INDUCTION OF CYTOTOXIC T LYMPHOCYTES AGAINST *I*-REGION-CODED DETERMINANTS: IN VITRO EVIDENCE FOR A THIRD HISTOCOMPATIBILITY LOCUS IN THE MOUSE*

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Murine T lymphocytes (responder cells) when cocultivated in the mixed lymphocyte culture (MLC)[1] together with *H-2* incompatible lymphocytes (stimulator cells) undergo a phase of cell proliferation which ultimately culminates in the generation of cytotoxic T lymphocytes (CTL) (1–4). Recent results obtained with inbred strains carrying intra *H-2*-recombinant chromosomes suggest that the cytotoxic activity of the CTL generated is directed against products coded by the *H-2K* or *H-2D* region of the *H-2* complex (i.e. serologically defined [SD] *H-2* private specificities, or products closely linked to them) (5–7). In contrast antigens coded by the *I* region of the *H-2* complex effectively induce proliferative responses in the MLC, yet appear not to be recognized as target by the CTL generated (6–12).

The view of functional differences between peripheral (*H-2D* and *H-2K*) regions and the central (*I*) region of the *H-2* complex has recently been challenged by the observation that *I*-region-different mice carrying no known histocompatibility differences nevertheless reject each others' skin graft (13–15). Moreover cytotoxic lymphocytes were detected in the spleen of mice immunized with skin grafts from *I*-region-incompatible mice (16). These paradoxical findings prompted us to reinvestigate the genetic requirements for the in vitro induction of CTL. We now report that products coded by the *I* region of the *H-2* complex are capable to trigger in vitro the generation of CTL.

Materials and Methods

Animals. All mice were used at an age of between 6 to 10 wk. C57BL/10Sn(B10), B10.D2/nSn, B10.BR/SgSn, B10.A/SgSn, B10.A(2R)/SgSn, B10.AKM/Sn, DBA/2J, DBA/1J, A/J, and A.SW/sn were purchased from Jackson Laboratories, Bar Harbor, Maine. A.TL, A.TH, B10.HTT, and B10.A(4R) mice were originally provided by Doctors D. C. Shreffler and C. S. David, University of Michigan, Ann Arbor, Michigan, and now maintained in our colony. The former was obtained from Dr. Holmes, Melbourne, Australia, the latter strain was kindly provided by Dr. J. Klein, University of Texas, Southwestern Medical School, Dallas, Texas, when it was in the fifth backcross generation to B10. In this study F4 of the N5 homozygous backcross were used. All hybrids used in this study were produced in our laboratory. The genetics of the strains regarding

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; CTL, cytotoxic T lymphocytes; DMEM, Dulbecco's modified Eagle's medium; LPS, lipopolysaccharide; ME, mercaptoethanol; MLC, mixed lymphocyte culture; PHA, phytohemagglutinin.

their *H-2* haplotype are indicated in terms of the allelic origin of the *K, I-A, I-B, I-C, S,* and *D* regions, respectively (Table I).

Antisera. Antiserum to theta alloantigen of C3H mice (anti-Thy 1.2) was raised as reported (17) and used in the complement (C)-dependent cytotoxicity assay (treatment with anti-Thy 1.2 serum and C) as described (17).

Separation by Velocity Sedimentation at 1 g. The technique of Miller and Phillips (18) in the modification of McDonald et al. (19) was used. Mitogen-stimulated cells out of which the blast cells had to be separated, were harvested from the culture, applied (150×10^6 cells) to the gradient consisting of 7–30% horse serum in phosphate-buffered saline, and allowed to sediment for 4 h at 4°C. Blast cells sedimenting faster than 4.5 mm/h (20) were pooled and used as target cells.

In Vivo Immunization. Mice were immunized by skin grafting (sex identical animals) followed 1 wk later by intraperitoneal injection of 15×10^6 splenic lymphocytes of skin donor origin. The skin-grafting technique employed was described by Klein and Bailey (21). 4 wk after grafting the spleen cells of the mice were used as responder cells and re-exposed in a "secondary" MLC to the same allogeneic stimulus.

