ALLOTYPE-SPECIFIC ANALYSIS OF ANTI-(TYR,GLU)-ALA–LYS ANTIBODIES PRODUCED BY Ir-1A HIGH AND LOW RESPONDER CHIMERIC MICE*

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Katz et al. (1) have demonstrated a restriction in lymphoid cell interaction when the antigen used is under immune response (Ir) gene control. T cells from (low responder × high responder) F1 mice primed to the terpolymer L-glutamic acid, l-lysine, l-tyrosine (GLT) can collaborate with 2,4-dinitrophenyl (DNP)-primed B cells from the Ir-GLT high responder but not low responder strain in response to DNP-GLT (1). In contrast are the studies of Bechtol et al. and Bechtol and McDevitt (2, 3), who examined the antibody responses of tetraparental mice immunized with the synthetic polypeptide poly-L(Tyr,Glu)-poly d,L-Ala–poly-L-Lys ((T,G)-A–L), an antigen under Ir-lA genetic control. Several tetraparental mice produced anti-(T,G)-A–L antibody of low responder strain immunoglobulin (Ig) allotype (2, 3). These results indicated that the Ir-lA gene was not expressed in B cells and implied that interactions among genetically dissimilar cell populations could occur when tolerance existed to H-2 antigenic differences. Recent studies with bone marrow cell chimeric mice have shown that chimeric T cells can interact with H-2 histoincompatible B cells in response to antigens not under Ir gene control (4-6).

To clarify whether lymphoid cell chimerism, with presumed tolerance to H-2 incompatibility, would permit effective cell interactions in response to antigens under Ir gene control, bone marrow cell chimeric mice were prepared by using strains differing both for Ig allotype and for high versus low responsiveness to (T,G)-A–L. An antigen-specific and allotype-specific antibody assay was used to discriminate the responses produced by high and low responder strain B cells in these chimeras. The results suggest that lymphoid cell chimerism per se is not sufficient to obviate Ir gene-mediated restrictions in cell interaction.

Materials and Methods

Animals, Immunization. C3H-SW (CSW, H-2a, Igα), CWB (H-2b, Igα), CKB (H-2k, Igβ), C3H/ DiSn (H-2k, Igα), (CSW × C3H)F1, and (CKB × CSW)F1 congenic mice were obtained from the breeding colonies maintained at Stanford University. CKB ↔ CSW bone marrow cell chimeric mice were prepared by the method of von Boehmer et al. (4). 8 wk old (CSW × C3H)F1 mice were irradiated with 800 rads from a 250 kV, 15 mA Phillips X-ray generator. Bone marrow cells were flushed from the femurs of CKB and CSW adults by using Dulbecco's phosphate-buffered saline (PBS) containing 5% fetal calf serum (FCS). T cells were eliminated by incubating 10⁶ bone marrow...
cells with 1 ml of a 1:5 dilution of AKR anti-C3H (anti-Thy 1.2) thymocyte serum in PBS-FCS for 25 min at 4°C. The anti-Thy 1.2-treated bone marrow cells were washed and incubated in 3 ml of a 1:8-1:12 dilution of agarose absorbed rabbit complement in Dulbecco's modified Eagles medium for 45 min at 37°C. Irradiated (CSW × C3H)F~ mice were injected with 5 × 10^6 washed bone marrow cells from each donor (CKB, CSW) i.v. 8 wk later, these CKB ↔ CSW chimeric mice were bled for preimmune sera, then immunized i.p. with 10 μg of (T,G)-A - L-52 in complete Freund's adjuvant. As controls, 8 to 12-wk old CKB, CSW, and (CKB × CSW)F~ mice were also immunized. 1 mo after priming, a secondary challenge of 10 μg of (T,G)-A - L-52 was administered i.p. in PBS, and the mice were bled for secondary sera 8 days later.

