A NEW LYMPHOCYTE-ACTIVATING DETERMINANT LOCUS EXPRESSED ON T CELLS, AND MAPPING IN *I-C* SUBREGION*

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The *H-2* gene complex of the mouse is comprised of four distinct regions, *K* and *D*, marked by genes which control histocompatibility antigens, *S*, defined by a gene(s) for the Ss serum protein (C'4 component) and Slp allotype traits, and *I*, which include genes controlling immune responses, lymphocyte-activating determinants (*Lad*),[1] graft-versus-host reaction, immune response-associated (Ia) antigen, and a histocompatibility locus (1-3). The *I* region has been further divided into subregions, *I-A*, *I-B*, *I-J*, *I-E*, and *I-C*, which control different sets of specific immune responses or suppression (4).

I-C subregion was defined by specificity Ia.6 which differentiated $H\text{-}2^k$ (Ia.6−) and $H\text{-}2^d$ (Ia.6+) haplotypes for this subregion (2). *I-E* subregion was defined by specificity Ia.22, positive in $H\text{-}2^k$ and negative in $H\text{-}2^d$ (4). Recently, we identified a new private Ia specificity (Ia.23) of $H\text{-}2^d$ origin, also mapping in *I-E* subregion.[2] Sequential precipitation studies suggest that specificities Ia.7, 22 ($H\text{-}2^k$) and Ia.7, 23 ($H\text{-}2^d$) are expressed on products coded by *I-E* subregion gene(s).[3] That leaves Ia.6 as the only specificity mapping in the *I-C* subregion.

Previous investigations have shown that genes controlling products which cause stimulation in the mixed lymphocyte reaction (MLR) map in a number of different regions of the *H-2* gene complex (5, 6). The strongest MLR stimulation has been seen with *I* region differences. Specific loci controlling *Lad* were identified in the *I-A* and *I-C* subregions (7). Recently, we identified a *Lad* locus expressed exclusively on T cells and mapping in the *I-J* subregion (8). Previous MLR studies with the *I-C* subregion also included incompatibility at *I-J* and *I-E* subregions (9).

By using recombinant strains of mice which differ for *I-C* (including *S* and *G*) subregion (A and A.AL; B10.AM and B10.A(2R)) or combinations with only *I-C* incompatibility [(A × C3H.OL)F_1 and A.AL], we have attempted to test for the existence of a *Lad* locus in this subregion. Our results show that *I-C* subregion gene(s) code for MLR determinants on T cells. *I-C* subregion also code for genes involved in the alloantigen-mediated MLR suppressor factors (10, 11), α-gene

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[1] *Abbreviations used in this paper:* AS, agarose and spleen cell absorbed, EDTA-treated; EHAA, modified Eagles-Hanks medium; FcR^+, Fc receptor positive, Glø, random linear terpolymer of L-glutamic acid, L-lysine, and L-phenylalanine; HBSS, Hanks balanced salt solution; Ia, immune response-associated; *Lad*, lymphocyte-activating determinant; MLR, mixed lymphocyte reaction; NMS, normal mouse serum.

[2] C. S. David et al. Manuscript submitted for publication.

[3] Cullen et al. Manuscript in preparation.

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for response to random linear terpolymer of L-glutamic acid, L-lysine, and L-phenylalanine (GLϕ) (12), determinants on Fc receptor positive (FcR$^+$)T cells (13) and possibly other T-cell functions.

Materials and Methods

Mice and Alloantisera. Inbred strains, recombinants, and F_1 hybrids used in this study were all produced in the Department of Genetics mouse colony at Washington University School of Medicine, St. Louis, Mo. A battery of anti-*Ia* and anti-*H-2* antisera were produced in our laboratories, as previously described (14).

Cell Preparation. Thymus cells, spleen cells, and lymph node cells were used as T-cell sources. Purification of T cells was performed by passing the lymph node cell suspensions through nylon wool columns (15).

