

SECONDARY IN VITRO RESPONSES OF T LYMPHOCYTES TO NON-H-2 ALLOANTIGENS

Self-H-2-Restricted Responses Induced in Heterologous Serum are not Dependent on Primary-Stimulating Non-H-2 Alloantigens*

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In cell-mediated cytotoxic reactions involving thymus-dependent (T) lymphocytes pre-sensitized against virus-infected (1-5) or chemically modified (6-8) cells, the ability of the immunocompetent cell(s) to respond against the foreign substance depends most likely on a complex system of receptors capable of recognizing both the major histocompatibility (H-2) antigens and the "classical" antigen. Such a "two-step" interaction between killer cell and target cell apparently is a necessary prerequisite for efficient cytolysis to be effected. Similarly, additional studies (9-12) have now shown that cytotoxic reactivity against non-H-2 alloantigens in mouse also belong to this category of reactions. In all these systems, susceptibility of target cells to lysis has required sharing of H-2-associated serologically defined (SD)¹ antigens by the sensitizing and subsequent target cells. This phenomenon, referred to as H-2-restricted cytolysis, has resulted in the development of two major groups of hypotheses: the "altered-self" or H-2 modification hypothesis (3, 7, 9), and the dual recognition or intimacy hypothesis (13). The altered-self hypothesis states that antigen capable of inducing cytotoxic T lymphocytes (CTLs) do so through modification of H-2 SD molecules on the same cell and thereby create the necessary point for cytotoxic attack. The dual recognition hypothesis, on the other hand, argues that a CTL is equipped with at least two distinct receptors, one specific for the relevant major histocompatibility SD molecule, and the second specific for the "classical" antigen (e.g., the virus-specific or chemical-modification determinant). As yet, however, no conclusive experiment exists which decides between these two groups of hypotheses as to how immunocompetent T lymphocytes recognize and respond to immunogens.

In the present report we show how different *in vitro* culture conditions can yield completely different patterns of T-cell reactivity against non-H-2-associated gene products in mouse. Our primary aim has been to determine what role non-H-2 alloantigens play in initiation and restimulation of mixed leukocyte culture (MLC) and cell-mediated lympholysis (CML) reactions. Results indicate

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¹ *Abbreviations used in this paper:* CML, cell-mediated lympholysis; CTL, cytotoxic thymus-dependent lymphocyte; FBS, fetal bovine serum; ³H-TdR, tritiated thymidine; LPS, lipopolysaccharide; MLC, mixed leukocyte culture; PBS, phosphate-buffered saline; SD, serologically defined.

that either H-2-restricted or non-H-2 antigen-specific reactions can be generated in vitro depending only on the primary culture conditions: heterologous serum induces H-2-restricted reactions, while homologous serum permits development of specific anti-non-H-2 alloantigen reactions. These findings have important bearing on theoretical discussions of how proliferative and cytotoxic T lymphocytes recognize self-H-2 structures, as well as carry a warning as to how H-2-restricted reactions may be generated in vitro, and perhaps in vivo, without actual intention by the investigator.

Materials and Methods

Mice. Inbred strains of mice used in this study and maintained in this laboratory are A.AL, A.BY, A.TL, B10.A, B10.AKM, B10.BR, B10.M, B10.RIII(71NS), B10.S, B10.S(7R), CBA/H, CBA/J, C3H/HeJ, C57BL/6Bom, and C57BL/10Sn. Strain C57BL/10Sn and its congenic resistant partner strains were established from breeding pairs generously provided by Dr. F. H. Bach (Immunobiology Research Center, University of Wisconsin, Madison, Wis.). Strains A.AL and A.TL were kindly provided by Dr. D. C. Shreffler (Department of Genetics, Washington University, St. Louis, Mo.). Both male and female mice were utilized and ranged in age from 4 to 20 wk.

Culture Medium. The serum-free medium as described by Click et al. (14) and Heber-Katz et al. (15) was supplemented with either 0.5% normal mouse serum according to the protocol of Peck and Click (16), or with 2.5% fetal bovine serum (FBS). The mouse serum was homologous with the responding strain and pooled from a minimum of six animals. FBS was purchased from GIBCO-BIO-CULT, Glasgow, Scotland or Flow Laboratories Svenska AB, Solna, Sweden. Six different batches of sera were used in this study.

MLC Combinations. Primary MLCs were performed in tissue culture flasks (no. 3013, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). Each flask contained $20\text{--}25 \times 10^6$ responding cells cultured with an equal number of X-irradiated (1,500–2,000 R) stimulating cells in 12 ml of medium. At various times of culture the cells were dispersed from the bottom of the flask with a Pasteur pipette; 0.2 ml of this cell suspension was removed to microtiter plate wells for radiolabeling.

Secondary MLCs were performed in microtiter plate cultures according to the procedures described by Peck and Bach (17). Cultures consisted of 0.05×10^6 or 0.075×10^6 responding cells (T-cell blasts) cultured with 0.5×10^6 X-irradiated stimulating cells.

At appropriate times of culture, as indicated in the text and figure legends, $0.8 \mu\text{Ci}$ tritiated thymidine ($^3\text{H-TdR}$) (Amersham, London, England) in a volume of 0.02 ml was added to each well for 4 h. Cells were filtered through glass-fiber filters using a multiple-sample harvester (Skatron; Flow Laboratories, Oslo, Norway) and total $^3\text{H-TdR}$ incorporation was determined by liquid scintillation procedures. Data are expressed in counts per minute of the mean of triplicate cultures. In general, standard deviations of primary MLC's are less than 10% of the means and those of the secondary MLCs are less than 5%.

Preparation of Responding T Lymphocytes. The responding cells in primary MLC were prepared by passage of whole spleen cell suspensions whose contaminating erythrocytes had been lysed in a 0.84% NH_4Cl treatment through Ig-anti-Ig glass bead columns, according to the protocol of Wigzell et al. (18). This procedure removes Ig-bearing B cells. After passage through the columns, the nonadherent cells are washed twice in phosphate-buffered saline (PBS) and resuspended to a density of 100×10^6 cells/ml. The resulting cell population can be shown to be >98% theta antigen-bearing T cells and <0.5% Ig-bearing B cells.

