T-CELL-MEDIATED CYTOTOXIC IMMUNE RESPONSES TO F9 TERATOCARCINOMA CELLS: CYTOLYTIC EFFECTOR T CELLS LYSE H-2-NEGATIVE F9 CELLS AND SYNGENEIC SPERMATOGONIA*

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Most if not all immune functions mediated by thymus-derived (T) lymphocytes appear to be associated with cell surface structures coded for by the major murine histocompatibility complex (MHC).[1] For example the central I-region of the MHC is critical for Ly 1-positive T helper cells (1–3), T cells transferring DTH reactions (4) and antibacterial immunity (5), and T cells proliferating in the MLC (6). In contrast Ly 2.3-positive cytotoxic T lymphocytes (CTL) specific for alloantigens (6–9), virus antigens (10–11), cell associated non-MHC-histocompatibility antigens (12–14), and haptens (15, 16) are functionally associated with the peripheral *H-2K* or *H-2D* region of the MHC. In the case of CTL it has been argued that their lytic function can take place only towards H-2-positive targets. This argument is derived from results showing that both hapten and virus-specific H-2 restricted CTL are unable to lyse virus infected or hapten conjugated H-2-negative targets (17–18).

If T cells must recognize *H-2* gene products in addition to foreign antigens on cell surfaces to become sensitized or to mediate lytic effector functions, it should not be possible to induce CTL with specificity for H-2-negative target cells. In the experiments reported here this question has been tested using the H-2-negative embryonal carcinoma cell line F9 (19) as stimulator cells.

Materials and Methods

Mice. CBA (H-2^k), BALB/c (H-2^d), and C57BL/6 (H-2^b) mice were obtained from G.I. Bomholtgaard, Ry, Denmark, and 129/Sv (H-2^{bc}) mice from The Jackson Laboratory, Bar Harbor, Maine.

Teratocarcinoma Cell Lines. The characteristics of the embryonal carcinoma cell lines used have been published (19, 20). Some features of the cells are summarized in Table I. The F9 cells

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[1] *Abbreviations used in this paper:* C, complement; Con A, concanavalin A; CTL, cytotoxic T lymphocytes; DMEM, Dulbecco's modified Eagle's medium; DTH, delayed type hypersensitivity; EC, embryo carcinoma; EID_{50}, embryo infectivity dose; LPS, lipopolysaccharide; MCC, mixed cell cultures; ME, mercaptoethanol; MHC, major murine histocompatibility complex; MLC, mixed lymphocyte culture; PBS, phosphate-buffered saline; R α-MIg, rabbit anti-mouse Ig; TNP, trinitrophenol.

TABLE I
Published Characteristics of the Teratocarcinoma Cell Lines Used

Cell types (all originated from 129 [H-2^{bc}] mice)	Cell line designation	In vitro differentiation	Detectable antigens	
			H-2	F9
EC*	F9	0	−	+
EC	PCC3	++	−‡	+§
EC	PCC4	±	−‡	+§
Parietal yolk sac	PYS-2	0	Not tested	−

* The data are taken from Jacob (20).
‡ Appears under conditions of differentiation.
§ Disappears under conditions of differentiation.

were cultivated in tissue culture dishes coated with gelatin in Dulbecco's modified Eagle's medium containing 15% fetal calf serum in an atmosphere of 10% CO_2. The PCC3, PCC4, and PYS cell lines do not require gelatine. By subcultivating F9, PCC3, and PCC4 cells each 3rd day care was given that the cells did not grow to confluencing monolayers. From a frozen stock of cells kept in liquid nitrogen each 6th wk fresh cultures of F9 cells were started. Using alloimmune CTL generated in a primary mixed lymphocyte culture (see below) to test for the presence of H-2 antigens on the tumor cell lines used, results as given in Table II were obtained. Accordingly both the F9 cells and PYS cells were not lysed by H-2^k anti-H-2^{bc} immune CTL. However sometimes the PCC3 and almost regularly the PCC4 cells were lysed significantly, suggesting that some of the cells were H-2^{bc}-positive, under the conditions used.