Separation of B cells from spleen cells was performed as follows: spleen cells (5×10^7) were reacted with anti-Thy-1.2 serum (provided by the Transplantation Branch, National Institutes of Allergy and Infectious Diseases, Bethesda, Md.) at a final concentration of 10% antiserum in 5% fetal calf serum-containing RPMI 1640 medium. After 30 min at 37°C, the cells were washed twice with Hanks' balanced salt solution (HBSS). Rabbit complement was added and the mixture was incubated for an additional 40 min at 37°C, in a humidified atmosphere containing 5% carbon dioxide. For absorption of anti-Ia sera, bone marrow cells were used as a source of B cells (16).

Macrophages were collected by washing the normal mouse peritoneal cells with HBSS. Thereafter, the peritoneal cell suspension was placed into tissue culture dishes (Corning Glass Works, Corning, N.Y.) for 60 min and nonadherent cells removed. The adherent cells were collected and after one more 60 min incubation, were used as the source of macrophages.

Cytotoxic Test. Two-stage dye-exclusion microcytotoxic tests were performed as previously described (14).

Absorption of Anti-Ia Antisera. 100 λ of antiserum (B10.K × A.TL)F_1 anti-A (anti-*I-C^d*, *S^d*, *G^d*) was diluted 1/10 with a modified Eagles'-Hanks' medium (EHAA) (17) containing 2% human plasma, and adsorbed with 1×10^8 cells of (*a*) macrophages, (*b*) B cells (bone marrow cells), and (*c*) T cells, respectively, of B10.A(2R) (*I-C^d*) mice, at 37°C for 60 min. After incubation, supernates were assayed for their ability to inhibit MLR response by using B10.AM (*I-C^k*) T cells as responder and B10.A(2R) (*I-C^d*) T cells as stimulator.

Mixed Lymphocyte Reaction Assay. The medium employed in this assay was an EHAA medium supplemented with 2% human plasma. 5×10^5 viable responder cells were cultured with 1.0×10^6 mitomycin C-treated stimulator cells (18) in 0.2 ml of medium in microculture plates (Falcon 3040, BioQuest, BBL & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.). Incubation was performed at 37°C in a 5% carbon dioxide, 95% air, humidified atmosphere for 72 h. 2 μCi of [^{3}H]thymidine (sp. act 1.9 Ci per mmol, New England Nuclear, Boston, Mass.) was then added and the culture was harvested after a further 16 h. Within each experiment the mean and SD are calculated from three replicate wells. The experiments were repeated three to six times to check for repeatability of results and stimulation index.

Inhibition of MLR. MLR assays were done by the technique described above. 5×10^5 nylon wool-purified responder T cells were cocultured with 1.0×10^6 mitomycin C-treated, nylon wool-purified stimulator cells in the presence of 1.25% heat-inactivated alloantiserum, or normal control serum, in a total vol of 200 μl. Percent inhibition was calculated according to the following formula:

Percent inhibition =

$$\left(1 - \frac{\text{cpm of allogeneic culture in antiserum-cpm of syngeneic culture in NMS}}{\text{cpm of allogeneic culture in NMS-cpm of syngeneic culture in NMS}}\right) \times 100.$$

Treatment of Ia-Antisera and Complement. Nylon wool-purified T cells were reacted with antiserum or control normal mouse serum (NMS), at a final concentration of 10%, in RPMI-1640 containing 2.5% heat-inactivated human plasma. After incubation for 30 min at 37°C, the cells were washed and suspended in agarose- and spleen cell-absorbed, EDTA-treated (AS-absorbed)

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rabbit complement (19). After incubation for 90 min at 37°C, the dead cells were counted and separated from live cells on a Ficoll-Hypaque gradient. Dead cells in these tests were scored by a dye exclusion test. Only surviving cells, after the treatment by antisera and complement (and followed by mitomycin C treatment), were used as stimulator cells in these MLR tests. Culture media, cell numbers of stimulators and responders, the period of culture, and the method of harvesting were all the same as those described above. No antisera were added to the media for elimination studies. Percent inhibition was estimated by the formula:

$$\text{Percent inhibition} = \left(1 - \frac{\text{cpm of allogeneic culture of treated cells-cpm of syngeneic culture}}{\text{cpm of allogeneic culture-cpm of syngeneic culture}}\right) \times 100.$$