Mixed Lymphocyte Culture. Spleen cells of the various strains were used as source of responding and stimulating cells. Before culture, the stimulating cells received a dose of 3,000 rads (Philips machine RT 200) at a dose rate of 620 rads/min. The cells were prepared as described (22) and 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, 2×10^{-5} M mercaptoethanol (ME), and 5% fetal calf serum (20). Splenic responder cells (4×10^6) and stimulator cells (1.5×10^6) were cultured in a vol of 2 ml (20) in a humified atmosphere of 10% CO_2 in air for the desired period of time. At the end of the incubation period, the cells were collected, washed, resuspended to 5×10^5 viable cells/ml (as determined by eosin dye exclusion), and tested for cytotoxic activity.

Target Cells. Phytohemagglutinin (PHA)-, lipopolysaccharide (LPS)-, and concanavalin A (Con A)-transformed lymphoblasts were used as target cells. In selected experiments only target cells purified according to their sedimentation rate at 1 *g* (see above) were used. Lymphoblasts were obtained by pooling replicate cultures containing 5×10^6 spleen cells which had been cultured for 72 h in DMEM (without ME) containing either PHA-M(no. R 15-0576, Grand Island Biological Co., Grand Island, N. Y.) at a final dilution of 1:500, or 5 μg/ml LPS (Difco Laboratories, Detroit, Mich.), or 5 μg/ml Con A (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). Usually $3–5 \times 10^6$ blast cells were labeled with 150 μCi ^{51}Cr (Radio Chemical Centre Amersham, Buchler, Frankfurt, Germany) as described (22).

Cytotoxicity Assay. Various numbers of viable MLC cells were incubated with a constant number (2×10^4) ^{51}Cr-labeled target cells. Percent specific ^{51}Cr release was calculated as described (23). For each lymphocyte population tested a dose response curve was established. The SD of triplicate assays was calculated. The data are given without SD since in all experiments listed the SD of percent lysis was less than 5%.

Results

Secondary In Vitro Cytotoxic Responses against Products Coded by Subregions of the H-2 Complex. Spleen cells of mice immunized in vivo against alloantigens controlled by distinct regions of the *H-2* complex were cocultivated in a "secondary" MLC together with stimulator cells which were *H-2* identical to the cells used for in vivo immunization. After 5 days of culture the responder cells were tested for cytotoxic activity against ^{51}Cr-labeled LPS-induced blast lymphocytes. Representative results obtained are given in Table II and clearly suggest that cytotoxic activity was detectable against determinants controlled by either the *H-2K, H-2D,* or the *I* region. No cytotoxic activity was observed against products coded by the *I-B* or *I-C* subregion. In these strain combinations the skin grafts were not rejected within 25 wk (Table II).

Primary In Vitro Cytotoxic Responses against Products Coded by the I Region. Representative results obtained in a primary MLC are listed in Table III

TABLE I
H-2 Haplotype of the Mice Used

Strain	*H-2* haplotype (allelic origin)*					
	K	*I-A*	*I-B*	*I-C*	*S*	*D*
AQR	*q*	*k*	*k*	*d*	*d*	*d*
B10.HTT	*s*	*s*	*s*	*k*	*k*	*d*
B10.A	*k*	*k*	*k*	*d*	*d*	*d*
B10.A(4R)	*k*	*k*	*b*	*b*	*b*	*b*
B10.A(2R)	*k*	*k*	*k*	*d*	*d*	*b*
A.TH	*s*	*s*	*s*	*s*	*s*	*d*
A.TL	*s*	*k*	*k*	*k*	*k*	*d*
B10.BR	*k*	*k*	*k*	*k*	*k*	*k*
A/J	*k*	*k*	*k*	*d*	*d*	*d*
B10	*b*	*b*	*b*	*b*	*b*	*b*
B10.D2	*d*	*d*	*d*	*d*	*d*	*d*
C57BL/6	*b*	*b*	*b*	*b*	*b*	*b*
A.SW	*s*	*s*	*s*	*s*	*s*	*s*
CBA	*k*	*k*	*k*	*k*	*k*	*k*
DBA/1	*q*	*q*	*q*	*q*	*q*	*q*

* The data are derived from reference 11.

and demonstrate that cytotoxic effector cells against *I*-region-coded determinants can be induced in vitro. In five combinations differing for the *I-A* and *I-B*, or the former and the *I-C* region, CTL cells were generated. Since there is no combination available to investigate the effect of a separate *I-A* subregion difference we attempted tentatively to map by exclusion the *I* subregion controlling the target antigen for CTL. Thus, A.TH cells stimulated by A.TL were tested for their ability to lyse cells identical with the stimulator for the whole *I* region (A.TL), for the *I-A* and *I-B* subregions (AQR), or the *I-A* subregion [B10.A(4R)]. In each instance a comparable lysis was observed. It appears therefore that the *I-A* subregion controlled the main target of the CTL generated in this combination. This conclusion is strengthened by the negative results obtained with combinations differing for the *I-B* or *I-C* region only [the latter one might not be a complete but only partial difference, since the *I-C* region of the $H\text{-}2^d$ and $H\text{-}2^k$ haplotype are either cross-reactive or partially identical (24)].