Cell Cultures. Alloantigen reactive CTL were induced in mixed lymphocyte cultures (MLC) as described (21). In short, spleen cells of the various mouse strains were used as source of responding and stimulating cells. Before culture the stimulating cells received a dose of 2,000 rads (Philips machine RT 200, Philips Electronic Instruments, Inc., Mount Vernon, N. Y.) at a dose rate of 620 rads/min. The cells were cultured in multi dish culture trays (Linbro FB-24Tc, Linbro Chemicals, New Haven, Conn.) using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 mM Hepes, 5×10^{-5} M mercaptoethanol (ME), and 5% fetal calf serum. Splenic responder cells (4×10^6) and stimulator cells (1.5×10^6) were cultured in a vol of 2 ml in a humidified atmosphere of 10% CO_2 for 5 days. Thereafter the cells were collected, washed, resuspended to 1×10^6 viable cells/ml (as determined by eosin dye exclusion) and tested for cytotoxic activity.

Anti-F9 cell immune cytotoxic effector cells were induced in mixed cell cultures (MCC). If not stated differently responder spleen cells (4×10^6) were cultured with 0.25×10^6 X-irradiated (5,000 rads) F9 cells in multi dish culture trays under the conditions described above. After 5 days the cells were harvested, their viability was determined, and subsequently tested for cytotoxic activity.

Target Cells. In initial experiments we used as H-2-positive target cells the P815 (H-2^d) and EL4 (H-2^b) tumor cell lines. Because irregular results were obtained, we subsequently used only lipopolysaccharide (LPS) or concanavalin A (Con A) transformed lymphoblasts as H-2-positive targets. Lymphoblasts were obtained by pooling replicate cultures containing 4×10^6 spleen cells which had been cultured for 72 h in DMEM (without ME) containing either 5 μg/ml Con A (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) or 5 μg/ml LPS (Difco Laboratories, Detroit, Mich.). The F9, PCC3, PCC4, or PYS-2 cells were harvested using 1 mM EDTA in phosphate-buffered saline (PBS) (pH 7.3) free of Mg^{++} and Ca^{++}. The spermatogonia were prepared as described below. Usually $1\text{–}2 \times 10^6$ cells were labeled with 150 μCi ^{51}Cr (Radio Chemical Centre, Amersham, England), as described (21).

Cytotoxicity Assay. Various numbers of viable MLC- or MCC cells were incubated with a constant number (2×10^4) ^{51}Cr-labeled target cells. Percent specific ^{51}Cr-release was calculated according to the formula

TABLE II
Test for H-2^{bc} Alloantigens on F9, PCC3, PCC4, and PYS Cells

CTL	Specific lysis of ^{51}Cr-labeled target cells*					
	F9	PCC3	PCC4	PYS	H-2^{bc} blasts	H-2^{d} blasts
			%			
H-2^{k} anti-H-2^{bc}‡	−2	17	29	1	62	14
H-2^{k} anti-H-2^{d}‡	1	3	−1	0	22	75

* Ratio attacker to target cells of 20:1, background lysis of the targets during the 4 h cytotoxicity assay was: F9 = 18%, PCC3 = 24%, PCC4 = 4%, LPS induced H-2^{bc} blasts = 27%, H-2^{d} blasts = 22%.

‡ Alloimmune CTL were induced in a primary MLC.

% specific lysis = ^{51}Cr-release by immune cells
− ^{51}Cr-release by responder cells cultured without stimulator cells/maximal ^{51}Cr-release
− ^{51}Cr-release by responder cells cultured without stimulator cells × 100.

For each lymphocyte population tested a dose-response curve was established. For simplicity reasons the data are given without SD, since in the ^{51}Cr-assays performed the SD of percent lysis was less than 5%.

Antisera. Antiserum to theta alloantigen of C3H mice (anti-Thy 1.2) was raised as reported (22) and used in the complement (C)-dependent cytotoxicity assay (treatment with anti-Thy 1.2 serum and C) as described (22). The mouse 129 anti-F9 cell hyperimmune antiserum used was a gift of Dr. Rolf Kemler, Institut Pasteur, Paris. Its cytotoxic titer to F9 cells was 1:2,400, its properties have been described (23). The H-2^{k} anti-H-2^{b} antiserum was raised by repeated intraperitoneal injections of C57BL/6 spleen cells into CBA mice. Its cytotoxic titer was 1 to 128.

Inhibition Experiments. The "cold inhibition" assays were performed according to Herberman et al. (24), the only modification being that the assay was performed in tubes instead of plastic dishes. As inhibitor cells LPS induced blast cells were used. In the antibody inhibition experiments the target cells (2×10^4 in 100 μl medium) were first incubated for 10 min at 37°C with 100 μl of antiserum diluted with normal culture medium. Thereafter the effector cells (suspended in 200 μl medium) were added. The cell mixture was centrifuged for 1 min at 200 *g* and incubated for the assay time required at 37°C in 10% CO_2 and processed as described (21).