**Allotype-Specific Radioimmunoassay for Anti-(T,G)-A - L Antibody.** A modification of the antigen-specific plate radioimmunoassay described by Klinman et al. (7) was used to measure serum anti-(T,G)-A - L antibodies. The wells of flexible plastic microtiter plates (Scientific Products Inc.) were coated with 20 μl of (T,G)-A - L-52 at a concentration of 0.05 mg/ml in PBS. This antigen concentration was sufficient to saturate the wells for antigen, since higher concentrations did not alter the antibody binding curves. After incubation with antigen for 2 h at room temperature, the antigen-coated plates were washed twice with PBS containing 5% agamma horse serum (PBS-HS). The wells were then incubated with 20 μl of serum dilutions prepared in PBS containing 1% bovine serum albumin (PBS-BSA) for 3 h at room temperature, washed twice with cold PBS-HS, and incubated overnight at 4°C with 20 μl per well of the 125I-labeled detecting reagent (anti-allotype antibody).

After washing the plates four times with cold PBS-HS, the wells were cut out of the plates and counted individually in a gamma counter for 0.4 or 1.0 min. Variation in duplicate 125I-counts bound generally did not exceed 15%.

Allotype-specific, 125I-labeled mouse antibodies against Ig-1a (IgG2a), Ig-1b (IgG2a), Ig-4a (IgG1), and Ig-4b (IgG1) Ig allotypes were prepared and generously provided by Dr. Len Herzenberg, Mr. Vernon Oi, and Mr. Derek Hewgill (Stanford University School of Medicine) who have described the methodology for the preparation of these reagents in a separate communication (8). The specificity of Ig allotype detection with these reagents was analyzed in the (T,G)-A - L-specific antibody binding assay, and the results are described below. Pooled secondary sera from CSW and CKB mice immunized with (T,G)-A - L-52, which have been used routinely in this laboratory as standards for antibody analysis by a modified Farr assay (2, 3) were used as the source of standard anti-(T,G)-A - L antibodies for the allotype-mixing studies described below.

**Results**

An analysis of the Ig allotype-specific detection of anti-(T,G)-A - L antibodies in mixtures of CSW (Ig a) and CKB (Ig b) secondary sera, is shown in Table I. CSW and CKB mice, both H-2 b, are high-responders to (T,G)-A - L, whereas CKB mice (H-2 k) are low-responders. The 100% Ig a (CSW) and 100% Ig b (CWB, CKB) columns represent the counts bound of the four iodinated anti-allotype reagents, beginning with a 1:100 anti-(T,G)-A - L serum dilution, with doubling dilutions thereafter. The 125I-labeled anti-Ig a allotype antibodies do not bind significantly to (T,G)-A - L-specific antibodies from CSW (Ig a) mice, nor do the iodinated anti-Ig a allotype antibodies bind to Ig a allotype anti-(T,G)-A - L antibodies from either the high-responder (CWB) or low-responder (CKB) mice. There is a quantitative difference in the amount of anti-(T,G)-A - L antibody produced by CWB versus CKB mice, in both the IgG1 (Ig-4 b) and IgG2a (Ig-1 b) class. The values in Table I obtained for the binding and detection of 100% Ig a (CSW) and 100% Ig b (CWB) allotype anti-(T,G)-A - L antibodies can also be plotted as standard curves, such that 20 μl of a 1:100 antisera dilution is designated 20 × 10⁻² μl, a 1:200 dilution is 10 × 10⁻³ μl, and so forth, versus 125I-anti-allotype-counts bound. A linear correlation is then observed between iodinated anti-allotype counts bound and antibody concentration at high serum dilutions, where the detecting
### Table I

**Allotypic Detection of Anti-(T,G)-A-L Antibody in Serum Mixtures**

| mAB-detecting reagent | Log serum dilution | 100% Igα | 100% Igβ | 20% Igα/80% Igβ | 40% Igα/60% Igβ | 60% Igα/40% Igβ | 80% Igα/20% Igβ |
|------------------------|-------------------|---------|---------|-----------------|-----------------|-----------------|-----------------|
|                        |                   | CSW     | CWB     | CKB             | CSW + CKB       | CSW + CKB       | CSW + CKB       |
| Anti-Igα in 1:1 dilution |                   | 1.1775  | 1.925   | 1.688           | 1.986           | 1.889           | 2.027           |
|                        |                   | 1.1845  | 1.982   | 1.746           | 1.959           | 1.847           | 2.009           |
|                        |                   | 1.1774  | 1.951   | 1.765           | 1.938           | 1.830           | 1.957           |
|                        |                   | 1.1803  | 1.997   | 1.787           | 1.970           | 1.867           | 2.005           |
|                        |                   | 1.1872  | 2.019   | 1.809           | 2.001           | 1.889           | 2.037           |
|                        |                   | 1.1717  | 1.986   | 1.818           | 2.076           | 1.855           | 2.025           |
|                        |                   | 1.1778  | 2.013   | 1.815           | 2.054           | 1.878           | 2.034           |
|                        |                   | 1.1840  | 2.027   | 1.835           | 2.096           | 1.897           | 2.066           |
|                        |                   | 1.1817  | 2.048   | 1.852           | 2.170           | 1.944           | 2.136           |
|                        |                   | 1.1776  | 2.061   | 1.864           | 2.213           | 1.987           | 2.207           |
|                        |                   | 1.1836  | 2.087   | 1.885           | 2.275           | 2.013           | 2.267           |