Separation of MLC-Reactive T Blast Cells from Primary Cultures. The MLC-reactive T lymphocytes were separated as T blast cells from the nonresponsive population using 1 g velocity sedimentation procedures as described by Miller and Phillips (19) and Andersson and Häyry (20). Primary MLC cells were collected from the culture flasks following maximum $^3\text{H-TdR}$ incorporation, resuspended in PBS supplemented with 4% FBS, then layered over a linear 15–30% FBS gradient. The cells were permitted to fall through the gradient for 3–4 h. Fractions of 12 ml each were collected and microscopically examined for numbers of blast and nonblast cells. Only those fractions containing >95% blast cells were kept. After two to four washes with PBS, the resulting pooled T-lymphocyte blast cell suspension was divided into two aliquots: the first was immediately

dispensed to microtiter plate wells and restimulated with appropriate stimulating cell populations, while the second was placed in tissue culture flasks (nos. 3013 or 3024; Falcon Plastics, Div. of BioQuest) for 3 days before restimulation. This 3-day incubation permitted the blast cells to revert to small lymphocytes.

CML Assay. CML was performed according to procedures detailed elsewhere (21) and is a modification of the techniques described by Alter et al. (22). The effector cells were generated in secondary MLC, after restimulation periods of 48 or 60 h. The secondary primed lymphocytes were collected from the mixed cultures, pooled, centrifuged, and washed twice in serum-free medium or medium supplemented with 2.5% FBS.

LPS-stimulated spleen cells were used as the target cells. Approximately 48 h before the CML assay, appropriate target cell cultures containing 10.0×10^6 spleen cells in 5.0 ml serum-free medium or medium supplemented with FBS were established in culture dishes (no. 3002; Falcon Plastics, Div. of BioQuest). Target cells were incubated 2 h then stimulated with 25–50 $\mu\text{g/ml}$ lipopolysaccharide (LPS) (prepared by Dr. G. Möller, Laboratory of Immunobiology, Karolinska Institute, Stockholm, Sweden). At time of assay the target cells were collected, centrifuged, resuspended in 0.4 ml of supernate removed from the centrifuged cells, and labeled for 1 h with 250–400 $\mu\text{Ci Na}_2^{51}\text{CrO}_4$. The labeled cells were washed three times in medium. Trinitrophenyl (TNP)-conjugated target cells were prepared according to the procedures of Shearer (6).

Cell destruction was performed in round-bottomed Sterilin microtiter plates (Sterilin Ltd., Teddington, Middlesex, England) at various effector cell numbers plus 1.0×10^4 labeled target cells. Cell destruction proceeded 4 h, after which time the plates were centrifuged, the supernate collected, and the quantity of released ^{51}Cr determined. Percent cytotoxicity is expressed as

$$\frac{[^{51}\text{Cr released}_{(\text{exp. 1})} - ^{51}\text{Cr released}_{(\text{spontaneous})}]}{[^{51}\text{Cr released}_{(\text{maximum})} - ^{51}\text{Cr released}_{(\text{spontaneous})}} \times 100.$$

Results

Development of Specific Memory against *Mls* Locus Products in Cultures Supplemented with Mouse Serum. Splenic T lymphocytes from B10.BR mice ($H-2^k$, Mls^b) devoid of Ig-bearing B cells and stimulated with whole spleen cells from CBA/J mice ($H-2^k$, Mls^d) exhibit strong primary MLC activation in cultures supplemented with homologous serum, as shown in Fig. 1 A. Since both strains possess the $H-2^k$ haplotype, MLC activation is believed to result primarily from different allelic products of the *Mls* locus (23, 24). On day 5 of primary culture (i.e., 24 h after maximum $^3\text{H-TdR}$ incorporation) B10.BR anti-CBA/J blast cells were separated from the nonreactive cells on a 1 g velocity sedimentation gradient, as described in the Materials and Methods. The isolated T-cell blasts were re-established as the responding cell population and stimulated with spleen cells from mice possessing various genetic differences. Results, presented in Fig. 1 B, clearly show a specific, secondary-like reactivity by B10.BR anti-CBA/J blast cells against CBA/J spleen cells. No restimulation is observed when cells from B10.BR (the responding strain), CBA/H ($H-2^k$, Mls^b —similar to B10.BR), A.AL ($H-2^d$, Mls^c), or B10.RIII(71NS) ($H-2^r$, Mls^b) are used as the stimulating populations. In addition, C3H/HeJ ($H-2^k$, Mls^c), B10.AKM ($H-2^m$, Mls^b), and C57BL/10 ($H-2^b$, Mls^b) also failed to cause strong reactivation, although the data is not included here.

A highly specific anti-CBA/J pattern of MLC reactivation is also observed if before restimulation the B10.BR anti-CBA/J blast cells are first permitted to revert to small lymphocyte-like cells (determined by morphological examination in a microscope) through incubation in tissue culture flasks for 3 days in the absence of stimulating cells (Fig. 1 C). Moreover, the reverted B10.BR