B-Cell Removal Procedure. Ig-positive cells within splenic lymphocytes were killed acording to L. Herzenberg (personal communication) by treatment of the cells with rabbit anti-mouse Ig serum (Behring-Werke AG, Marburg Lahn W.-Germany) diluted 1:4 with PBS containing 0.2% Na-azide. The cells were washed and incubated with agarose absorbed guinea pig serum (22) containing 0.2% Na-azide to inhibit capping. Dead cells were removed according to the method described by Von Boehmer and Shortman (25). After this regimen only 2–6% of the viable cells were Ig positive as detected using direct immunofluorescence techniques. Viable cells were used as responder cells in MCC.

Induction of Trinitrophenol (TNP)-Specific CTL. The method described by Shearer (26) has been used. In short, X-irradiated (3,000 rads) splenic lymphocytes (10^7) were incubated for 10 min at 37°C in PBS containing 10 mM trinitrobenzol sulfonic acid (TNBS) (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y.) 1×10^6 TNP modified mouse 129 cells (stimulator cells) were cocultured over 5 days together with 4×10^6 mouse 129 derived splenic responder cells. Cytotoxic activity was tested towards LPS induced TNP-conjugated mouse 129 derived LPS induced splenic blast cells or towards TNP-conjugated F9 cells.

Induction of Parainfluenza (Sendai) Virus-Specific CTL. Sendai virus-specific CTL were obtained by injecting 10^4 embryo infectivity dose (EID_{50}) of Sendai virus (kindly provided by Dr. Pünter, Farbwerke Hoechst AG, Frankfurt, W.-Germany) into the hind footpad of mouse 129 mice. After 5 days the draining lymph node cells were prepared and the cells were cultured for additional 72 h in vitro (27, 28). In the ^{51}Cr-cytotoxicity, the target cells used were either syngeneic macrophages obtained as peritoneal excudate cells (28) or F9 cells. The target cells were infected with Sendai virus (at a multiplicity of 10 EID_{50}/cells for 2 h) and simultaneously labeled with ^{51}Cr-chromate.

Preparation of Spermatogonia. The gonads of 4- to 6-day-old CBA and 129 mice were dissected, the capsules were cut with a small scissor, and resuspended in 5 ml of PBS containing 0.1% trypsin. The organ suspension was incubated at 37°C and repeatedly forced through Pasteur pipettes to disrupt mechanically the organs. After 10–15 min at 37°C the supernate containing the nonsedimenting cell content was taken, centrifuged, and the resulting cell pellet was washed twice. Providing the pipetting had been performed gently, about 80–90% of the spermatogonia were viable according to eosin dye exclusion criteria. About 1–2 × 10^6 spermatogonia were labeled with 150 μCi ^{51}Cr in a total vol of 1 ml. The background release of ^{51}Cr labeled spermatogonia containing about 80% viable cells was in the order of 30–40% during a 5 h incubation period.

In Vivo Sensitization of Mice to F9 Cells. About 2–5 × 10^6 viable F9 cells were injected s.c. In the majority of mice a palpable tumor developed which sometimes did regress. However the transplantation characteristics have not been studied in detail. The mice were used experimentally 4–6 wk after injection with F9 cells.

Results

Induction of Anti-F9 Cell Immune CTL. In agreement with published work (17, 18) we noted that mouse 129 derived TNP-specific or Sendai virus-specific H-2 restricted CTL were unable to lyse TNP conjugated or Sendai virus infected H-2-negative F9 target cells (Table III). Since however F9 cells are lysed when linked via lectins to CTL sensitized against any H-2 haplotype (29, 30), the negative results might be explained with the antigen specificity of antigen primed H-2 restricted CTL. Accordingly the inability to lyse H-2-negative targets would not be a matter of resistance to lysis, but would merely reflect a need for H-2 to trigger the recognition phase of antigen-specific CTL. Consequently we tested the possibility to induce anti-F9 specific CTL. CBA mice (H-2^k) were immunized in vivo against F9 cells. 4 wk after priming the spleen cells were used as responder cells and cocultivated in a MCC together with irradiated F9 stimulator cells. After 5 days of culture the responder cells were tested for cytotoxicity against ^{51}Cr labeled F9 cells. Representative results obtained are incorporated in Table III and clearly suggest that cytotoxic activity was detectable against F9 targets, irrespective whether they were TNP conjugated or Sendai virus infected. It should be stressed that the F9 target cells lysed by anti-F9 immune CTL were not affected by anti-H-2^{bc} reactive CTL thus confirming that the F9 target cells used were H-2 negative.

