0	0		ND	
RLδ1							0	0	0		ND	
B. Con A blasts												
BALB/c	*d*	*d*	*d*	*d*	*d*	*d*	0.0	0.0	0.0	0.0	0.0	0.0
DBA/2	*d*	*d*	*d*	*d*	*d*	*d*	0	0	0	4	5	5
B10.D2	*d*	*d*	*d*	*d*	*d*	*d*	0	0	0	0	5	5
C3H	*k*	*k*	*k*	*k*	*k*	*k*	82	70	36	74	78	74
B10.BR	*k*	*k*	*k*	*k*	*k*	*k*	68.65	62.60	28.42	68	75	62
B10	*b*	*b*	*b*	*b*	*b*	*b*	0	0	0	40	28	5
C57BL/6	*b*	*b*	*b*	*b*	*b*	*b*	10	0	0	10	0	0
B10.S	*s*	*s*	*s*	*s*	*s*	*s*	8	2	2	52	53	15
A.SW	*s*	*s*	*s*	*s*	*s*	*s*	0	0	0	58	47	10
N.ZW	*z*	*z*	*z*	*z*	*z*	*z*	25.37	10.10	0.8	70.75	65.66	30.31
A.CA	*f*	*f*	*f*	*f*	*f*	*f*	20	8	0	35	20	5
S.WR	*q*	*q*	*q*	*q*	*q*	*q*	0	5	7	22	20	7
B10.A	*k*	*k*	*k*	*d*	*d*	*d*	49.22	30.8	0.10	78	68	23
A.J	*k*	*k*	*k*	*d*	*d*	*d*	22	8	2	64	63	23
B10.A(2R)	*k*	*k*	*k*	*d*	*d*	*b*	55	31	10	71	65	65
B10.A(4R)	*k*	*k*	*b*	*b*	*b*	*b*	55	32	14	71	74	50
C3H.OH	*d*	*d*	*d*	*d*	*d*	*k*	60.42	40.12	2.13	68	66	37
D2.GD	*d*	*d*	*d*	*b*	*b*	*b*	0.0	0.0	0.0	8	2	0
B10.A(3R)	*b*	*b*	*b*	*d*	*d*	*d*	4	0	0	56	44	14
B10.A(5R)	*b*	*b*	*b*	*d*	*d*	*d*	0.0	0.0	0.0	54	38	6
A.TH	*s*	*s*	*s*	*s*	*s*	*d*	0	0	0	31	19	4
A.TL	*s*	*k*	*k*	*k*	*k*	*d*	0	0	0	38	22	8
A.AL	*k*	*k*	*k*	*k*	*k*	*d*	45	22	8	61	62	50
B10.HTT	*s*	*s*	*s*	*k*	*k*	*d*	0	0	0	22	10	4
B10.WB	*j*					*bja*	15	10	0	25	15	3

* Percent ^{51}Cr released after 4-hr coincubation with BALB/c anti-MCG4 cytotoxic T cells; the spontaneous ^{51}Cr release from the target cells has been subtracted; ratios of effector to target cells are indicated; the numbers show mean values (four samples per group) from individual experiments; CTL were derived from spleen cells either of normal BALB/c mice (primary stimulation) or of BALB/c MCG4 regressor mice (secondary stimulation); ND, not done.

† For etiology of these lines see ref. 16.

‡ Values from two separate experiments.

that a possible mix-up with H-2^k-type cells had to be excluded. This was achieved by an isoenzyme analysis kindly performed by H. H. Krog. The tumor-derived enzymes esterase-3 (ES-3) and isocitrate dehydrogenase 1 (Id.1) showed identity in sodium dodecyl sulfate/polyacrylamide gel electrophoresis with those of normal BALB/c tissue, thus confirming the BALB/c origin of the tumor. Another important piece of information was obtained from a chromosome analysis, kindly performed by A. Radbruch, Cologne, West Germany. It revealed a normal diploid genotype with two chromosomes 17, the chromosomes that carry the genes of the *H-2* complex.