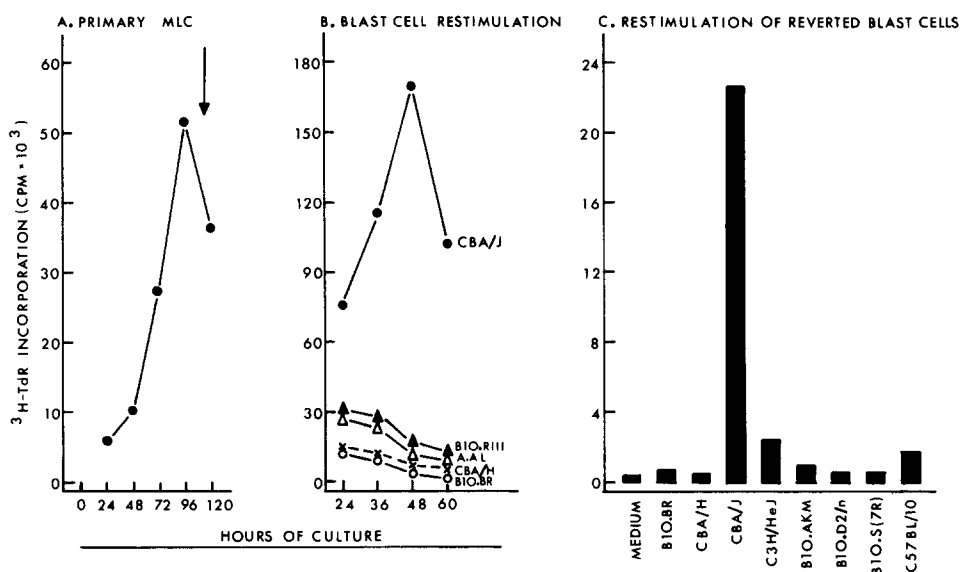


FIG. 1. Specific memory responses against *Mls* locus products in cultures supplemented with mouse serum. (A) Kinetics of primary MLC activation of B10.BR T lymphocytes stimulated with CBA/J spleen cells. Cultures were carried out in tissue culture flasks and the arrow indicates time at which the activated cells were collected for secondary MLC. (B) Kinetics of secondary MLC activation of purified B10.BR anti-CBA/J blast cells stimulated with CBA/J (●—●), A.AL (Δ — Δ), B10.RIII(71NS) (\blacktriangle — \blacktriangle), CBA/H (\times — \times), and B10.BR (○—○) spleen cells. MLC were carried out in microtiter plates. (C) $^3\text{H-TdR}$ incorporation of B10.BR anti-CBA/J cells stimulated with spleen cells from indicated strains after reversion of blast cells to small lymphocyte-like cells. Activation was measured at 60 h of culture. All cultures contained medium supplemented with 0.5% normal mouse serum. Stimulating cells were inactivated by X irradiation (1,750 R). $^3\text{H-TdR}$ (0.8 $\mu\text{Ci/well}$) was added to each culture 4 h before termination of MLC at times indicated.

anti-CBA/J cells elicit a distinctly secondary-like response against CBA/J cells with maximum $^3\text{H-TdR}$ incorporation occurring around 60 h of culture (unpublished observation). All other combinations examined, including B10.BR, CBA/H, C3H/HeJ, B10.AKM, B10.D2/n (*H-2^d*, *Mls^b*), B10.S(7R) (*H-2th*, *Mls^b*), and C57BL/10, fail to cause strong reactivation, although a small, but reproducible, stimulation occurs with C3H/HeJ and C57BL/10. Thus, B10.BR anti-CBA/J T lymphocytes generated in homologous mouse serum are strongly, and specifically, restimulated by cells of the primary stimulating strain, CBA/J, and exhibit a specific memory response.

Similarly, a specific anti-CBA/J secondary response is obtained if, instead of B10.BR, CBA/H T lymphocytes are used as the responding population and the primary MLC contains homologous mouse serum (data not included here). B10.BR and CBA/H, both possessing *H-2^k* and *Mls^b*, are mutually nonresponsive toward one another in MLC.

Development of H-2-Restricted Responses after Activation by Mls Locus Products in Cultures Supplemented with Heterologous Serum. The experiments of the previous section were repeated using identical culture conditions except in place of homologous mouse serum, FBS at a concentration of 2.5% was used to

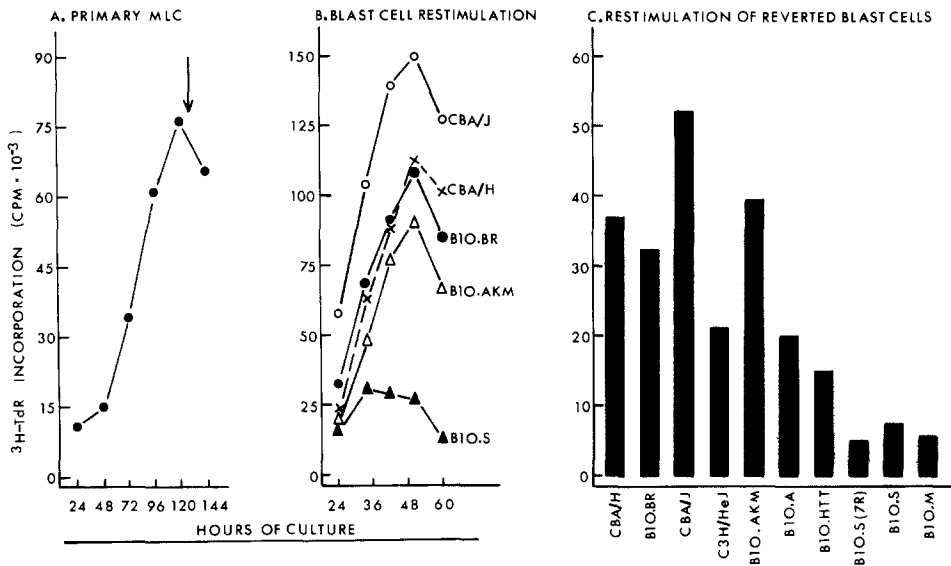


FIG. 2. H-2-restricted secondary responses of cells primed in vitro against the *Mls* locus in cultures supplemented with FBS. (A) Kinetics of primary MLC activation of CBA/H T lymphocytes stimulated with CBA/J spleen cells. Cultures were carried out in tissue culture flasks. Arrow indicates time at which the activated cells were collected for secondary MLC. (B) Kinetics of secondary MLC activation of purified CBA/H anti-CBA/J blast cells stimulated with CBA/J (○—○), CBA/H (×---×), B10.BR (●—●), B10.AKM (△—△), and B10.S (▲—▲) spleen cells. MLC were performed in microtiter plates. (C) ³H-TdR incorporation of CBA/H anti-CBA/J cells stimulated with spleen cells from various strains of mice after reversion of the blast cells to small lymphocyte-like cells. MLC activation was measured at 60 h of culture. All reactions were performed in medium supplemented with 2.5% FBS. Stimulating cells were inactivated by X irradiation (1,650 R). ³H-TdR (0.8 μCi) was added to each culture 4 h before termination of MLC at times indicated.

supplement the medium. Splenic T lymphocytes from CBA/H mice (*H-2^k*, *Mls^b*) stimulated by whole spleen cells from CBA/J mice (*H-2^k*, *Mls^d*) exhibit a strong primary MLC response under these conditions, as depicted in Fig. 2A. Again, this MLC activation is considered to be primarily against the *Mls* locus product(s). The CBA/H anti-CBA/J blast cells were isolated after peak ³H-TdR incorporation in primary MLC on a 1 g velocity sedimentation gradient and re-established as responding cells in microcultures. Again spleen cells from various mouse strains were used as stimulating cells and the kinetics of MLC proliferation were followed. Results are presented in Fig. 2B. X-irradiated CBA/J cells, syngeneic with the specific primary MLC activator, induces strong secondary-like reactivation with maximum ³H-TdR incorporation occurring 48 h after restimulation. However, strong secondary responses are not restricted to CBA/J cells: MLC reactivation is induced by cells from CBA/H (syngeneic with the responding population, i.e., self), B10.BR (*H-2^k*, *Mls^b*), and B10.AKM (*H-2^m*, *Mls^b*). In contrast, B10.S (*H-2^s*, *Mls^b*), a strain not carrying the *H-2^k* haplotype, induces little if any reactivation of the CBA/H anti-CBA/J T blast cells. Since B10.AKM is an H-2 recombinant strain possessing part of the *H-2^k* haplotype, it appears that CBA/H anti-CBA/J cells

generated in cultures containing heterologous serum respond against any cell-type carrying a part of or the whole $H-2^k$ haplotype, irrespective of the *Mls* locus allele.

