Brief Definitive Report

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

By JOAN L. PRESS‡ and HUGH O. McDEVITT

(From the Division of Immunology, Department of Medicine, Stanford University, School of Medicine, Stanford, California 94305)

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) F_1 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-1A* 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-1A* 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-2*^{b}, *Ig*^{a}), CWB (*H-2*^{b}, *Ig*^{b}), CKB (*H-2*^{k}, *Ig*^{b}), C3H/DiSn (*H-2*^{k}, *Ig*^{a}), (CSW × C3H)F_1, and (CKB × CSW)F_1 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)F_1 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 adult mice by using Dulbecco's phosphate-buffered saline (PBS) containing 5% fetal calf serum (FCS). T cells were eliminated by incubating 10^8 bone marrow

* Supported by National Institutes of Health grant no. AI 07757.

‡ Recipient of an Arthritis Foundation Postdoctoral Fellowship, present address: Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Mass. 02154.

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_1 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_1 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 ^{125}I-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 ^{125}I-counts bound generally did not exceed 15%.

Allotype-specific, ^{125}I-labeled mouse antibodies against *Ig-1*^{a} (IgG_{2a}), *Ig-1*^{b} (IgG_{2a}), *Ig-4*^{a} (IgG_1), and *Ig-4*^{b} (IgG_1) 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 CWB mice immunized with (T,G)-A–L-509, 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 CWB (*Ig*^{b}), and mixtures of CSW and CKB (*Ig*^{b}) secondary sera, is shown in Table I. CSW and CWB 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 ^{125}I-labeled anti-Ig^b 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^b 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 IgG_1 (Ig-4^b) and IgG_{2a} (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 antiserum dilution is designated 20×10^{-2} μl, a 1:200 dilution is 10×10^{-2} μl, and so forth, versus ^{125}I-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 Mixures**

^{125}I-detecting reagent‡	Log_2 serum dilution§	100%Ig^a	100%Ig^b	100%Ig^b	20%Ig^a/80%Ig^b		40%Ig^a/60%Ig^b		60%Ig^a/40%Ig^b		80%Ig^a/20%Ig^b	
		CSW	CWB	CKB	CSW + CWB	CSW + CKB	CSW + CWB	CSW + CKB	CSW + CWB	CSW + CKB	CSW + CWB	CSW + CKB
Anti-Ig-4^a	1	2,107	124	79	1,732	1,807	1,914	1,847	1,895	1,869	1,886	2,027
	2	1,845	91	78	1,595	1,686	1,972	1,883	1,905	1,897	1,747	1,838
	3	1,774	95	94	1,382	1,383	1,645	1,656	1,680	1,836	1,778	1,757
	4	1,821	90	67	996	873	1,478	1,467	1,627	1,584	1,727	1,739
	5	1,472	88	72	631	589	990	894	1,305	1,195	1,517	1,446
	6	1,171	67	73	395	342	614	593	814	778	1,035	913
	7	776	106	66	213	239	388	375	520	554	672	609
	8	548	66	87	188	193	315	258	353	332	471	452
Anti-Ig-4^b	1	194	3,213	706	3,257	668	3,213	552	3,024	384	2,797	319
	2	172	3,044	361	3,004	417	2,966	347	2,704	291	2,522	261
	3	112	2,999	237	2,873	255	2,594	244	2,374	190	1,861	173
	4	101	2,612	167	2,498	152	2,180	120	1,835	102	1,246	116
	5	55	2,016	89	1,749	114	1,372	87	1,040	108	664	84
	6	56	1,260	82	1,070	96	850	74	589	71	385	64
	7	53	791	77	670	74	505	75	343	58	205	64
	8	52	490	49	450	54	340	58	233	61	181	58
Anti-Ig-1^a	1	11,851	126	101	4,588	4,775	7,202	7,544	9,187	8,906	9,847	10,389
	2	8,013	80	61	2,848	2,842	4,434	4,605	5,961	5,835	6,745	6,863
	3	5,620	86	50	1,724	1,603	2,999	3,013	3,861	3,700	4,627	4,213
	4	3,604	74	87	1,026	1,025	1,745	1,607	2,406	2,260	2,716	2,687
	5	2,123	75	73	666	641	1,020	1,025	1,434	1,313	1,779	1,747
	6	1,358	90	69	428	409	741	643	949	880	1,107	1,016
	7	889	61	62	297	272	469	474	609	595	669	688
	8	621	87	77	218	215	353	381	479	447	583	508
Anti-Ig-1^b	1	98	9,854	1,278	8,467	1,655	7,094	1,604	4,930	1,172	3,350	634
	2	161	6,358	742	5,269	787	4,147	851	2,921	700	1,950	349
	3	108	3,703	373	3,095	403	2,529	405	1,559	319	1,052	246
	4	99	2,167	279	1,919	252	1,344	233	974	224	671	147
	5	102	1,200	162	1,256	173	745	141	572	148	323	115
	6	77	695	108	613	132	434	104	327	84	249	80
	7	72	426	105	371	104	277	87	228	90	155	64
	8	80	322	108	264	90	212	104	172	88	148	82