Results

Mixed Lymphocyte Reaction Across I-C Subregion. Strain combinations A-A.AL and B10.AM-B10.A(2R) were used in one-way MLR tests to detect *Lad* determinants controlled by the *I-C* subregion. These strains differ in *I-C*, *S*, and *G* regions (Table I). As shown in Table II, when lymph node cells were used as stimulators and responders, a significant MLR response was seen. In general, $I\text{-}C^d$ strains were better stimulators than the $I\text{-}C^k$ strains. To rule out the contribution of *S* and *G* regions (as well as *TL* and *Q* regions), (A × C3H.OL)F_1 animals were used as the responding strains and A.AL as the stimulating strains. Substantial stimulation was detected which should be due only to MLR determinants mapping in the *I-C* subregion. This experiment was repeated six times and similar results were obtained.

The Origin of the Stimulator Cell. Mitomycin C-treated stimulator cells from strain A and untreated responder lymph node cells from A.AL mice were cocultured to detect the population of cells stimulating across the *I-C* subregion. The stimulator cell populations used, were spleens, lymph nodes, thymuses, purified T cells and B cells. As shown in Table III thymus cells showed very weak stimulation and spleen cells displayed intermediate stimulation. Cocultures with the lymph node cells yielded stimulation index of 2.79.

Purified T cells and B cells were used in subsequent experiments. T cells were purified by nylon wool passage while B cells were purified by treatment with anti-Thy-1.2 serum. The stimulation index was about 1.05 when B cells were used. When nylon wool-purified T cells were employed as stimulators, the stimulation index increased to about 3.3. This result suggests that *Lad* determinants of *I-C* subregion are primarily expressed on T cells. This experiment was repeated three times with similar results.

Inhibition of MLR Response by Anti-Ia Sera. Nylon wool-purified T cells of (A × C3H.OL)F_1 hybrids were cocultured with nylon wool-purified mitomycin C-treated A.AL T cells in the presence of various anti-Ia sera (Table IV). Strong inhibition was observed in the presence of antisera A anti-A.AL (anti-$I\text{-}C^k$, S^k, G^k) and B10.S(9R) anti-B10.HTT (anti-$I\text{-}J^s$, $I\text{-}C^k$, S^k, G^k). Both of these antisera lack cytotoxic activity against unfractionated spleen cells but could contain antibodies against $I\text{-}C^k$ region products. However, antisera (C3H.Q × B10.D2)F_1 anti-AQR (anti-$I\text{-}A^k$, $I\text{-}B^k$, $I\text{-}J^k$, $I\text{-}E^k$) and C3H.OH anti-C3H.OL (anti-S^k, G^k), which lacked antibodies against antigens coded by *I-C* subregion, failed to give inhibition. These antisera could possibly contain antibodies to antigens coded

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TABLE I
H-2 Gene Complex of Selected Recombinants

Strain	H-2 Haplotype	H-2 Regions									
		K	*A*	*B*	*J*	*E*	*C*	*S*	*G*	*D*	*TL*
A	*a*	*k*	*k*	*k*	*k*	*k*	*d*	*d*	*d*	*d*	*a*
A.AL	*al*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*d*	*c*
C3H.OL	*o1*	*d*	*d*	*d*	*d*	*d*	*d*	*k*	*k*	*k*	*b*
C3H.OH	*o2*	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*k*	*b*
B10.AM	*h3*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*b*	*b*
B10.A(2R)	*h2*	*k*	*k*	*k*	*k*	*k*	*d*	*d*	?	*b*	*b*
B10.HTT	*t3*	*s*	*s*	*s*	*s*	*k*	*k*	*k*	*k*	*d*	*c*
B10.S(9R)	*t4*	*s*	*s*	?	*k*	*k*	*d*	*d*	*d*	*d*	*a*