When the CBA/H anti-CBA/J T blasts are permitted to revert to small lymphocyte-like cells before restimulation, the pattern of reactivation (Fig. 2C) is similar to that observed when blast cells are directly restimulated. Stimulation with CBA/J cells elicits the strongest response, but again strong MLC reactivity is induced by cells carrying the $H-2^k$ haplotype (e.g., CBA/H, B10.BR, and C3H/HeJ) or even a part of $H-2^k$ [e.g., B10.A, B10.AKM, and B10.HTT ($H-2^u$, Mls^b)]. Once more, only slight, if any, MLC activity is observed with strains possessing non- $H-2^k$ -derived haplotypes [e.g., B10.M ($H-2^f$, Mls^b), B10.S(7R), and B10.S]. Thus, CBA/H anti-CBA/J cells generated in heterologous serum exhibit H-2-restricted, secondary-like responses after priming against *Mls* locus antigen.

Data in Fig. 2C also suggests that H-2-restricted MLC activation can be determined by genes located solely in $H-2 I$. Two of the stimulating strains—B10.HTT, whose $H-2$ derivation is *ssskkd*,² and B10.S(7R), whose derivation is *sssssd*—presumably possess genetic differences only at $H-2 I-C$ and $H-2 S$. Despite this, B10.HTT cells stimulate the CBA/H anti-CBA/J blasts, whereas B10.S(7R) cells do not. Assuming that the $H-2 S$ region products are inactive in MLC activation (25, 26), the $H-2$ restriction must result from $H-2 I-C$ subregion identity between B10.HTT and CBA/J. In any event, the $H-2 K$ and $H-2 D$ regions appear irrelevant for H-2-restricted MLC responses against B10.HTT. It should also be noted that B10.A (*kkkddd*), which shares $H-2 I-A$ and $I-B$ subregions with CBA/J, and B10.AKM (*kkkkkq*), which shares the whole $H-2 I$ region with CBA/J, induce progressively stronger MLC responses, thus suggesting a correlation between *I* region sharing and strength of reactivation.

B10.BR anti-CBA/J T blast cell responses have also been examined in the presence of FBS. A similar $H-2^k$ -restricted MLC activity occurs (data is not presented here to avoid duplication), thereby indicating that *Mls* locus specific and H-2-restricted responses are not strain-dependent phenomena.

H-2-Restricted MLC Activation: Dependence on Primary Culture Conditions. *Mls* locus-primed T lymphocytes can exhibit either an H-2-restricted response or a specific anti-*Mls* locus memory response. This difference apparently depends only on whether the primary MLC contains heterologous or homologous serum, respectively. Of interest, then, was to test if the H-2-restricted secondary MLC activation results from specific modification of H-2 products by FBS. In order for this phenomenon to be due to H-2 modification, two requirements must be fulfilled: (a) the responding cells must be generated in primary cultures containing FBS, and (b) secondary cultures must also contain FBS so as to permit an identical modification of secondary stimulating cells.

Splenic B10.BR T lymphocytes were stimulated with CBA/H spleen cells in primary MLC containing either homologous mouse serum or FBS. Strong MLC

² Denotes the *K*, *I-A*, *I-B*, *I-C*, *S*, and *D* regions of $H-2$ and from which haplotype they were derived.

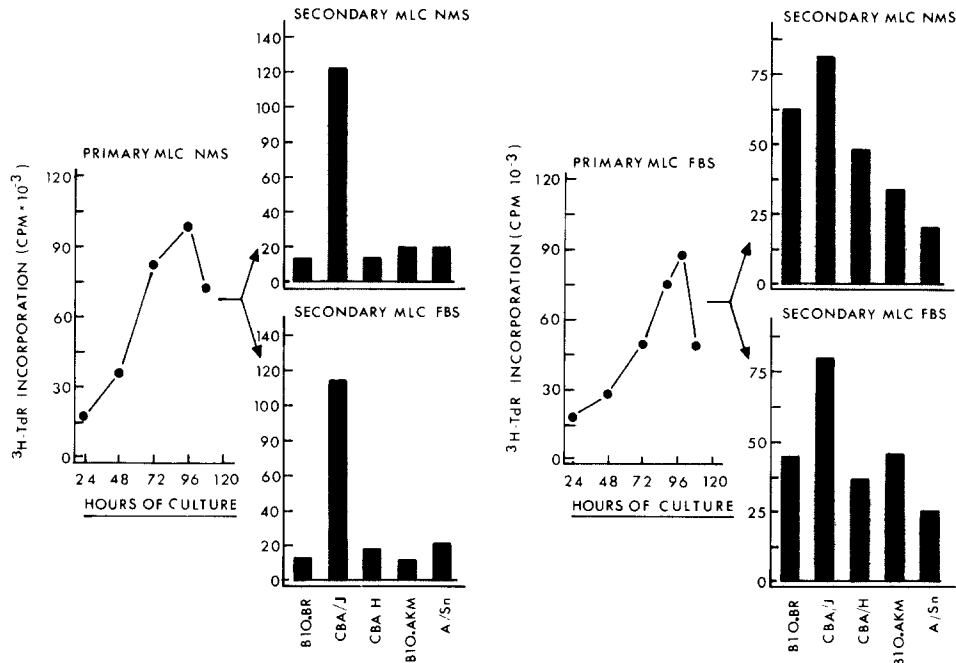


FIG. 3. Dependence on primary MLC for development of anti-*Mls* locus or H-2-restricted responses. B10.BR T lymphocytes were stimulated with CBA/J spleen cells in primary MLC containing either 0.5% normal mouse serum (NMS) or 2.5% FBS. At 108 h of culture each activated population of B10.BR anti-CBA/J blast cells was collected and divided into two aliquots. Each aliquot was re-established as responding cells in secondary MLC: one set in medium containing mouse serum, the other set in medium containing FBS. The B10.BR anti-CBA/J blast cells were stimulated with X-irradiated (2,000 R) spleen cells from various strains and $^3\text{H-TdR}$ incorporation was measured at 48 h of culture, after a 4 h pulse with $0.8 \mu\text{Ci/culture}$.