It is known from studies with F_1 hybrids that the *H-2* genes from both parental chromosomes are usually codominantly expressed on the cell surface. If this is the case also for MCG4 tumor cells, then any theory on the changes in H-2 antigen expression by these cells has to explain the disappearance of H-$2K^d$ and H-$2D^d$ molecules coded for by four genes on two identical chromosomes and the appearance of two new H-2 molecules (K and D), both of which belong to one particular foreign H-2 haplotype. The apparent genetic linkage between the new K and D molecules suggests a genetic rather than an epigenetic mechanism as the basis for the observed changes in expression of H-2 molecules. A mutation within a structural gene could result in the disappearance of certain self H-2 determinants and the appearance of new H-2 determinants, but such changes would be restricted to one gene, whereas our findings involve changes in at least four genes. We believe that a disturbance in the regulation of gene expression could more easily explain our findings and therefore favor the derepression hypothesis of *H-2* genes (7–10). We are aware, however, that the exact chemical nature and genetic basis of the H-2-like alloantigens on our tumor cells remains to be elucidated.

It is difficult to estimate how frequently tumor cells may express H-2-like alloantigens, but similar findings have been reported from chemically induced (18, 19), virus-induced (20, 21), and spontaneous tumors of AKR (22), SJL (23, 24) and other strains of mice (25). It is interesting to notice that on several occasions the tumor cells seemed to express new H-2K-like and H-2D-like molecules of the same foreign haplotype (18, 25). If the genetic information for H-2 alloantigens exists within the genome of each cell of the mouse, we have to assume that alloantigens can also become expressed occasionally on normal cells. The recent observation that H-2 alloantisera can be raised occasionally in individual mice by immunization with syn-

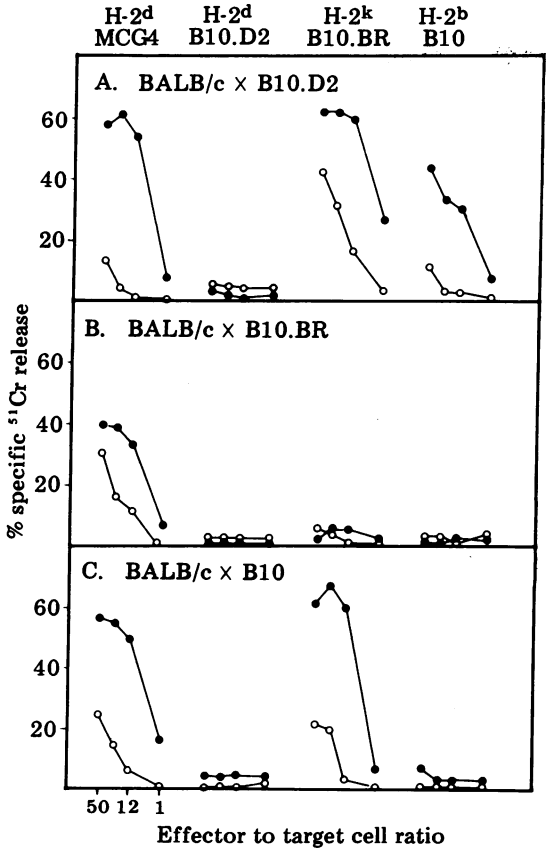

FIG. 3. Specificity of anti-MCG4 tumor cytolytic T lymphocytes derived from F_1 hybrids between BALB/c and B10.D2 (H-2^d) (*A*), BALB/c and B10.BR (H-2^k) (*B*), and BALB/c and B10 (H-2^b) (*C*) mice. Spleen cells from mice that had rejected a subcutaneous inoculum of 10^6 tumor cells were cultured for 5 days in the absence (○) or presence (●) of mitomycin C-treated MCG4 tumor cells. They were then tested for cytolytic activity against ^{51}Cr-labeled MCG4 tumor cells or Con A blasts from B10, B10.D2, or B10.BR mice.

geneic normal tissue (26) could be interpreted along these lines. We here suggest a hypothesis for the significance of alloreactive T lymphocytes: The high frequency of alloreactive T cells is the result of an exposure of the immune system during ontogeny to H-2 alloantigens coded for by multiple genes rather than alleles. Disturbance in regulation of *H-2* gene expression is suggested to occur in every individual with a low but finite frequency. Alloreactive T cells may serve to eliminate cells expressing wrong H-2 antigens in order to maintain the integrity of the body. Our hypothesis differs from Burnet's immune surveillance theory (27), which is based on somatic mutation, and also from the one recently suggested by Finberg *et al.* (3), in which exposure to self H-2 antigens modified by foreign antigens is suggested to result in the expansion of the alloreactive T cell pool. The observation that tumor cells can express immunogenic H-2-like alloantigens is highly relevant for our understanding of the genetic basis and the biological significance of major transplantation antigens.

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