TABLE II
MLR Responses of Lymph Node Cells among I-C Subregion Differing Strains

Responder	Stimulator*	Incompatible region	cpm of [^{3}H]TdR‡ (mean ± SD)	Stimulation indices
B10.AM	B10.AM	–	970.8 ± 131	–
B10.AM	B10.A(2R)	C, S, G	4,832.0 ± 420	5.0
B10.A(2R)	B10.A(2R)	–	3,201.6 ± 481	–
B10.A(2R)	B10.AM	C, S, G	5,442.9 ± 1,966	1.7
A	A	–	1,609.5 ± 224	–
A	A.AL	C, S, G	5,020.1 ± 439	3.1
A.AL	A.AL	–	2,381.6 ± 293	–
A.AL	A	C, S, G	7,918.5 ± 386	3.3
(A × C3H.OL)F_1	(A × C3H.OL)F_1	–	2,526.7 ± 174	–
(A × C3H.OL)F_1	A.AL	C	10,733.8 ± 709	4.2

* Mitomycin C-treated cells.
‡ Represents mean and SD of three replicate wells.

by *TL* and *Q* loci, but the stimulator and responder are compatible for these two regions. Further, anti-Thy-1.2 antiserum as well as anti-$H\text{-}2K^k$ and anti-$H\text{-}2D^d$ failed to inhibit. These results also suggest that MLR observed in the combination (A × C3H.OL)F_1 versus A.AL is also due to *I-C* gene products and that the antisera made across *I-C* incompatibility contain antibodies against *Lad* gene products of this region.

To confirm the above results with anti-$I\text{-}C^d$ sera, B10.AM cells were used as responders and B10.A(2R) cells as stimulators. In this combination *S* and *G* regions (and *TL* and *Q*) are also incompatible. All the antisera that could contain antibodies against $I\text{-}C^d$ region products inhibited the MLR. To test the target cell involved in this inhibition, antiserum (B10.K × A.TL)F_1 anti-A (anti-$I\text{-}C^d$, S^d, G^d) was absorbed with purified B cells, T cells, and macrophages, respectively. Antisera absorbed with bone marrow cells (B cells) and peritoneal macrophages of B10.A(2R) ($I\text{-}C^d$) still gave substantial levels of MLR inhibition. When the antiserum was absorbed with nylon wool-purified T cells from B10.A(2R) lymph nodes, no inhibition was observed. These results also suggest

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TABLE III
MLR Responses of Lymph Node Cells Stimulated by Thymocytes, Spleen Cells, Lymph Node Cells, B Cells, and T Cells Incompatible for I-C Subregion

Responder (lymph node cells)	Stimulator*	cpm of [^{3}H]TdR Incorporation	
		Experiment‡ (mean ± SD)	Stimulation indices
A.AL	A.AL (Spleen)	4,571.6 ± 283	–
A.AL	A (Spleen)	10,015.1 ± 734	2.19
A.AL	A.AL (Thymus)	1,786.3 ± 481	–
A.AL	A (Thymus)	1,933.2 ± 260	1.08
A.AL	A.AL (Lymph node)	4,467.9 ± 235	–
A.AL	A (Lymph node)	12,480.6 ± 791	2.79
A.AL	A.AL (B Cells)§	4,950.1 ± 312	–
A.AL	A (B Cells)	5,218.3 ± 1,071	1.05
A.AL	A.AL (T Cells)‖	4,162.6 ± 223	–
A.AL	A (T Cells)	13,766.0 ± 1,942	3.30

* Mitomycin C-treated cells.
‡ Mean and SD of three replicate wells.
§ Prepared by treatment of spleen cells with anti-Thy-1.2 + C′. About 90% cells were immunoglobulin-positive cells.
‖ Prepared by nylon wool column. About 90% cells were Thy-1.2 antigen positive.

that *I-C* gene products are expressed on T cells and not on B cells and macrophages.

In an attempt to rule out the possibility that antibodies against *S* and *G* region products are involved in the above MLR inhibitions, the antisera were absorbed with C3H.OL lymphocytes. C3H.OL ($I\text{-}C^d$, S^k, G^k) removes the inhibiting capacity of (B10.K × A.TL)F_1 anti-A (anti-$I\text{-}C^d$, S^d, G^d) but not B10.S(7R) anti-B10.HTT (anti-$I\text{-}E^k$, $I\text{-}C^k$, S^k, G^k). Thus, removal of antibodies against the *S* and *G* regions does not affect inhibition of *I-C* incompatible MLR. These results also indicate that putative antibodies against *TL* and *Q* region antigens do not cause inhibition.