* A 1:100 dilution of secondary CSW anti-(T,G)-A-L serum was mixed with a 1:100 dilution of either CWB or CKB anti-(T,G)-A-L serum to give allotype ratios indicated.

‡ 20 µl of detecting reagent added per assay: anti-Ig-4α, 20136 counts; anti-Ig-4β, 20,886 counts; anti-Ig-1α, 18,171 counts; and anti-Ig-1β, 19,931 counts, all per 0.4 min.

§ First serum dilution is 1:100; doubling dilutions thereafter.

The binding of Igα allotype anti-(T,G)-A-L antibody to the antigen-coated plate can thus be detected in the presence of binding by Igβ allotype antibody.
**Table II**

**Analysis of Anti-(T,G)-A-L Antibody Produced by Chimeric Mice**

| Serum | Anti-(T,G)-A-L antibody | Production of LR allotype |
|-------|------------------------|---------------------------|
| Donor | Log 10 Anti-1^a | Log 10 Anti-1^a | Log 10 Anti-1^a | Log 10 Anti-1^a | Log 10 Anti-1^a | Log 10 Anti-1^a | Log 10 Anti-1^a | Log 10 Anti-1^a |
|       | reagent                |                           |                           |                           |                           |                           |                           |                           |
| CKB → CSW Chimeras | |
| No. 1 | 1 278 148 206 51  | 2 168 184 149 57  | 3 148 144 154 56  | 4 98 145 144 69  | |
| No. 2 | 1 1,604 168 272 140  | 2 870 108 488 111  | 3 517 159 282 65  | 4 306 202 210 76  | |
| No. 3 | 1 2,174 223 777 77  | 2 1,197 168 430 48  | 3 649 184 275 57  | 4 469 142 247 57  | |
| No. 4 | 1 2,129 214 1,880 116  | 2 1,247 197 1,530 66  | 3 679 182 1,049 61  | 4 446 155 548 49  | |
| No. 5 | 1 963 246 1,080 71  | 2 556 206 285 74  | 3 343 200 355 65  | 4 230 176 311 47  | |
| CKB × CSW F1 | |
| No. 1 | 1 379 661 237 1,185  | 2 239 390 145 650  | 3 152 254 127 370  | 4 158 246 159 247  | |
| No. 2 | 1 5,630 175 1,998 124  | 2 6,076 267 2,164 161  | |

*Chimeric mice were immunized 8 wk after reconstitution with 10 μg (T,G)-A-L in PBS i.p. and bleed 8 days later. F1, CKB, and CSW mice immunized at same time. Starting serum dilution 1:100; doubling dilutions thereafter.

1 Counts for anti-1^a and anti-1^a are counts per minute; for anti-4^a and anti-4^a, counts per 1 min. 20 μl of detecting reagent (anti-allotype antibody) added per well; anti-1^a and anti-1^a, 36,836 and 9,530 counts/minute, respectively; anti-4^a and anti-4^a each 20,990 counts/0.4 min.

Furthermore, at least 20% of either Ig allotype can be detected in these mixtures. For example, there is a clear distinction in the counts bound of either anti-Ig-1^a or Ig-4^a antibody for the 80% Ig^a/20% Ig^a mixture of (CSW + CKB) versus (CSW + CKB). When standard curves are plotted as described above, there is a good correlation between the observed and expected anti-allotype counts bound in the two mixtures at varying Ig allotype ratios, by using the serum dilutions which correspond to the linear portions of the curves.