In two combinations, i.e. the (DBA/1 × B10.D2)F_1 anti-AQR and the A.TH anti-A.TL combination, besides the indicated *I*-region difference (Tables II and III) there exists an additional difference at the *TLa* locus (25, 26). To diminish the probability that products of this locus represent the target antigens of the CTL generated in these combinations, A.TH (TLa^a) effector cells generated against A.TL (TLa^c) were tested against target cells differing with respect to the TLa allele from A.TL, namely AQR (TLa^a) and B10.A(4R) (TLa^b) cells. The observed almost equal lysis of LPS blasts derived from these strains make it improbable, although do not rule out, that *TLa*-gene products are the target antigens.

It should be noted that the data reported in Tables II and III represent a summary of several independent experiments performed over a time period of 4 mo. Therefore it is not possible to compare the magnitude of cytotoxic responses

TABLE II

Secondary Cytotoxic In Vitro Responses against Products Coded by Various Regions of the H-2 Complex

"Immune" responder cells	Stimulator cells	Stimulating *H-2* region	^{51}Cr release assay		
			Origin of LPS-induced target cells	% Specific lysis ratio of effector cells to target cells	
				30:1	3:1
(DBA/1 × B10.D2)F_1*	AQR	*I-A, I-B*	AQR	73	34
			B10.A	54	25
			B10.D2	2	0
(A.TL × B10.D2)F_1*	B10.HTT	*I-A, I-B*	B10.HTT	64	29
			A.TH	48	21
			A.TL	3	1
(B10.A(4R) × DBA/2)F_1‡	B10.A	*I-B*	B10.A	4	1
(CBA × B10)F_1‡	B10.A(2R)	*I-C*	B10.A(2R)	−2	0
(A.SW × B10.A)F_1‡	A.TL	*I-C, S*	A.TL	0	−3
(A.TL × B10.D/2)F_1*	B10.BR	*K, D*	B10.BR	84	47
			A.TH	5	1
(A.TL × B10.D/2)F_1*	A/J	*K*	A/J	66	44
(ASW × CBA)F_1*	ATL	*D*	ATL	87	66

Mice were first immunized in vivo by skin grafting followed by intraperitoneal injection of 15 × 10^6 splenic lymphocytes. After 4 wk, the spleen cells were used as responder cells and cultured togehter with stimulator cells carrying the same *H-2* haplotype against which the responder cells have been immunized. After 5 days of culture the responder cells were tested for cytotoxic activity against various ^{51}Cr-labeled LPS-induced blast lymphocytes. Background lysis of the LPS blast cells ranged between 18–32%. The strain combinations listed were tested at least three times. Representative experiments are given.

* Skin graft rejected after 2 wk.

‡ Skin graft not rejected after 25 wk.

obtained across the experiments listed. For example, the cell combination A.TH anti-A.TL (Table III) was tested seven times. In five experiments the cytotoxic activity obtained ranged between 24–53% lysis (at a ratio of 30:1), and in two of the experiments no significant cytotoxic activity was detectable.

Lytic Activity of I-Region-Induced CTL against LPS, ConA, and PHA Target Cells. Next it was tested whether cytotoxic lymphocytes generated in *I*-region-stimulated MLC's are equally cytotoxic for LPS-, ConA-, and PHA-induced blast lymphocytes. In order to ensure that the results obtained are not obscured by contamination with the heterogeneous population of small lymphocytes, only blast cells purified by velocity sedimentation at 1 *g* were used as target cells. The results obtained (Table IV) clearly suggest that *I*-region-induced cytotoxic lymphocytes were equally cytotoxic for LPS and ConA blast cells, but barely for PHA blasts.