* A 1:100 dilution of secondary CSW anti-(T,G)-A–L-509 serum was mixed with 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-4^a, 20136 counts, anti-Ig-4^b, 20,986 counts, anti-Ig-1^a, 18,171 counts, and anti-Ig-1^b, 19,931 counts, all per 0.4 min.

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

reagent is in excess. Saturated (plateau) conditions are observed at lower serum dilutions (e.g. 1:100) (curves not shown).

Table I also demonstrates that in mixtures of anti-(T,G)-A–L antibodies from two high-responder strains differing in Ig allotypes (CSW + CWB mixtures), the Ig^a and Ig^b allotype antibodies can be clearly distinguished. The binding of Ig^b allotype anti-(T,G)-A–L antibody does not appear to interfere with or displace the binding of Ig^a allotype antibody. This is seen most clearly by comparing the counts bound by either anti-Ig-1^a or anti-Ig-4^a allotype antibody in the mixtures (CSW + CWB) versus (CSW + CKB). For example, at a 1:100 dilution of total serum antibody in a 20% Ig^a/80% Ig^b mixture, there are 1,732 counts of anti-Ig-4^a bound for (CSW + CWB), and 1,807 counts bound for (CSW + CKB).

The binding of Ig^a allotype anti-(T,G)-A–L antibody to the antigen-coated plate can thus be detected in the presence of binding by Ig^b allotype antibody.

TABLE II

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

Donor*	Serum dilution‡	^{125}I-labeled anti-allotype reagents§				Production of LR allotype‖
	Log^2	Anti-1^a	Anti-1^b	Anti-4^a	Anti-4^b	Anti-(T,G)-A–L antibody
CKB ↔ CSW Chimeras						
No. 1	1	278	148	206	51	–
	2	168	184	148	57	
	3	148	144	154	56	
	4	98	145	144	69	
No. 2	1	1,604	168	761	140	No
	2	870	186	488	111	
	3	517	159	282	65	
	4	306	202	210	78	
No. 3	1	2,174	232	777	77	No
	2	1,197	168	409	48	
	3	649	184	275	57	
	4	469	142	247	57	
No. 4	1	2,129	214	1,860	116	No
	2	1,247	197	1,530	66	
	3	679	182	1,049	61	
	4	446	155	548	49	
No. 5	1	983	246	1,030	71	No
	2	556	206	593	74	
	3	343	202	355	65	
	4	230	170	311	47	
CKB × CSW F_1						
No. 1	1	379	661	237	1,185	Yes
	2	239	395	145	660	
	3	152	254	127	370	
	4	158	246	109	247	
CKB × CSW F_1						
No. 2	1	2,556	8,657	2,081	3,562	Yes
	2	1,370	4,641	1,449	3,147	
	3	798	2,465	942	2,352	
	4	496	1,542	484	1,533	
No. 3	1	153	390	141	240	–
	2	123	198	108	132	
	3	126	183	119	95	
	4	105	174	94	72	
No. 4	1	4,127	6,761	1,980	3,048	Yes
	2	2,745	4,260	1,841	2,920	
	3	1,476	2,274	1,549	2,541	
	4	975	1,265	969	1,663	
No. 5	1	3,181	9,043	2,026	3,253	Yes
	2	2,052	5,382	2,004	2,836	
	3	1,313	3,233	1,740	2,436	
	4	850	1,791	1,059	1,741	
CKB						
No. 1	1	89	290	119	130	
No. 2	1	71	310	93	128	
No. 3	1	67	336	93	103	
CSW						
No. 1	1	5,830	175	1,998	124	
No. 2	1	6,076	267	2,164	161	

* Chimeric mice were immunized 8 wk after reconstitution with 10 μg (T,G)-A–L-52 i.p. in CFA, challenged 1 mo later with 10 μg (T,G)-A–L i.p. in PBS, and bled 8 days later. F_1, CKB, and CSW mice immunized at the same time.

‡ Starting serum dilution 1:100; doubling dilutions thereafter.

§ Counts for anti-1^b and anti-1^a are counts per minute; for anti-4^b and anti-4^a, counts per 0.4 min. 20 μl of detecting reagent (anti-allotype antibody) added per well: anti-1^b and anti-1^a, 26,836 and 9,530 counts/minute, respectively; anti-4^b and anti-4^a each 20,000 counts/0.4 min.