Next we established the parameters required for the induction of anti-F9 cytotoxic effector cells. First the ratios of responder cells and X-irradiated F9 stimulator cells were defined which were required to induce optimal cytotoxic anti-F9 responses. Using both normal and in vivo primed splenic CBA responder cells the highest cytotoxic activities were induced at a ratio of about 20 to 1 (Fig. 1). At this ratio of responder cells to stimulator cells a 4–5 day culture period of in vivo primed responder cells yielded higher cytotoxic anti-F9 responses compared to normal responder cells (Fig. 2). That the cytotoxic activity observed was mediated by T lymphocytes was suggested by two observations. First in vivo primed splenic lymphocytes enriched for T cells by treatment with R α-MIg and complement in the presence of Na-azide, and subsequently cultured in vitro together with F9 stimulator cells were lytic for F9 target cells (Table IV). Second, treatment of anti-F9 immune cytotoxic effector cells with anti-Thy 1.2 serum plus complement abrogated their cytotoxic

TABLE III
Lytic Activity of Anti-Sendai Virus, Anti-TNP, and Anti-F9 Cell Immune Effector Cells

Effector cells	Specific lysis of targets*					
	F9	F9-TNP	F9 Sendai	H-2^{bc} Blasts	H-2^{bc} Blasts-TNP	H-2^{bc} Macrophages Sendai virus infected
	%					
H-2^{bc} Anti-TNP immune CTL‡	0	2	ND	6	34	ND
H-2^{bc} Anti-Sendai virus immune CTL‡	−1	ND	3	5	ND	27
H-2^{k} Anti-F9 cell sensitized effector cells	39	31	34	1	ND	ND
H-2^{k} Anti-H-2^{bc} immune CTL‡	2	0	3	67	ND	ND

ND = not done.
* Ratio effector to target cells of 50:1; 6 h cytotoxicity assay; background lysis of the target cells was less than 34%.
‡ For details see Materials and Methods.

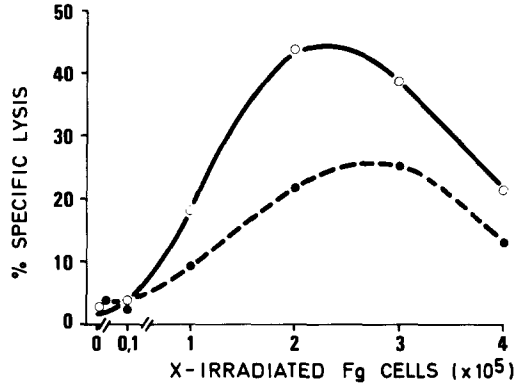

FIG. 1. Titration of the ratio responder to stimulator cells. Spleen cells (4 × 10^6) of CBA primed in vivo to F9 cells (○—○—○) or spleen cells (4 × 10^6) of unprimed CBA mice (●- -●- -●) were cultured with a graded number of X-irradiated (4,000 rads) F9 cells for 5 days. The cells were harvested and assayed at a ratio of 50:1 for cytotoxicity against ^{51}Cr-labeled F9 cells. Background lysis of the F9 cells during the 5 h cytotoxicity assay was 24%.

activity (Table IV). Representative results of a comparison of the in vitro responsiveness of T cells derived from various mouse strains are summarized in Table V. Secondary in vitro cytotoxic anti-F9 responses could be induced using CBA (H-2^k), BALB/c (H-2^d), C57BL/6 (H-2^b), and syngeneic mouse 129 (H-2^{bc}) derived T responder cells. In contrast so far we succeeded only with CBA mouse derived T responder cells to induce primary cytotoxic anti-F9 responses (Table V). At present the reason for these differences is unknown.