TABLE III
Primary Cytotoxic In Vitro Responses against Products Coded by Regions of the H-2 Complex

Responder cells	Stimulator cells	Stimulating *H-2* region	^{51}Cr release assay		
			Origin of LPS-induced target cells	% Specific lysis ratio of effector cells to target cells	
				30:1	3:1
(DBA/1 × B10.D2)F_1	AQR	*I-A, I-B*	AQR	47	21
			B10.D2	5	0
(A.TL × B10.D2)F_1	B10.HTT	*I-A, I-B*	B10.HTT	32	15
			B10.BR	−1	0
A.TH	A.TL	*I + S*	A.TL	38	17
			AQR	42	11
			B10.A(4R)	34	12
			A.TH	2	0
A.TL	B10.HTT	*I-A, I-B*	B10.HTT	23	11
(A.SW × B10.D2)F_1	A.TL	*I + S*	A.TL	44	19
(B10.A(4R) × DBA/2)F_1	B10.A	*I-B*	B10.A	5	1
(A.SW × B10.A)F_1	A.TL	*I-C, S*	A.TL	0	−4
(CBA × B10)F_1	B10.A(2R)	*I-C, S*	B10.A(2R)	1	−3
(A.TL × B10.D2)F_1	A/J	*K*	A/J	27	14
(A.SW × CBA)F_1	A.TL	*D*	A.TL	31	19
(A.TL × B10.D2)F_1	B10.BR	*K, D*	B10.BR	40	22

Responder and stimulator were cultured in a MLC over 5 days. Cytotoxic activity generated was tested against LPS-induced blast lymphocytes (target cells) at various ratios of lymphocytes to target cells. Background lysis of the target cells was less than 29%. Each cell combination was tested at least three times. Representative experiments are given.

That the cytotoxic lymphocytes generated in an *I*-region-stimulated MLC were T cells in type, was suggested by the fact that pretreatment with anti-Thy 1.2 serum plus C abolished their cytotoxic activity (Table V).

Attempts to Compare the Relative Stimulating Capacity of Different Regions of the H-2 Complex. The relative stimulating capacity of the central *I* region and the peripheral *K* and *D* regions was compared to that of the complete *H-2* complex. Equal numbers of (A.TL × B10.D2)F_1 cells (responder cells) were cultured under identical conditions together with splenic stimulator cells derived from either B10.HTT mice (*I-A*- and *I-B*-subregion stimulation), or from B10.BR mice (*D*- and *K*-region stimulation) or from C57BL/6 mice ($H\text{-}2^b$ complex stimulation). After 5 days the cultured cells were harvested and tested for cytotoxic activity against both PHA- and LPS-induced blast lymphocytes. The results (Fig. 1) demonstrate that the anti-$H\text{-}2^b$ complex response resulted in the strongest cytotoxic activity. Moreover CTL generated in the anti-*H-2* complex

TABLE IV
Lytic Activity of I-Region-Induced CTL against PHA-, LPS-, and Con A-Induced Blast Cells

Responder cells	Stimulator cells	^{51}Cr release assay		
		Mitogen used for A.TL blast target cell induction	% Specific lysis	
			30:1	3:1
A.TH	A.TL	LPS	47	22
		Con A	32	15
		PHA	9	3
(A.SW × B10.D2)F_1	A.TL	LPS	21	12
		Con A	18	5
		PHA	6	1

A.TH or (A.SW × B10.D2)F_1 mouse-derived responder cells were cultured together with A.TL mouse-derived stimulator cells (*I*-region stimulation). After 5 days the responder cells generated were tested for cytotoxic activity against purified (velocity sedimentation at 1 *g*) LPS-, Con A-, or PHA-induced A.TL-derived blast lymphocytes. Background lysis of the A.TL target cells was: LPS blasts, 24%, Con A blasts, 27% and PHA blasts, 32%.

response lysed LPS and PHA blast almost equally well, this being in agreement with previous work (27). If one compared the ratio of effector cells to target cells required to achieve 50% lysis of the target cells, the cytotoxic activity triggered by the *H-2*^{b} complex incompatibility was about 30-fold stronger than that induced by a mere *D*- and *K*-region stimulation. Compared to the cytotoxic activity induced by *I-A*- and *I-B*-subregion stimulation the magnitude of cytotoxicity of the anti *H-2*^{b} complex response was about 40-fold stronger. This conclusion is based on the assumption that all the LPS target cells used carry the same amount of *I*-region-coded target antigens.