proliferation occurred in both systems, as shown in Fig. 3. Both sets of B10.BR anti-CBA/J blasts were then isolated by 1 g velocity sedimentation procedures and re-established as the responding populations in mixed cultures containing either mouse serum or FBS. The B10.BR anti-CBA/J blast cells generated in homologous serum exhibited a highly specific secondary anti-CBA/J response whether the secondary cultures contained homologous serum or FBS (Fig. 3). In a similar manner, B10.BR anti-CBA/J cells generated in FBS exhibited strong *H-2^k*-restricted secondary responsiveness (including anti-self reactivity) in either FBS or mouse serum containing cultures. Thus, specificity of reactivity in restimulation of B10.BR anti-CBA/J blast cells is determined by the primary culture conditions and is independent of secondary culture conditions.

Heterologous Serum-Dependent H-2-Restricted Cytolysis after Secondary MLC Activation of T Lymphocytes Primed In Vitro against Mls Locus Products. Results of the previous sections indicate that H-2-restricted MLC responsiveness develops after priming of T cells against the *Mls* locus product(s) in the presence of FBS. We therefore wished to examine the cytolytic activity of secondary MLC-activated T lymphocytes to determine, first, if effector T cells can be generated by this protocol, and second, if so, will the subsequent cytotox-

icity be *Mls* locus specific or H-2 restricted. One point of interest is that CTLs are normally not generated in primary MLC against *Mls* locus products (21, 24, 27).

Splenic T lymphocytes from either B10.BR mice or CBA/H mice were primed *in vitro* with whole spleen cells from CBA/J mice. The B10.BR anti-CBA/J and CBA/H anti-CBA/J blast cells were isolated 1 day after peak MLC activation on 1 *g* velocity sedimentation gradients and restimulated in secondary MLC with X-irradiated CBA/J spleen cells. After secondary MLC, the activated lymphocytes were tested in CML against various target cells. Identical culture conditions were used throughout except that B10.BR activation was performed in the presence of homologous serum while CBA/H activation was performed in cultures containing FBS. Results are presented in Table I. B10.BR anti-CBA/J cells failed to elicit detectable killing of CBA/J target cells regardless of the effector to target cell ratios examined. To control for the cytotoxic capacity of B10.BR cells per se, an anti-*H-2* reaction—B10.BR anti-C57BL/10—was included and showed positive CML. In contrast to the negative CML activity on CBA/J targets by cells activated in mouse serum, the CBA/H anti-CBA/J cells lysed CBA/J targets, as well as CBA/H (i.e., self) and B10.BR cells. These reactions required only 4:1 effector to target cell ratios for strong CML. No cytolysis occurred against B10.S(7R) targets, which carry the *H-2^h* haplotype.

Several variations, including restimulation of primed CBA/H anti-CBA/J blasts in secondary MLC with CBA/H cells in place of CBA/J cells, proved just as efficient in producing anti-*H-2^k* CTLs (Table I). Target cell destruction occurred against CBA/H, B10.BR, and CBA/J—strains possessing the *H-2^k* haplotype. Little or no killing occurred against B10.S(7R).

Anti-Self-H-2 CTLs: Dependence on FBS for their Generation, but not for Subsequent Activity. It is possible that FBS induces generation of anti-self-*H-2*-reactive T cells through the introduction of new antigens onto the surface of growing cells *in vitro*, a situation somewhat analogous to the virus-infected or hapten-modified cell systems of anti-“self” reactivity (1-8). To test this, purified splenic B10.BR anti-CBA/J T blast cells, generated in primary MLC containing FBS and then restimulated in secondary MLC with CBA/J spleen cells for 48 h, were tested for CML activity on various target cells generated either in medium supplemented with FBS or in serum-free medium. Target cells generated in serum-free medium should lack the possible FBS-induced antigens. Results of two such experiments are presented in Table II. B10.BR and CBA/J target cells were lysed by B10.BR anti-CBA/J CTLs irrespective of whether the target cells were generated in the presence or absence of FBS. Note, however, that nonstimulated, fresh B10.BR cells were not lysed, suggesting that target antigens do not develop during the 4 h of the assay despite the presence of FBS. Thus, the CTLs apparently do not require recognition of FBS-induced target antigens to kill target cells.

Also presented in Table II are data consistent with the concept that FBS-dependent anti-self-*H-2* cytolysis is determined by the SD regions of *H-2*. The *H-2^k*-restricted nature of the CML activity is demonstrated in the positive killing of target cells carrying the *H-2^k* haplotype, e.g., CBA/J and B10.BR. In addition, B10.BR anti-CBA/J CTLs elicit strong killing against B10.RIII(71NS) target cells (*H-2^r*), reflecting the strong cross-reactivity of *H-2^r* and *H-2^k* observed in

TABLE I
Anti-Self-H-2 Restricted Cytolytic Activity of T Lymphocytes against Mls Locus Product(s) in Medium Containing Heterologous Serum

Effector cells*	Serum source for I ^o and II ^o MLC	Secondary MLC-stimulating cells	Target cell	Effector:target cell ratios	CML
					%
B10.BR anti-CBA/J‡	Mouse serum	CBA/J	CBA/J§	100:1	0.5
			CBA/J	50:1	-2.8
			CBA/J	25:1	-2.1
			CBA/J	12:1	-2.8
			CBA/J	6:1	0.3
B10.BR anti-C57BL/10	Mouse serum	C57BL/10	C57BL/10	6:1	48.8
CBA/H anti-CBA/J	FBS	CBA/J	CBA/J	4:1	23.4
			B10.BR	4:1	21.5
			CBA/H	4:1	29.3
			B10.S(7R)	4:1	0.0
		CBA/H	CBA/H	4:1	34.9
			B10.BR	4:1	32.3
			CBA/J	4:1	16.8
			B10.S(7R)	4:1	5.2

* Effector cells collected 48 h after secondary MLC activation.