Elimination of Stimulating Cells with Anti-Ia +C′. We next investigated the ability of various anti-Ia sera to eliminate the stimulating cell population in the presence of complement. Nylon wool-purified T cells of B10.AM mice were incubated with anti-Ia sera and rabbit complement. The dead cells were eliminated by centrifugation on Ficoll-Hypaque and the viable T cells were used as stimulators. As shown in Table V antisera directed against $I\text{-}C^k$ region products eliminated the stimulating cells. Antiserum (B10.A(4R) × C3H.OL)F_1 anti-B10.K could not contain antibodies against *TL* or *Q* region antigens. Antisera directed against $I\text{-}A^k$, $I\text{-}B^k$, $I\text{-}J^k$, $I\text{-}E^k$, S^k, G^k had no effect. These results suggest that the *I-C* gene products are expressed on a subpopulation of T cells which does not express the other *I* subregion products. As expected anti-$H\text{-}2K^k$, anti-$H\text{-}2D^b$, and anti-Thy-1.2 eliminated the MLR response.

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TABLE IV

Effects of Anti-Ia Sera on MLR Responses between A.AL T Cells as Stimulator and T Cells of (C3H.OL × A)F_1 Hybrids as Responder

Responder*	Stimulator‡	Treatment§	Region	[^{3}H]TdR Incorporation (mean ± SD)‖	Inhibition¶
					%
A × C3H.OL	A × C3H.OL	NMS		635.0 ± 120	–
A × C3H.OL	A.AL	NMS		3,281.3 ± 278	–
A × C3H.OL	A.AL	B10.S(7R) Anti-B10.HTT	(E, C, S, G)^k	1,660.5 ± 234	61
A × C3H.OL	A.AL	A Anti-A.AL	(C, S, G)^k	2,071.1 ± 563	46
A × C3H.OL	A.AL	B10.S(9R) Anti-B10.HTT	J^s (C, S, G)^k	1,254.7 ± 310	77
A × C3H.OL	A.AL	(C3H.Q × B10.D2)F_1 Anti-AQR	(A, B, J, E)^k	2,890.9 ± 237	15
A × C3H.OL	A.AL	A.TL Anti-A.AL	K^k	2,658.3 ± 325	24
A × C3H.OL	A.AL	(B10.K × A.TL)F_1 Anti-A	(C, S, G)^d	2,850.3 ± 180	26
A × C3H.OL	A.AL	C3H.OH Anti-C3H.OL	(S, G)^k	2,973.1 ± 268	12
A × C3H.OL	A.AL	(B10.AKM × ASW)F_1 Anti-A.TH	D^d	3,146.0 ± 519	5
A × C3H.OL	A.AL	Anti-Thy.1.2		3,011.7 ± 271	10

* Nylon wool-purified T cells.
‡ Mitomycin C-treated T cells.
§ Serum at 1.25% final dilution in the culture.
‖ Mean and SD from three replicate wells.
¶ $\text{Inhibition} = \left(1 - \frac{\text{allogeneic MLR in anti-Ia sera-syngeneic MLR in NMS}}{\text{allogeneic MLR in NMS} - \text{syngeneic MLR in NMS}}\right) \times 100.$

Discussion

Specificity Ia.6 was defined as a private specificity of *H-2^d* haplotype mapping in the *I-C^d* subregion. Haplotype *H-2^a* was positive for Ia.6 which suggested the expression of *I-C^d* segment in that strain. Ia.6 specificity exhibited several unique qualities. Only 15–20% of lymph node cells were lysed by anti-Ia.6 and the antibodies were detected only in the early bleedings and were IgM in nature (20). The antisera reacted with some T-cell lymphomas. After the original antisera was exhausted we were unable to produce other sera with cytotoxic activity. Further, we were unable to detect Ia.6 by immunoprecipitation assay by using spleen cells (Cullen, unpublished observations). These results suggested that Ia.6 may be a T-cell specificity. Recent results indicate that Ia.6 is expressed on a subpopulation of T cells from *I^d* strains mapping in *I-C*. *H-2^a* haplotypes and the recombinants derived from it express this antigen.