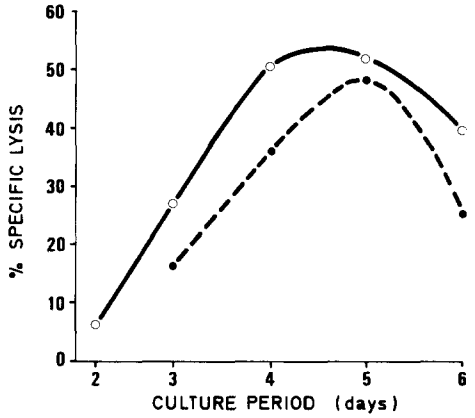

FIG. 2. Kinetics of the generation of anti-F9 cytotoxic effector cells. Spleen cells (4×10^6) of CBA mice primed in vivo with F9 cells (○—○—○) or spleen cells (4×10^6) of normal CBA mice (●—●—●) were cultured together with 0.25×10^6 X-irradiated F9 cells. At daily intervals cells were harvested and tested for cytotoxicity to ^{51}Cr-labeled F9 target cells at a ratio of 50:1. Background lysis of the target cells during the 5 h cytotoxicity assay was less than 32%.

TABLE IV
Effect of Anti-Thy 1.2 Serum Plus Complement upon Anti-F9 Cytotoxic Effector Cells

Treatment of anti-F9 effector cells generated in vitro using purified T cells as responder cells*	Specific lysis of F9 targets	
	100:1‡	10:1
	%	
No treatment	34	17
Complement	38	12
Anti-Thy 1.2 serum	36	19
Anti-Thy 1.2 serum plus complement	4	−1

* Splenic lymphocytes of CBA mice primed in vivo with F9 cells were treated with R α-Mlg antiserum plus complement in the presence of Na-azide to kill Ig-positive lymphocytes. After dead cell removal the viable cells (4×10^6 responder cells) were cultured together with 0.25×10^6 X-irradiated F9 stimulator cells and cultured for 5 days.

‡ Ratio effector cells to target cells. Background lysis of the target cells during the 5 h cytotoxicity assay was less than 16%.

Specificity of Anti-F9 Immune CTL. To explore the antigen specificity of anti-F9 immune CTL two approaches have been used. First the lytic activity of anti-F9 immune CTL was tested towards various target cells with known cell surface antigen characteristics (see Tables I and II). As can be seen in Table VI anti-F9 immune CTL were cytotoxic for F9, PCC3, and PCC4 target cells, yet did not lyse syngeneic mouse 129 targets (H-2 positive) nor the mouse 129 parietal yolk sac-like PYS-2 target cells (F9 negative). It was interesting to note that the PYS-2 target cells were not lysed by anti-H-2^{bc} CTL, suggesting that they may be devoid of H-2 antigens. However in further experiments it

TABLE V
In Vitro Cytotoxic Anti-F9 Responses of Cells Derived from Different Mouse Strains

In vitro stimulation*	Specific lysis of F9 cells 70:1§	7:1
	%	
CBA_p‡ → F9	75	19
CBA → F9	42	12
$BALB/c_p$ → F9	27	4
BALB/c → F9	3	0
$C57BL/6_p$ → F9	24	6
C57BL/6 → F9	2	−1
129_p → F9	19	4
129 → F9	0	0

* Splenic responder cells (4×10^6) were cultured together with 0.25×10^6 X-irradiated F9 stimulator cells for 5 days. Examples of results obtained in independent experiments are given.

‡ p refers to splenic lymphocytes derived from mice primed in vivo to F9 cells.

§ Ratio attacker cells to target cells; background lysis of F9 cells was less than 28%; assay time was 5 h.

TABLE VI
Specificity of Anti-F9 Immune CTL

In vitro stimulation*	Specific lysis of target cells‡ F9	PCC3	PCC4	H-2^{bc} blasts	PYS	H-2^d blasts
			%			
CBA_p anti-F9§	60	32	29	2	−1	0
CBA anti-F9	36	20	ND	4	2	3
CBA anti-H-2^{bc}	3	8	19	57	−2	12

* Splenic responder cells (4×10^6) were cultured together with 0.25×10^6 X-irradiated F9 stimulator cells for 5 days.

‡ The data refer to a ratio of attacker to target cells of 50:1. Background lysis of the target cells during the 5 h cytotoxicity assay was F9 = 24%, PCC3 = 14%, PCC4 = 27%, H-2^{bc} blasts = 32%, PYS = 11%, H-2^d blasts = 35%.

§ Splenic CBA cells were derived from CBA mice injected s.c. 3 wk earlier with 2×10^6 viable F9 cells.

has to be firmly established, that the PYS cells are not resistant to CTL-mediated cytolysis.