‡ Genetic designations for strains in this table: B10.BR - *H-2^k*, *Mls^b*; B10.S(7R) - *H-2^h*, *Mls^b*; CBA/H - *H-2^k*, *Mls^b*; CBA/J - *H-2^k*, *Mls^d*; C57BL/10 - *H-2^b*, *Mls^b*.

§ Spontaneous release averaged between 24 and 34% of the maximum release.

allogeneic CML (28). This cross-reactivity is believed due to specificity *H-2.25*. We also observe a slight, but consistent cross-reactivity of *H-2^s* and *H-2^k*, another cross-reacting combination in allogeneic CML (28, 29). This is reflected in the CML observed against A.TL cells (possessing haplotype *H-2^u* whose derivation is *skkkkd*), and to a lesser degree against B10.S(7R) cells (whose *H-2^h* haplotype derivation is *sssssd*). In the case of A.TL, both the *H-2 I* and *H-2 S* regions are derived from *H-2^k*, yet the CML activity remains well within the range of *H-2^k* and *H-2^s* cross-reactive responses (28). Thus, these data, although limited by the availability of proper genetic strains, suggest that the *H-2 K* and *H-2 D* region products exert the strongest influence on the development of *H-2*-restricted CML.

Appearance of H-2-Restricted CTLs in Nonstimulated Cultures after Incubation in Heterologous Serum. No evidence exists for an anti-*Mls* locus antigen cytolytic reactivity in vitro. It is thus possible that MLC activation against the *Mls* locus products merely serves a catalytic helper function for development of anti-self-*H-2* T-cell cytotoxicity. No requirement at all may exist for *Mls* locus activation: FBS per se might be able to induce anti-self-*H-2* CTL development, especially in light of the recent demonstration that nonspecific killer cells can be induced by FBS (30).

Splenic T lymphocytes were established in nonstimulated cultures containing

TABLE II
Anti-Self-H-2 CML of Specific Target Cells Generated in Serum-Free Medium or in Medium Supplemented with Heterologous Serum

Exp.	Effector cells*	Secondary MLC-stimulating cells	Target cells‡	Serum source in target cell cultures	Effector:target cell ratios	CML %
1	B10.BR anti-CBA/J§	CBA/J	B10.BR	FBS	17:1	16.1
			CBA/J	FBS	17:1	32.0
			CBA/J	Serum-free	17:1	29.8
			B10.RIII(71NS)	FBS	17:1	22.5
2	B10.BR anti-CBA/J	CBA/J	B10.BR	FBS	11:1	39.4
			B10.BR	Serum-free	11:1	58.5
			CBA/J	FBS	11:1	65.6
			CBA/J	Serum-free	11:1	47.6
			A.TL	FBS	11:1	11.3
			B10.S(7R)	FBS	11:1	4.7
		Fresh B10.BR	—	11:1	0.8	

* Effector cells collected from secondary MLC after 48 h of restimulation by CBA/J cells.

‡ LPS-stimulated 48 h before CML assay.

§ Genetic designations for strains in this table: A.TL — *H-2^l* (*skkkkd*), *Mls^a*; B10.BR — *H-2^k* (*kkkkkk*), *Mls^b*; B10.RIII(71NS) — *H-2^r* (*rrrrrr*), *Mls^b*; B10.S(7R) — *H-2^h* (*sssssd*), *Mls^a* CBA/J — *H-2^k* (*kkkkkk*), *Mls^l*.

2.5% FBS. This serum concentration induces a minor T-cell blast response and after 5 days in culture 42×10^6 blast cells from an original population of 400×10^6 T cells were collected. These blast cells were "restimulated" for 48 h with X-irradiated syngeneic spleen cells in secondary MLC. The cultures were harvested and tested for cytotoxicity on various target cells. Results are presented in Table III. Positive CML was observed on target cells possessing the "self"-*H-2^k* haplotype, i.e., B10.BR and CBA/J. No killing occurred against B10.S(7R) or A.TL targets, two strains not possessing *H-2^k* haplotype-derived *H-2* SD regions. Again, CBA/J target cells generated in serum-free cultures were sensitive to cytolysis.

A similar pattern of CML is exhibited by "FBS-activated" T cells when tested on a panel of TNP-conjugated target cells (Table III): only target cells possessing *H-2* SD regions derived from the *H-2^k* haplotype were killed. TNP conjugation, therefore, does not prevent the anti-self-*H-2* CTLs from recognizing and killing on the appropriate target antigen(s).

Because all CML assays in this study have been carried out in medium supplemented with FBS, it remained possible that target cells, including those generated in serum-free medium, could be modified by FBS during the 4 h of the CML assay, thereby rendering them sensitive to cytolysis. To eliminate this possibility we have examined *H-2*-restricted cytolysis in a totally serum-free CML, as well. CBA/H effector cells were generated in nonstimulated primary cultures containing 2.5% FBS. After isolation by velocity sedimentation and

TABLE III
 Generation of CTLs Exhibiting Anti-Self-H-2 CML after Incubation in Nonstimulated Cultures Supplemented with Heterologous Serum

Exp.	Effector cells*	Secondary MLC-stimulating cells	Target cells‡	Serum source in target cell cultures	Effector:target cell ratios	CML %	
1	B10.BR§	B10.BR	B10.BR	FBS	10:1	18.4	
			CBA/J	FBS	10:1	9.6	
			B10.S	FBS	10:1	-11.1	
2	B10.BR	B10.BR	B10.BR	FBS	25:1	20.3	
			CBA/J	FBS	50:1	25.7	
					25:1	24.9	
					12:1	17.8	
					6:1	15.2	
			CBA/J	Serum-free	25:1	19.4	
			A.TL	FBS	25:1	-0.9	
			B10.S(7R)	FBS	25:1	-3.1	
			B10.BR	B10.BR-TNP	FBS	25:1	21.6
				CBA/J-TNP	FBS	25:1	28.7
				CBA/J-TNP	Serum-free	25:1	20.5
				A.TL-TNP	FBS	25:1	-0.9
	B10.S(7R)	FBS	25:1	0.2			

* Effector cells generated in nonstimulated primary culture supplemented with 2.5% FBS. Blast cells isolated on day 5 and "stimulated" with X-irradiated syngeneic B10.BR cells for 48 h.

‡ LPS-stimulated 48 h before CML assay.

