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), 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-2K (Ia.6−) and H-2D (Ia.6+) haplotypes for this subregion (2). I-E subregion was defined by specificity Ia.22, positive in H-2K and negative in H-2D (4). Recently, we identified a new private Ia specificity (Ia.23) of H-2D origin, also mapping in I-E subregion. Sequential precipitation studies suggest that specificities Ia.7, 22 (H-2K) and Ia.7, 23 (H-2D) are expressed on products coded by I-E subregion gene(s). 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]F1 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, Glc, 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.

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for response to random linear terpolymer of L-glutamic acid, L-lysine, and L-
phenylalanine (GLcb) (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 F1 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 µl of antisera (B10.K × A.TL)F1 anti-A (anti-I-C^b, S^b,
G^b) 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^v) 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
[3H]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 alloantisera, or normal
control serum, in a total vol of 200 µl. Percent inhibition was calculated according to the following
formula:

\[
\text{Percent inhibition} = \left(1 - \frac{\text{cpm of allogeneic culture in antiserum-cpm of syngeneic culture in NMS}}{\text{cpm of allogeneic culture in NMS-cpm of syngeneic culture in NMS}}\right) \times 100.
\]

**Treatment of Ia-Antisera and Complement.** Nylon wool-purified T cells were reacted with
antisera 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)
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:

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

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-C\(d\) strains were better stimulators than the I-C\(k\) strains. To rule out the contribution of S and G regions (as well as TL and Q regions), \((A \times C3H.OL)F_1\) 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 \((A \times C3H.OL)F_1\) 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-C\(k\), S\(k\), G\(k\)) and B10.S(9R) anti-B10.HTT (anti-I-J\(k\), I-C\(k\), S\(k\), G\(k\)). Both of these antisera lack cytotoxic activity against unfractonated spleen cells but could contain antibodies against I-C\(k\) region products. However, antisera \((C3H.Q \times B10.D2)F_1\) anti-AQR (anti-I-A\(k\), I-B\(k\), I-J\(k\), I-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
### TABLE I

| Strain      | H-2 Haplotype | H-2 Regions |
|-------------|---------------|-------------|
|             | K  | A  | B  | J  | E  | C  | S  | G  | D  | TL |
| A           | a  | k  | k  | k  | k  | d  | d  | d  | d  | a  |
| A.AL        | a1 | k  | k  | k  | k  | k  | k  | d  | d  | c  |
| C3H.OL      | a2 | d  | d  | d  | d  | k  | k  | k  | b  |
| C3H.OH      | a3 | d  | d  | d  | d  | d  | d  | d  | k  | b  |
| B10.AM      | a4 | k  | k  | k  | k  | d  | d  | d  | b  | b  |
| B10.A(2R)   | a5 | k  | k  | k  | k  | d  | d  | ?  | b  | b  |
| B10.HTT     | a6 | s  | s  | s  | s  | k  | k  | k  | d  | c  |
| B10.S(9R)   | a7 | s  | s  | ?  | k  | d  | d  | d  | a  |  

### TABLE II

| 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.AL             | 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)F₁  | (A × C3H.OL)F₁ | – | 2,526.7 ± 174 | – |
| (A × C3H.OL)F₁  | 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-2K$^a$ and anti-H-2D$^d$ failed to inhibit. These results also suggest that MLR observed in the combination (A × C3H.OL)F₁ 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-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-C$^d$ region products inhibited the MLR. To test the target cell involved in this inhibition, antiserum (B10.K × A.TL)F₁ anti-A (anti-I-C$^d$, S$^d$, G$^d$) was absorbed with purified B cells, T cells, and macrophages, respectively. Antiserum absorbed with bone marrow cells (B cells) and peritoneal macrophages of B10.A(2R) (I-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...
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-C\(^{a}\), S\(^{a}\), G\(^{a}\)) removes the inhibiting capacity of (B10.K \(\times\) A.TL)\(F_{1}\) anti-A (anti-I-C\(^{a}\), S\(^{a}\), G\(^{a}\)) but not B10.S(7R) anti-B10.HTT (anti-I-E\(^{k}\), I-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-C\(^{k}\) region products eliminated the stimulating cells. Antiserum (B10.A(4R) \(\times\) C3H.OL)\(F_{1}\) anti-B10.K could not contain antibodies against TL or Q region antigens. Antisera directed against I-A\(^{k}\), I-B\(^{k}\), I-J\(^{k}\), I-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-2K\(^{k}\), anti-H-2D\(^{k}\), and anti-Thy-1.2 eliminated the MLR response.
**Table IV**

*Effects of Anti-Ia Sera on MLR Responses between A.AL T Cells as Stimulator and T Cells of (C3H.OL × A)F₁ Hybrids as Responder*

| Responder* | Stimulator‡ | Treatment§ | Region | [³H]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) | 1,660.5 ± 234 | 61 |
| A × C3H.OL | A.AL        | Anti-A.AL  | (C, S, G) | 2,071.1 ± 563 | 46 |
| A × C3H.OL | A.AL        | B10.S(9R) Anti-B10.HTT | J*(C, S, G) | 1,254.7 ± 310 | 77 |
| A × C3H.OL | A.AL        | (C3H.Q × B10.D2)F₁ Anti-AQR | (A, B, J, E) | 2,890.9 ± 237 | 15 |
| A × C3H.OL | A.AL        | A.TL Anti-A.AL | K* | 2,658.3 ± 325 | 24 |
| A × C3H.OL | A.AL        | (B10.K × A.TL)F₁ Anti-A | (C, S, G) | 2,850.3 ± 180 | 26 |
| A × C3H.OL | A.AL        | C3H.OH Anti-C3H.OL | (S, G) | 2,973.1 ± 268 | 12 |
| A × C3H.OL | A.AL        | (B10.AKM × ASW)F₁ Anti-A.TH | D² | 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.

Discussion

Specificity Ia.6 was defined as a private specificity of H-2d haplotype mapping in the I-C² subregion. Haplootype H-2a was positive for Ia.6 which suggested the expression of I-C² 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 cytototoxic 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 Ia strains mapping in I-C. H-2a 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
A NEW LYMPHOCYTE-ACTIVATING DETERMINANT LOCUS

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 | [%H]Tdr In- | 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)F₁ Anti-B10.HTT + C' | (B, J, E, C)⁺ | 4,056.6 ± 108 | 100       |
| B10.A(2R)  | B10.AM      | B10.S(7R) Anti-B10.HTT + C' | J⁺(C, S, G) | 4,418.5 ± 317 | 97        |
| B10.A(2R)  | B10.AM      | C3H.OL Anti-C3H.OL + C' | (S, G)⁺ | 7,035.8 ± 561 | 11        |
| B10.A(2R)  | B10.AM      | (A.TH × B10.HTT)F₁ Anti-A.TL + C' | (A, B, J)⁺ | 6,703.2 ± 837 | 22        |
| B10.A(2R)  | B10.AM      | B10.A(3R) Anti-B10.A(3R) + C' | J⁺ | 6,814.1 ± 710 | 19        |
| B10.A(2R)  | B10.AM      | A.TL Anti-A.AL + C' | K⁺ | 3,976 ± 710 | 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. Further, Ia.23 and Ia.7 are also expressed on the same molecule. 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 Iₖ and I₄ 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)F₁ 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.
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 GL6; (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-2k and H-2d 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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