Recent results on the mapping of Ia.22 and Ia.23 further clarify the definition

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TABLE V
Anti-Ia Elimination of MLR Response Among B10.A(2R) T Cells as Responder and B10.AM as Stimulator

Responder*	Stimulator‡	Pretreatment§	Region	[³H]TdR Incorporation (mean ± SD)	Inhibition
					%
B10.A(2R)	B10.A(2R)	NMS + C′	(−)	4,324.3 ± 459	–
B10.A(2R)	B10.AM	NMS + C′	(−)	7,386.0 ± 521	–
B10.A(2R)	B10.AM	(B10.A(4R) × C3H.OL)F_1 Anti-B10.K + C′	$(B, J, E, C)^k$	4,056.6 ± 108	100
B10.A(2R)	B10.AM	B10.S(7R) Anti-B10.HTT + C′	$J^s(C, S, G)^k$	4,418.5 ± 317	97
B10.A(2R)	B10.AM	C3H.OH Anti-C3H.OL + C′	$(S, G)^k$	7,035.8 ± 561	11
B10.A(2R)	B10.AM	(A.TH × B10.HTT)F_1 Anti-A.TL + C′	$(A, B, J)^k$	6,703.2 ± 837	22
B10.A(2R)	B10.AM	B10.A(3R) Anti-B10.A(5R) + C′	J^k	6,814.1 ± 710	19
B10.A(2R)	B10.AM	A.TL Anti-A.AL + C′	K^k	3,976	100
B10.A(2R)	B10.AM	Anti-Thy-1.2 + C′		3,033	100

* Nylon wool-purified T cells.
‡ Mitomycin C-treated T cells.
§ Stimulator cell treated with 1/10 serum plus AS-absorbed rabbit complement, then washed.

of *I-E* and *I-C* subregions. Sequential immunoprecipitation experiments suggest that Ia.22 and Ia.7 are expressed on the same molecule.[3] Further, Ia.23 and Ia.7 are also expressed on the same molecule.[2] The map positions of specificities Ia.7, 22 and 23 seem to fall within the *I-E* subregion, Ia.7 being a public specificity while Ia.22 and 23 are private specificities of I^k and I^d strains, respectively. Thus specificities with predominant expression on B cells map within the *I-E* subregion while Ia.6 expressed on T cells map within the *I-C* subregion.

The mixed lymphocyte reaction studies in strain combination of A-A.AL and B10.AM-B10.A(2R) indicate a *Lad* locus controlled by the *I-C* subregion. When (A × C3H.OL)F_1 is used as responder and A.AL as stimulator, the incompatibility should only be at *I-C* subregion. Antisera made across *I-C* incompatible strains which do not exhibit cytotoxic activity on spleen cells can inhibit the MLR reaction. Even though some of these antisera could contain antibodies against gene products of *TL* and *Q* regions, they do not seem to be involved in the inhibition. The antibodies are directed against the MLR determinants on the stimulator cells. Further, absorption of these antisera with purified T cells removes the inhibiting ability while purified B cells and macrophages do not seem to play a role.

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Our results and those of others suggest that *I-C* subregion gene(s) codes for products expressed selectively on a subpopulation of T cells. The following immunological traits have been mapped to the *I-C* subregion; (*a*) α-gene for response to GLϕ; (*b*) gene controlling generation and acceptance of alloantigen-mediated suppressor factors; (*c*) determinants on FcR^+ T cells; (*d*) specificity Ia.6, and (*e*) MLR simulating determinants. The number of genes involved in the control or regulation of the above phenomena remain to be seen.

Summary

A new lymphocyte-activating determinant (*Lad*) locus expressed on T cells was identified, mapping in the *I-C* subregions of $H\text{-}2^k$ and $H\text{-}2^d$ haplotypes. The mixed lymphocyte reaction stimulation could be inhibited by anti-Ia sera made in strains incompatible for this chromosomal segment. Experiments with purified lymphocyte cell populations suggested that this *Lad* locus was expressed on T cells. Further, only purified T cells were able to remove the inhibiting activity from the anti-Ia sera. *I-C* subregion gene(s) seem to code for products selectively expressed on a subpopulation of T cells.

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