§ Genetic designations for strains in this table: A.TL - $H-2^l$ (*skkkkd*), *Mls^c*; B10.BR - $H-2^k$ (*kkkkkk*), *Mls^b*; B10.S - $H-2^s$ (*sssss*), *Mls^b*; B10.S(7R) - $H-2^{th}$ (*sssssd*), *Mls^b*; CBA/J - $H-2^k$ (*kkkkkk*), *Mls^d*.

|| Target cells conjugated with TNP according to the procedures of Shearer (6).

restimulation in secondary MLC with CBA/J cells, these blasts were tested for their ability to kill LPS-stimulated targets generated either in serum-free medium or medium supplemented with mouse serum. The effector cells as well as the target cells were washed extensively in serum-free medium before CML assay. Results of one experiment are presented in Table IV. In general, the CBA/H CTLs elicited the same $H-2^k$ -restricted cytotoxicity as seen throughout this study - CBA/H, CBA/J, and B10.BR cells were killed to variable degrees, while B10.S(7R) cells were not. Target cells generated in both serum-free medium or medium supplemented with mouse serum were lysed. CML in serum-free medium, as expected, is less than reactions observed in CML run in the presence of serum (Table IV). A type of interaction between FBS and mouse serum components or activity is suggested by the limited reactivity seen in the control CML against CBA/J target cells generated in mouse serum. Nevertheless, cytotoxicity of target cells never exposed to FBS make it highly improbable that the target antigens are induced by FBS per se.

Discussion

The primary purpose of this study was to determine more precisely the

TABLE IV
Anti-Self-H-2-Restricted Cytotoxicity in a CML Assay Using Serum-Free Medium

Effector cells*	Secondary MLC-stimulating cells	Target cells‡	Serum source in target cell cultures	Serum source in CML	CML§
CBA/H	CBA/J	CBA/H	Mouse serum	Serum-free	3.7
		CBA/J	Mouse serum	Serum-free	15.7
		B10.BR	Mouse serum	Serum-free	12.3
		B10.S(7R)	Mouse serum	Serum-free	-7.8
		CBA/H	Serum-free	Serum-free	9.3
		CBA/J	Serum-free	Serum-free	11.8
		B10.BR	Serum-free	Serum-free	6.3
		CBA/J	Mouse serum	FBS	5.5
		CBA/J	Serum-free	FBS	28.8

* Effector cells generated in nonstimulated primary culture supplemented with 2.5% FBS. Blast cells were isolated on day 5 and stimulated with X-irradiated CBA/J spleen cells for 48 h.

‡ LPS-stimulated 48 h before CML assay.

§ Effector: target cell ratio, 20:1.

|| Genetic designation of strains in this table: B10.BR - $H-2^k, Mls^b$; B10.S(7R) - $H-2^h, Mls^b$; CBA/H - $H-2^k, Mls^b$; CBA/J - $H-2^k, Mls^d$.

importance of non-H-2 alloantigens in initiation and restimulation of T-cell-mediated cytotoxicity. Recent reports (9-11) have shown that lymphoid cells presensitized in vivo against non-H-2 alloantigens develop anti-"self"-H-2-restricted cytotoxic reactivity which is demonstrable in vitro, thus analogous with the "self"-H-2-restricted cytolysis against virus-infected (1-5) and chemically-modified (6-8) cells. In each of these systems the sensitizing and subsequent target cells must share $H-2$ K or D region-associated (SD) specificities for cytolysis to be effected.

To facilitate the study of this phenomenon we have chosen a completely in vitro model utilizing mouse strain combinations which possess identity at the $H-2$ locus, but which have genetic differences at non- $H-2$ loci that result in MLC activation (i.e., the Mls locus). This eliminates the requirement for in vivo sensitization, which no doubt involves a variety of direct and indirect activation mechanisms. In addition, we have utilized three relatively simple procedures to insure that the major part of the observed reactivity is due to T lymphocytes: (a) Ig-anti-Ig-coated glass bead column separation (18) of the primary MLC-responding cells which provides an initial responding population containing >98% T and <0.5% B cells, (b) 1 g velocity sedimentation gradient separation (19, 20) of antigen-reactive T blast cells from nonresponding cells, and (c) secondary mixed cultures (20, 31, 32) which greatly enhance the sensitivity of in vitro responses. In this way we have selected for highly purified, antigen-activated T-lymphocyte populations with specific responsiveness against the sensitizing antigen(s).

It is clear from the results of this study that such isolated T-cell clones, presumably activated by non-H-2 alloantigens residing on the stimulating cells, react in two distinct ways. First, they can exhibit a reactivity which we refer to as specific anti- Mls (or non- $H-2$) alloantigen responsiveness. This reaction apparently requires the recognition of identical Mls locus antigens on cells used

as primary and secondary stimulating populations. Second, the isolated T-cell clones can exhibit a specific anti-self-H-2-restricted reactivity in which the *Mls* locus difference apparently is irrelevant and in which there is no requirement for a known genetic difference between stimulating and responding cells (further discussion of specificity follows).

This dichotomy of T-cell reactivity is controlled completely by the culture environment used during the primary-activation phase. Anti-*Mls* locus-specific T cells appear to be the only T population activated in cultures containing homologous mouse serum. In contrast, the dominating reactivity of T-cell blasts from cultures supplemented with a heterologous serum like FBS is anti-self-H-2-restricted in nature. These data seriously question certain aspects of earlier work on H-2-restricted cytotoxicity and force a re-examination of such basic points as (a) the nature of the antigen(s) recognized by T cells in anti-self-H-2-restricted vs. specific anti-non-H-2 reactions, (b) the factors which induce development of anti-self-H-2 reactions, (c) the level at which an apparently specific anti-non-H-2 reaction is lost in favor of an anti-self-H-2-restricted response, (d) whether or not the same T-cell clones are involved in these two types of activities, and (e) the degree to which H-2-restricted cytotoxicity can represent a type of culture artifact, especially when adequate controls are lacking. Unfortunately, only part of these questions can be answered at this time.

We have shown in Fig. 1 (the secondary MLC responses of B10.BR anti-CBA/J) that T-cell clones generated in cultures supplemented with homologous serum exhibit a highly discriminatory reactivity with respect to restimulation by relevant and irrelevant antigens. MLC activation of B10.BR lymphocytes with CBA/J cells results primarily from different allelic products at the *Mls* locus, therefore, the specific nature of the secondary reaction indicates development of specific memory in vitro to the *Mls* locus products per se. We have found, in addition, that H-2 identity between the primary and secondary stimulating cells is required for successful recognition of the specific *Mls* locus product.³ Despite this, it must be pointed out that such memory T cells remain incapable of effecting target cell destruction against *Mls* locus antigens or against "self-H-2-associated" products (see Table I).