To further analyze the antigen specificity of anti-F9 immune CTL, in a second approach inhibition experiments were performed using either hyperimmune mouse 129 anti-F9 antiserum or unlabeled (cold) inhibitor cells. As shown in Table VII, H-2^{bc} anti-F9 antiserum blocked the lytic actions of H-2^k anti-F9 immune CTL to F9 target cells, yet did not effectively interfere with the lytic activity of alloimmune anti-H-2^{bc} reactive CTL. The observed blocking effect of mouse 129 anti-F9 antisera ruled out antibody-dependent cell cytotoxicity as explanation for the anti-F9 cytotoxicity observed, and suggested that the antiserum used (which contains anti-F9 antigen antibodies [23]) blocked

TABLE VII

Inhibition of Anti-F9 Cell Cytotoxicity in the Presence of Mouse 129 Anti-F9 Antiserum

Effector cells*	Antisera present		Specific lysis			
	Mouse 129 Anti-F9 cell antiserum	H-2^k Anti-H-2^b Antiserum	F9 cells		$H\text{-}2^{bc}$ blasts	
			70:1‡	7:1	50:1	5:1
			%			
H-2^k Anti-F9 cell immune CTL	–	–	47	12	3	0
	+ (1:20)§	–	19	2	ND	
	+ (1:120)§	–	38	9	ND	
	–	+ (1:20)§	44	13	ND	
H-2^k Anti-H-2^{bc} immune CTL	–	–	6	2	67	36
	–	+ (1:20)§	ND		12	3
	+ (1:20)§	–	ND		59	22

ND = not done.

* H-2^k anti-F9 cell immune CTL were induced by culturing in vivo sensitized CBA spleen cells together with X-irradiated F9 cells for 5 days. H-2^k anti H-2^{bc} CTL were induced in a primary MLC.

‡ Ratio attacker cells to target cells; background lysis of targets was less than 37% during the 5 h assay time.

§ Final dilution of anti-antiserum.

via competitive inhibition the lytic activity of anti-F9 immune CTL to F9 targets. A corollary to these findings was obtained using unlabeled (cold) F9 cells to block the cytolytic activity of H-2^k anti-F9 CTL to F9 targets. Cold F9 cells did not inhibit the lytic activity of anti H-2^{bc} alloreactive CTL yet blocked effectively the cytotoxic activity of anti-F9 immune CTL (Table VIII). On the other hand H-2^{bc} blasts, when used as inhibitor cells, did not alter the lytic reactivity to F9 targets, but to that towards H-2^{bc} target cells. Taken together these results further supported the conclusion that H-2 antigens are not involved during the lytic effector phase of anti-F9 cell immune CTL.

Cytolytic activity of H-2^k anti-F9 immune CTL to syngeneic spermatogonia: It has been reported that in adult C_3H and 129 mice the F9 antigen is expressed on spermatozoa but not on any of the somatic cells examined (20). In fact there is evidence that, in contrast to somatic cells, the whole male germ line from primordial germ cells up to spermatozoa expresses the F9 antigen (20). Since H-2^k anti-F9 cell immune CTL were cytotoxic for all F9 antigen-positive targets tested (F9, PCC3, PCC4 cells), and since mouse 129 anti-F9 antibodies blocked their cytolytic activity, the possibility existed that anti-F9 cell immune CTL recognized the F9 antigen on F9 cells as target antigen. To further substantiate this conclusion we tested whether H-2^k anti-F9 cell immune CTL would lyse ^{51}Cr-labeled spermatogonia prepared from 4- to 6-day-old CBA (H-2^k) and 129 (H-2^{bc}) mice. As can be seen in Table IX, H-2^k anti-F9 cell immune CTL lysed not only F9 cells, but also spermatogonia derived from young CBA and 129 mice. On the other hand H-2^d anti-H-2^k CTL did not lyse CBA mouse derived spermatogonia suggesting as already described by others (31) that the latter cells are H-2 negative.