Discussion

A strong argument in favor of the presently held view of functional differences between the peripheral *K* and *D* regions (7, 9, 12) and the central *I* region (5–7, 9, 12) of the *H-2* complex represents the observation that in the presence of *I*-region-incompatible stimulator cells the cytotoxic response generated in a MLC against *D*- and/or *K*-region-incompatible cells becomes amplified (22, 28–30). On the basis of these findings Schendel and Bach (22) and we (31) proposed the "two signal" hypothesis. Accordingly the *I* region is primarily involved in lymphocyte activation as detected by thymidine uptake techniques in the MLC. In contrast the peripheral regions code for serologically detectable (SD) transplantation antigens which function as target for the CTL generated. On the other hand the observation that *I*-region-incompatible mice reject each others' skin graft (13, 14) and generate cytotoxic lymphocytes (16), led to the postulate that *I*-region-coded determinants (Ia antigens) also function as transplantation antigens.

TABLE V

Susceptibility of Cytotoxic Lymphocytes Generated against I-Region-Coded Determinants to Treatment with anti-Thy 1.2 Serum Plus C

% Specific lysis		
No treatment	Treatment with anti-Thy 1.2 serum plus C	Treatment with AKR serum plus C
24	3	27

(DBA/1 × B10.D2)F_1 splenic lymphocytes (responder cells) were cultured in a MLC with AQR mouse-derived splenic stimulator cells for 5 days. The cells were harvested and divided into three parts. One cell fraction remained untreated, one was treated with anti Thy 1.2 serum plus C (17), and the third fraction was treated with normal AKR serum plus C. The cell fractions were tested for cytotoxicity against LPS-induced AQR blast cells at that cell dilution, which resulted in the case of untreated cells in a ratio of viable lymphocytes to target cells of 30 to 1. Background lysis of target cells was 28%.

So far the lytic activity of CTL generated in a MLC has been tested mostly on PHA-stimulated lymphoblast targets (5–7). Yet Ia antigens appear to be predominantly expressed on B cells (27, 32–35). Therefore in the present experiments LPS-induced blast cells (B-cell derived) were used as targets for detecting *I*-region-induced CTL. Moreover since the magnitude of cytotoxic activity induced in a secondary MLC exceeds that of a primary MLC (36, 37), it was concluded that by first immunizing mice in vivo against *I*-region-coded determinants it may be possible to generate CTL against these determinants in a subsequent MLC. Indeed, when using preimmunized responder cells strong lytic activity was triggered against *I-A, I-B* incompatible stimulator cells, provided LPS blast lymphocytes were used as targets (Table II). These results agreed with the finding that *I-A, I-B* incompatibility also resulted in skin graft rejection (Table II, and D. Götze, unpublished results). However no significant cytotoxic activity was detected against *I-B*- and *I-C*-coded determinants (these mouse combinations also failed to induce skin graft rejection). Essentially identical results were obtained in primary MLC's, although the magnitude of cytotoxic responses induced was reduced. Thus *I*-region- or *I-A, I-B*-subregion incompatibility induced lytic activity, whereas *I-B*- and *I-C*-subregion differences failed to do so under the experimental conditions used. *I*-region-coded determinants were recognized equally well on LPS- and Con A-induced lymphoblasts, but to a much lower extent on purified PHA blast cells (Table IV). These results may explain the previous failure to detect CTL in a *I*-region-stimulated MLC using PHA blast lymphocytes as targets (7, 9). While both *K/D*-region and *I*-region incompatibility triggered the generation of specific CTL in vitro (Tables 2 and 3), the magnitude of cytotoxic responses induced by complete *H-2*-complex incompatibility exceeded by far the sum of the individual responses against the *H-2K, H-2D,* and I region, respectively (Fig. 1). Although the interpretation of these results is limited by the fact that the stimulator cells used were incompatible to the