‖ LR (low-responder) allotype anti-(T,G)-A–L antibody production denoted as yes if counts bound with anti-1^b or anti-4^b are greater than those measured for CKB controls. Dashed line indicates little or no overall antibody production.

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^b or Ig-4^b antibody for the 80% Ig^a/20% Ig^b mixture of (CSW + CWB) 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^b allotype in their preimmune sera by Ouchterlony gel diffusion analysis. The plate assay was used to determine whether any of these chimeras made IgG_1 or IgG_{2a} anti-(T,G)-A–L antibody of low-responder allotype (Ig^b) in amounts greater than those produced by low-responder CKB mice. Secondary sera from CKB, CSW, and (CKB × CSW)F_1

mice were included as controls. Four of five F_1 mice made detectable anti-(T,G)-A–L antibody, and these four produced antibody of low-responder Ig allotype (Ig^b) 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 Ig^b (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 (Ig^a).

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-1A* 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-1A* gene-mediated restriction has also been observed in adoptive cell transfer studies with DNP-primed B cells from *Ir-1A* low responder BALB.K mice and (T,G)-A–L primed cells from either (BALB.B × BALB.K)F_1 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.[1] Since Ig^a and Ig^b 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-1A* 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 F_1 cells, but this has not been directly demonstrated. If the stimulation of chimeric high responder B cells occurs via interactions with F_1 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 tetraparental mouse studies by Bechtol et al. (2, 3). Several of the original C3H ↔ (CKB × CWB)F_1 tetraparental 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 Ig^a allotype in amounts greater than those detected in antisera of low responder C3H mice. However, some of the original (CWB × C3H)F_1 antisera also did not contain significant antibody levels of low responder Ig^a allotype, in contrast to the (CKB × CSW)F_1 control mice used in this study. The tetraparental antisera were originally analyzed by absorption onto and acid elution from anti-Ig^a 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.

(3). When the remaining acid eluted fractions of the tetraparental sera were analyzed by the allotype-specific plate assay, antibody of high responder Ig^b allotype was detected in varying amounts, but little antibody of low responder Ig^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.

Received for publication 12 August 1977.

References

1. Katz, D. H., T. Hamaoka, M. E. Dorf, P. H. Maurer, and B. Benacerraf. 1973. Cell interactions between histoincompatible T and B lymphocytes. IV. Involvement of the immune response (*Ir*) gene in the control of lymphocyte interactions in responses controlled by the gene. *J. Exp. Med.* **138**:734.
2. Bechtol, K. B., J. H. Freed, L. A. Herzenberg, and H. O. McDevitt. 1974. Genetic control of the antibody response to poly-L-(Tyr, Glu)-poly-D,L-Ala–poly-L-Lys in C3H ↔ CWB tetraparental mice. *J. Exp. Med.* **140**:1660.
3. Bechtol, K. B., and H. O. McDevitt. 1976. Antibody response of C3H ↔ (CKB × CWB)F_1 tetraparental mice to poly-L-(Tyr, Glu)-poly-D, L-Ala–poly-L-Lys immunization. *J. Exp. Med.* **144**:123.
4. von Boehmer, H., J. Sprent, and M. Nabholz. 1975. Tolerance to histocompatibility determinants in tetraparental bone marrow chimeras. *J. Exp. Med.* **141**:322.
5. von Boehmer, H., L. Hudson, and J. Sprent. 1975. Collaboration of histoincompatible T and B lymphocytes using cells from tetraparental bone marrow chimeras. *J. Exp. Med.* **142**:989.
6. Waldmann, H., H. Pope, and A. J. Munro. 1975. Cooperation across the histocompatibility barrier. *Nature (Lond.).* **258**:728.
7. Klinman, N. R., A. R. Pickard, N. H. Sigal, P. J. Gearhart, E. S. Metcalf, and S. K. Pierce. 1976. Assessing B cell diversification by antigen receptor and precursor cell analysis. *Ann. Immunol. (Inst. Pasteur)* **127C**:489.
8. Herzenberg, L. A., and L. A. Herzenberg. 1977. Mouse immunoglobulin allotypes: description and specific methodology. *In* Handbook of Experimental Immunology, 3rd edition. D. M. Weis, editor. Blackwell Scientific Publications Ltd., Oxford, England. In press.
9. Press, J. L., and H. O. McDevitt. 1977. *Ir-1A* gene influence on chimeric lymphoid cell interactions. *In* Proceedings of the Third *Ir* Gene Workshop. H. O. McDevitt, editor. Academic Press, Inc., New York. In press.