TABLE VIII
Inhibition of Anti-F9 Cell Cytotoxicity in the Presence of Unlabeled F9 Cells

Effector cells	Unlabeled inhibitor cells ($\times 10^4$)		Specific lysis			
	F9 cells	H-2^{bc} Blasts	F9 cells 50:1*	F9 cells 5:1	H-2^{bc} Blasts 50:1	H-2^{bc} Blasts 5:1
			%			
H-2^k Anti-F9‡ cell immune CTL	–	–	32	9	0	−1
	20	–	3	0	ND	
	5	–	16	3	ND	
	–	20	27	8	ND	
	–	5	31	10	ND	
H-2^k Anti-H-2^{bc}‡ immune CTL	–	–	4	1	87	42
	20	–	ND		82	39
	–	20	ND		37	12

ND = not done.
* Ratio attacker to target cells; 2×10^4 target cells; background lysis of the targets during the 5 h assay was less than 33%.
‡ H-2^k anti-F9 cell immune CTL were induced by culturing in vivo sensitized CBA mouse derived spleen cells together with X-irradiated F9 cells for 5 days. H-2^k anti-H-2^{bc} CTL were induced in a primary MLC.

TABLE IX
Cytolytic Activity of CBA Anti-F9 Immune CTL to CBA Mouse and 129 Mouse-Derived Spermatogonia

Effector cells	Specific lysis F9		Spermatogonia (CBA)		Spermatogonia (129)		H-2^k blasts	
	50:1*	5:1	50:1	5:1	50:1	5:1	50:1	5:1
				%				
CBA Anti-F9 cell‡ immune CTL	68	22	44	6	11	1	2	0
BALB/c Anti-CBA‡ immune CTL	ND		3	1	ND		52	25

ND = not done.
* Ratio effector cells to target cells; background lysis during the 5 h cytotoxicity assay was: F9 = 16%; CBA spermatogonia = 32%; 129 spermatogonia = 42%; H-2^k blast cells = 24%.
‡ In vivo anti-F9 sensitized CBA mouse spleen cells were cultured for 5 days together with X-irradiated F9 cells. BALB/c anti-CBA immune CTL were induced in a primary MLC.

Discussion

The new aspect of the results reported appears to be the finding that CTL precursors can be sensitized towards H-2-negative stimulator cells resulting in CTL, which in turn can recognize and lyse H-2-negative target cells. Thus H-2 associated CTL (8–16) and anti-F9 immune CTL differ with respect to the H-2 requirement for both the induction and expression of their cytolytic effector functions; the effector activity of anti-F9 immune CTL being H-2 independent in contrast to the functional activity of H-2K/D (9–14) or H-2I (21, 32, 33)

region restricted CTL. The finding that H-2^k anti-F9 immune CTL lyse syngeneic spermatogonia may indicate that the effector T cells are specifically sensitized towards the F9 antigen (19, 20), known to be linked with the T/t locus (20, 23).

As mentioned above so far most, if not all, immune functions of murine T cells are considered to be associated with *H-2* gene products (1–10, 34). One possibility to explain the observed *H-2* restriction of antigen primed T cells is to assume, that sensitization of T cells towards *H-2*-positive "stimulator cells" (which may be immunodominant) mainly results in the activation of clonally restricted, *H-2* associated T precursor cells. This argument implies that if non-*H-2* restricted, antigen-specific CTL precursors are present in a given T-cell population, their detection may be facilitated under conditions, where responder T cells are sensitized to *H-2*-negative stimulator cells. To test this prediction we used as stimulator cells embryonal carcinoma (EC) cells derived from mouse strain 129 (19). The nullipotential F9 cell line is known to be H-2 negative and F9 antigen positive (19, 20). Similar cell surface characteristics have been described for the multipotential EC cells PCC3 or PCC4, provided these cells had been grown under conditions preventing differentiation (20). In contrast the parietal yolk sac -like cell line PYS (35) is known to be devoid of F9 antigens (20).