Table II shows the results obtained from the Ig allotype-specific analysis of anti-(T,G)-A-L antibody produced by five CKB → CSW chimeric mice. All of the chimeric mice had Ig of both Ig^a and Ig^a allotype in their preimmune sera by Ouchterlony gel diffusion analysis. The plate assay was used to determine whether any of these chimeras made IgG1 or IgG2a anti-(T,G)-A-L antibody of low-responder allotype (Ig^a) in amounts greater than those produced by low-responder CKB mice. Secondary sera from CKB, CSW, and (CKB × CSW)F1.
mice were included as controls. Four of five F₁ mice made detectable anti-(T,G)-A-L antibody, and these four produced antibody of low-responder Ig allotype (Ig⁸) in amounts greater than the CKB control mice. Four of the five chimeric mice also made anti-(T,G)-A-L antibody, but none made antibody of Ig⁷ (low-responder) allotype in amounts greater than the CKB control mice. The anti-(T,G)-A-L antibody produced by these chimeras was of high-responder strain Ig allotype (Ig⁸).

Discussion

Lymphoid cell chimerism and antigen-priming in the presence of both high and low responder strain H-2 antigens do not appear sufficient to overcome the genetic restrictions on cell interaction imposed by Ir-IA gene regulation. None of the CKB ↔ CSW chimeras produced anti-(T,G)-A-L antibody of low responder strain Ig allotype in amounts significantly greater than those produced by control low responder CKB mice. This Ir-IA gene-mediated restriction has also been observed in adoptive cell transfer studies with DNP-primed B cells from Ir-IA low responder BALB.K mice and (T,G)-A-L primed cells from either (BALB.B × BALB.K)F₁ or BALB.B ↔ BALB.K chimeric mice in response to DNP-(T,G)-A-L (9). The chimeras used in this study were not examined for their ratios of parental H-2 haplotypes. However, in other studies with BALB.B ↔ BALB.K chimeras, the spleen cells routinely have been 50:50 mixes of both parental H-2 haplotypes. Since Ig⁻ and Ig⁷ allotypes could be detected in the CKB ↔ CSW chimeras’ preimmune sera, B cells from both strains were apparently reconstituted. It could be argued that due to concomitant allotypic as well as Ir-IA genetic disparity, there was insufficient repopulation by low responder T cells and/or macrophages, hence the inability of chimeric low-responder B cells to be stimulated by (T,G)-A-L. This argument would imply that chimeric low responder B cells cannot interact with chimeric histoincompatible high responder T cells and macrophages in response to an antigen under Ir gene control. It is presumed that the production of high responder strain Ig allotype anti-(T,G)-A-L antibody by the chimeras is due to the interaction of CSW lymphoid cells, and not endogenously repopulated F₁ cells, but this has not been directly demonstrated. If the stimulation of chimeric high responder B cells occurs via interactions with F₁ cells, then the results of this study are analogous to those reported by Katz et al. (1).

The results of this study differ from the tetrarenteral mouse studies by Bechtol et al. (2, 3). Several of the original C3H ↔ (CKB × CWB)F₁, tetrarenteral anti-(T,G)-A-L-509 sera were therefore analyzed by the allotype-specific plate assay. None contained anti-(T,G)-A-L antibody of low responder strain Ig⁸ allotype in amounts greater than those detected in antisera of low responder C3H mice. However, some of the original (CWB × C3H)F₁ antisera also did not contain significant antibody levels of low responder Ig⁸ allotype, in contrast to the (CKB × CSW)F₁ control mice used in this study. The tetrarenteral antisera were originally analyzed by absorption onto and acid elution from anti-Ig⁸ allotype-coupled Sepharose (2, 3). The passed and eluted fractions were then analyzed by an antigen-specific, but not allotype-specific Farr assay.

1 J. Press and H. McDevitt. Manuscript in preparation.
When the remaining acid eluted fractions of the tetraparental sera were analyzed by the allotype-specific plate assay, antibody of high responder Ig\(\text{b}\) allotype was detected in varying amounts, but little antibody of low responder Ig\(\text{a}\) allotype was observed (J. Press, unpublished observations). Further studies of new chimeric and tetraparental mice will be required to resolve this paradox and clarify the mechanisms underlying \(Ir\) gene regulation.

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