In complete contrast, T lymphocytes activated by *Mls* locus alloantigens in primary cultures supplemented with FBS, or even T lymphocytes merely cultured with FBS, exhibit strong anti-self-H-2 reactivity, both MLC and CML, with no significant specificity for the priming *Mls* locus antigens. It is most probable that FBS induces a set of T-cell clones distinct from those specifically activated by *Mls* locus determinants. The mechanism underlying the ability of FBS to activate a set of T cells into anti-self-H-2 reactivity is intriguing, especially in light of the recent report by Shustik et al. (30) which shows that spleen cells incubated in FBS may develop a totally nonspecific, yet strong, cytotoxic capability. Furthermore, it has been reported (33) that FBS can "induce" new antigenic determinants on cells growing in vitro, making them susceptible to immune lysis. But perhaps the most perplexing aspect is the

³ Peck, A. B., C. A. Janeway, Jr., and H. Wigzell. T lymphocyte responses to *Mls* locus antigens involve recognition of H-2 I region gene products. Manuscript submitted for publication.

requirement for fresh syngeneic cells to obtain restimulation of the blast cells (Fig. 2); cells within the culture itself appear incapable of stimulating.

Antigen-activated T cells generated in the presence of FBS display H-2-restricted reactivity in both MLC and CML. The H-2-restricted MLC activation could be shown to be controlled by *H-2 I* region determinants with no apparent activity against *H-2 SD* region determinants. On the other hand, H-2-restricted CTL development could be shown to be controlled by "self"-*H-2 SD* region structures with no detectable effect from "self"-*H-2 I* region components. This dichotomy of reactivity is similar to that observed in allogeneic T-cell-mediated cytotoxic reactions (22, 25, 26, 29). Also, the cross-killing of target cells in anti-self-H-2 CML reactions within this study, although limited to the *H-2^k* haplotype, are identical with those reported for allogeneic CML (28).

The altered-self hypothesis (3, 7, 9) proposes to explain H-2-restricted cytotoxicity as a response to surface component modification resulting from an interaction between a modifying principle and H-2 SD determinants. Arguments against the idea that FBS acts primarily as a modifying agent at the level of secondary activation in this system stem from experiments in Fig. 3 and Tables II-IV. First, no evidence was obtained suggesting that in H-2-restricted reactions FBS or FBS-induced determinants must be present on secondary MLC-activating cells or CML target cells. Anti-self-H-2 CML occurred with equal vigor on relevant target cells irrespective of whether the target cells were generated in serum-free medium or medium supplemented with FBS. Second, possible transfer of FBS or FBS-induced determinants by responding T cells to target cells seem quite improbable since the effector cells would have to carry saturating amounts of "FBS-modified structures" and transfer them to target cells during the short assay time (4 h). Rather, the target cells per se must already express the antigenic structures which function as relevant antigen in H-2-restricted cytotoxic reactions.

Although the *H-2*-restricted MLC and CML activity exhibit *H-2 I*-associated and *H-2 SD*-associated restrictions, respectively, antigenic specificities distinct from H-2 antigens, as well as distinct from FBS components, cannot be ruled out. For example, target cells were for the most part LPS-stimulated spleen cells since *Mls* locus determinants are expressed on B and not T cells (34), and thus LPS antigen most likely is present on target cells. However, LPS antigen alone does not activate T cells directly and would thus seem an unlikely CML target antigen. Likewise, *Mls* locus antigens or other non-H-2 alloantigens show no correlation with T-cell killing. More plausible antigens not so easily dismissed include various B-cell antigens like MBLA (35), MSpLA (36), Ia (37), Ala-1 (38), and Ly-8 (39) which can be recognized by T cells, including syngeneic T cells, or virus-associated determinants coded for by endogenous viruses which are activated during cell transformations (40-42).

It is clear from the above reasoning that it is important to delineate possible limitations for the types of target cells which function in anti-self-H-2 reactions. We suspect that the effector T cells and the subsequent target cells do possess some differences with respect to their surface structures. It is known that CTLs themselves are susceptible to cytolytic attack (43); consequently, should the anti-self-H-2-restricted CTL react against pure "self"-H-2 structures, then one

might expect a totally suicidal CML reaction. On the other hand, results of this study and others (30) force us to entertain the prospect that "self"-H-2 antigens may function just as efficiently as allogeneic H-2 antigens in controlling development of T-cell-mediated cytotoxic reactions.

In conclusion, we have presented data indicating how highly specific anti-self-H-2 reactivity against both *H-2 I* region determinants and *H-2 SD* region structures can be induced in vitro merely by activating T lymphocytes in cultures containing heterologous serum. These findings impose strict requirements for adequate controls in studies of "conventional" anti-self-*H-2*-restricted immune reactions carried out in vitro. On the other hand, they may provide us with some insight as to how T-cell-mediated autoimmune reactions may evolve.

Summary

The role of non-H-2 alloantigens, specifically *Mls* locus products, in secondary in vitro T-cell-mediated cytotoxicity has been studied. Splenic T lymphocytes, activated against *Mls* locus alloantigens in primary-mixed cultures and isolated by velocity sedimentation gradient separation techniques, were used as responding populations in secondary mixed leukocyte cultures (MLCs) and cell-mediated lympholysis (CML). Such T-cell clones could be shown to exhibit either "self"-H-2-restricted or anti-*Mls* locus-specific reactivity, with this dichotomy of reactivity depending only on the primary culture conditions. *Mls* locus-activated T lymphocytes generated in cultures supplemented with homologous serum exhibited specific memory responses in MLC, yet remained incapable of effecting target cell destruction against *Mls* locus antigens or against "self"-*H-2* structures in CML. In contrast, activated T-cell clones generated in the presence of heterologous serum displayed *H-2*-restricted reactivity in both secondary MLC and CML. *H-2*-restricted MLC activation was controlled by *H-2 I* region products, whereas *H-2*-restricted CML activity was controlled by products of the *H-2* serologically defined regions. Although heterologous serum was a necessary (and sufficient) entity for development of *H-2*-restricted responses, evidence argues against the possibility that heterologous serum acts via modification of cell surface components.

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