The data obtained strongly suggest that in vivo priming of mice with H-2-negative F9 cells followed by in vitro restimulation with F9 cells triggers the generation of anti-F9 immune CTL. Using this regimen anti-F9 immune CTL could be triggered in T cells derived from CBA (H-2^k), C57BL/6 (H-2^b), BALB/c (H-2^d), and 129 (H-2^{bc}) mouse stains. In contrast only CBA mouse derived T cells generated in a primary mixed cell culture cytotoxic anti-F9 cell responses. Since CBA responder T cells generated strong cytotoxic anti-F9 cell activity, such cells were used to study the antigen specificity of the effector cells generated. H-2^k anti-F9 cell immune CTL lysed efficiently F9 cells, and to a lesser extent PCC3 and PCC4 cells. The latter cells proved to be possibly H-2 positive under the conditions used (Table VI). However anti-F9 immune CTL were in no case cytotoxic for F9 antigen-negative PYS target cells, nor for LPS induced H-2^{bc} blast cells. Moreover mouse 129 anti-F9 antisera effectively blocked the cytotoxic activity of anti-F9 cell immune CTL against F9 targets. Finally, unlabeled F9 cells, but not H-2^{bc} blast cells inhibited the cytotoxic reaction against F9 target cells (Tables VI and VII). Taken together these results strongly support the conclusion that the CTL generated recognize cell surface antigens present on H-2-negative F9 cells (as well as on PCC3 and PCC4 cells). The data also imply that in principle the lethal effect of CTL upon target cells can take place in the absence of H-2 antigens. Thus cytotoxic anti-F9 cell responses appear to represent a model situation, in course of which CTL precursors are sensitized against H-2-negative cells, resulting in cytotoxic effector T cells capable to recognize and to lyse H-2-negative target cells. In regard to the two basic models for T-cell recognition, dual recognition or altered self (32, 36–38) these results imply, that the lytic activity of anti-F9 cell immune CTL takes place in the absence of H-2 self markers.

Results showing T-cell-mediated-specific cytolysis of H-2-negative target cells

have recently been obtained in other laboratories with rat (39) and mouse (S. R. Burakoff, personal communication) effector cells. If one argues that anti-F9 cytotoxic responses take place because the F9 cell antigen is processed by syngeneic macrophages during the sensitization phase, one has to explain why the CTL generated are cytotoxic for H-2-negative targets (lack of H-2 restriction). This argument also contradicts the remote possibility that we are observing CTL responses specific for FCS constituents (14). Such responses are *H-2* restricted (14). Alternatively it is conceivable that F9 cells do express a functional analogue to *H-2* gene products, which in turn would enable the sensitization of CTL in the absence of H-2 antigens. This in turn raises the question whether it is possible to induce F9 cell restricted virus or hapten-specific CTL. What then could be the nature of the target antigen recognized on F9 cells? First, out of the limited target cell panel tested only cells were lysed known to be F9 antigen positive. Second, mouse 129 anti-F9 cell antiserum, known to contain antibodies specific for F9 antigens (23), blocked the cytotoxic anti-F9 cell reactivity. Third, spermatogonia known to express F9 antigens (20), were lysed by H-2^k anti-F9 cell immune CTL. In fact, the latter system can be considered as an in vitro analogue of a T-cell-mediated cytotoxic autoimmune orchitis (40). Taken together these findings suggest, but do not prove, that anti-F9 cell immune CTL recognize the F9 antigen as target antigen. Since the F9 antigen is genetically linked to the T/t locus (19, 20, 23), the possibility has to be considered that we are observing CTL responses towards gene products coded for by the T/t locus. However recognition of EC antigens other than the F9 antigen has not yet been firmly excluded.

Summary

Murine thymus derived (T) lymphocytes primed in vivo to mouse 129 (H-2^{bc}) derived *H-2*-negative F9 embryonal carcinoma cells and rechallenged in vitro with X-irradiated F9 stimulator cells differentiated into anti-F9 cell immune cytotoxic T lymphocytes (CTL). Using CBA mouse derived splenic responder T cells, F9 stimulator cells triggered a primary cytotoxic anti-F9 response. The CTL generated lysed the F9 antigen-positive target cells F9, PCC3, and PCC4, but not the F9 antigen-negative mouse 129 derived PYS tumor cells, nor LPS induced H-2^{bc} blast cells. Mouse 129 anti-F9 cell antisera but not H-2^k anti-H-2^{bc} antisera blocked the lytic interaction with F9 target cells. Similarily unlabeled F9 cells but not H-2^{bc} blast cells inhibited the anti-F9 cell cytotoxicity. H-2^k anti-F9 cell immune CTL were found to be cytotoxic for syngeneic spermatogonia, known to express the F9 antigen.

The results suggest not only that CTL can recognize and lyse H-2-negative target cells, but also that CTL precursors can be sensitized against H-2-negative stimulator cells. From the data available it may be inferred that anti-F9 cell immune CTL recognize the F9 antigen, known to be linked with the T/t locus. Since anti-F9 cell immune CTL lyse syngeneic spermatogonia, the system may be useful to analyze in vitro the induction and effector phase of a T-cell-mediated cytotoxic autoimmune orchitis.

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