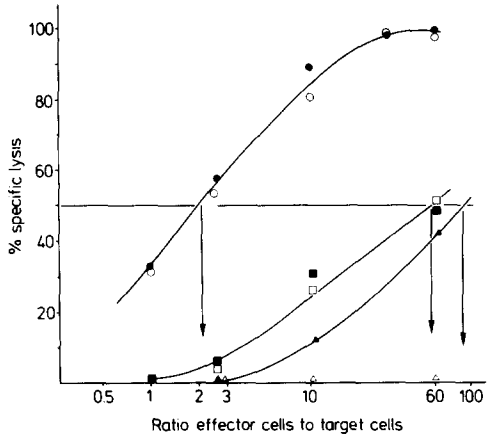

FIG. 1. (A.TL × B10.D2)F_1 splenic lymphocytes (responder cells) were stimulated in a MLC against either C57BL/6 (*H-2*^{b} complex stimulation), or B10.BR (*K, D* region stimulation) or B10.HTT (*I-A, I-B* subregion stimulation) mouse derived splenic stimulator cells. After 5 days of culture the responder cells were titrated for cytotoxic activity against purified (sedimentation velocity technique at 1 *g*) LPS and PHA-induced blast cells carrying the same *H-2* haplotype against which the responder cells were immunized. Background lysis of the target cells was less than 30%. C57BL/6 (○) PHA blasts and (●) LPS blasts; B10.BR (□) PHA blasts and (■) LPS blasts; and B10.HTT (△) PHA blasts (▲) LPS blasts.

responder cells at *H-2* regions of different *H-2* haplotypes, the results nevertheless are suggestive for a helper effect of an *I*-region incompatibility upon the generation of CTL reactive to *K*- and *D*-region-coded determinants (22, 27–30). These results complement independent evidence that a distinct T-cell subpopulation (T_1 cells), which can be characterized by functional (38), physical (39), and serological criterias (40), and which appears to be preferentially reactive to *I*-region-coded MLC determinants (40), exerts an amplifying effect on the induction of CTL reactive against *H-2K*- and *H-2D*-coded antigens.

The data presented here allow no answer in regard to the critical question whether *I*-region-coded determinants as detected by CTL are identical to either the MLC-stimulating determinants or to the serologically detectable Ia antigens. If it turns out that each determinant coded by the *I* region, which can be serologically detected, also is capable to provoke both cell proliferation and the generation of CTL in a MLC (alternative A, Fig. 2), then there is no necessity to postulate functional differences between peripheral and central regions of the *H-2* complex during the induction of a primary MLC. If, however, MLC-stimulating determinants are qualitatively different from target antigens recognized by CTL and if the recent evidence for T_1-T_2 cell synergism (39, 40) during allogeneic cell interactions is further substantiated, then the data presented here may be viewed as given in alternative B (Fig. 2). Accordingly, a third murine histocompatibility locus (*H-2I*) is situated in the *I-A* region. This *H* locus also has been detected by skin grafting (16). Since loci within the *I-A*, *I-B*, and *I-C* are controlling both MLC determinants and histocompatibility antigens the postulated *H-2I* locus within the *I-A* subregion at present is not separable from those controlling MLC determinants. The observation that certain MLC determinants fail to trigger CTL, (Tables II and III, and reference 20) yet provide a helper

	alternative A				alternative B			
Established regions of the H-2 complex	K	I (A B C)	S	D	K	I (A B C)	S	D
Multiplicity of loci of the H-2 complex	□	□□□□□□	□		□	□□□□□	□	□
Serologically detectable determinants coded by single loci of the H-2 complex	+	+ + + + + +	+		+	+ + + + +	+	+
Serologically detectable transplantation antigens	+	+ + + + + (+)	+		+ (SD1)	+ - - - - (SD3)	-	+ (SD2)
Target antigens for CTL	+	+ + + + + (+)	+		+	+ - - - -	-	+
MLC-determinants	+	+ + + + + (+)	+		(+)	+ + + + +	-	-(+)

FIG. 2. Present view of *H-2* functions in relation to genetic subregions.

effect upon the generation of CTL reactive to *K*- and *D*-region-coded determinants (41), however, provides additional support for the view of functional differences (alternative B) between MLC determinants and determinants for CTL in allogeneic lymphocyte interactions.

Summary

Determinants controlled by the *I* region of the murine *H-2* complex provoked the generation of cytotoxic T lymphocytes (CTL) in both a secondary and primary mixed lymphocyte culture. The stimulating determinants appeared to be controlled by loci within the *I-A* subregion. The target antigens of the CTL generated were present on both lipopolysaccharide- and concanavalin-induced blast lymphocytes, but were barely detectable on phytohemagglutinin-induced blast cells. The stimulating capacity for CTL induction of a complete *H-2* complex incompatibility by far exceeded the sum of *H-2D/K*-region and *I*-region incompatibility, respectively.

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