CELLULAR AND GENETIC CONTROL OF ANTIBODY RESPONSES IN VITRO

II. Ir Gene Control of Primary IgM Responses to Trinitrophenyl Conjugates of Poly-L-(Tyr,Glu)-Poly-D,L-Ala – Poly-L-Lys and Poly-L-(His,Glu)-Poly-D,L-Ala – Poly-L-Lys

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The use of synthetic polypeptides as immunogens has led to major insights into the genetic regulation of the immune response (1). In 1965, it was first demonstrated that the IgG responses to poly-L-(Tyr,Glu)-poly-D,L-Ala – poly-L-Lys [(T,G)-A – L][1] and poly-L-(His,Glu)-poly-D,L-Ala – poly-L-Lys [(H,G)-A – L] were under autosomal dominant genetic control in the mouse (2). By using the antigens (T,G)-A – L, (H,G)-A – L, and poly-L-(Phe,Glu)-poly-D,L-Ala – poly-L-Lys [(Phe,G)-A – L], the *Ir-1* locus controlling the IgG response to these antigens was described, and mapped within the murine major histocompatibility (*H-2*) complex (3). On the other hand, the primary IgM response to these antigens has generally been thought not to be under genetic control.

Mitchell et al. by using adult thymectomized, lethally irradiated, and syngeneic bone marrow reconstituted high and low responder mice immunized with (T,G)-A – L without adjuvant found the primary IgM response to be T-independent and not under genetic control, while the secondary IgG response was noted to be both T-dependent and genetically controlled (4). However, experiments involving adoptively transferred animals reconstituted with bone marrow cells and either antigen-primed thymocytes or antigen-specific T-helper factors implied that there was *H-2*-linked genetic control of the IgM response to (T,G)-A – L (5).

The ability to define more precisely the nature and function of immune response (*Ir*) genes has been limited by the relative lack of in vitro systems for analysis of genetic control and cellular requirements in the antibody response to synthetic polypeptides. Recently, an in vitro system was described for generating a true primary anti-hapten response to trinitrophenyl (TNP) conjugates of (T,G)-A – L [TNP-(T,G)-A – L] and keyhole limpet hemocyanin (TNP-KLH) which was T-cell and macrophage-dependent.[2] In the present report the ques-

[1] *Abbreviations used in this paper*: C, complement; (H,G)-A – L, poly-L-(His,Glu)-poly-D,L-Ala – poly-L-Lys; Ir, immune response; KLH, keyhole limpet hemocyanin; PFC, plaque-forming cells; (Phe,G)-A – L, poly-L(Phe,Glu)-poly-D,L-Ala – poly-L-Lys; RαMB, rabbit anti-mouse brain serum; SRBC, sheep erythrocytes; (T,G)-A – L, poly-L-(Tyr,Glu)-poly-D,L-Ala – poly-L-Lys; TNBS, 2,4,6-trinitrobenzene-sulfonate; TNP, trinitrophenyl.

[2] Hodes, R. J., and A. Singer. 1977. Cellular and genetic control of antibody responses in vitro. I. Cellular requirements for the generation of genetically controlled primary IgM responses to soluble antigens. *Eur. J. Immunol.*, in press.

tion of whether there is genetic control of the primary IgM response was examined utilizing this in vitro system. The primary IgM anti-TNP responses to both TNP-(T,G)-A–L and TNP-(H,G)-A–L were shown to be T-dependent and under autosomal dominant genetic control which was linked to the *H-2* complex and mapped to the *K* and *I-A* regions. In contrast, the response to TNP-KLH, while T-dependent, was not under demonstrable genetic control.

Materials and Methods

Animals. C57BL/10 (B10), B10.A, B10.BR, B10.D2, A/J, A.BY, C3H/HeJ, C3H.SW, and (B10 × B10.A)F_1 male mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. B10.A(4R), B10.A(5R), and (A/J × A.BY)F_1 male mice were kindly provided by Dr. David Sachs, National Institutes of Health, Bethesda, Md. All mice were used at 6–12 wk of age in all experiments. Pools of spleen cells from at least three syngeneic mice were used in each experiment.

Antigens. (T,G)-A–L was obtained from Yeda Research and Development Co., Ltd., Rehovot, Israel (lot number MC-3, mol wt 260,000). (H,G)-A–L was the generous gift of Dr. Edna Mozes, Weizmann Institute, Rehovot, Israel. KLH (lot number 530195) was obtained from Calbiochem, San Diego, Calif.

TNP Conjugation. Antigens were conjugated with 2,4,6-trinitrobenzene-sulfonate (TNBS) (Pierce Chemical Co., Rockford, Ill.) as previously described.[2] The degree of TNP modification of the antigens used was eight TNP groups per 100,000 daltons (T,G)-A–L unless otherwise stated, 5 TNP groups per 100,000 daltons (H,G)-A–L, 12 TNP groups per 100,000 daltons KLH, and 35 TNP groups per molecule bovine serum albumin. Sheep erythrocytes (SRBC) were conjugated with TNP (TNP-SRBC) by the method of Rittenberg and Pratt (6).

Cell Preparations and Culture Conditions. The preparation of cell suspensions and the evaluation of cell surface markers were as described elsewhere.[2] Preparation of a T-cell-enriched population (T cells) was accomplished by nylon wool column passage. Preparation of a T-cell-depleted population (B cells) was accomplished by the use of a T-cell-specific rabbit anti-mouse brain serum (RαMB) + complement (C).

Culture conditions used have also been described elsewhere.[2] Briefly, 5×10^5 spleen cells (or cell mixtures where specified) were cultured with indicated concentrations of TNP-(T,G)-A–L, TNP-(H,G)-A–L, TNP-KLH or no antigen in a total vol of 200 μl per flat bottom well of microtiter plates. Two or three microtiter wells were used per culture group, and replicate groups were employed as indicated. Cultures were incubated at 37°C in a 5% CO_2-humidified air atmosphere. Cultures were harvested on day 4 unless otherwise specified.

Hemolytic Plaque-Forming Cells (PFC) Assay. Direct PFC were assayed on TNP-SRBC by the slide modification (7) of the Jerne hemolytic plaque technique (8). Every culture group was assayed in the presence and absence of TNP-BSA (5×10^{-4} M final concentration of TNP) to determine the number of TNP-inhibitable PFC/10^7 cultured cells. Virtually all (>90%) direct PFC have been shown to be producing IgM antibody by inhibition with a specific goat anti-mouse μ-serum (6), which was kindly provided by Dr. Richard Asofsky, National Institutes of Health, Bethesda, Md. IgG PFC were determined by first blocking IgM PFC with a 1:250 dilution of goat anti-mouse μ-serum and then developing the IgG PFC with a 1:100 dilution of a pool of a specific rabbit anti-mouse γ_1- and γ_2-serum, which was also the generous gift of Dr. Richard Asofsky.

Results

In Vitro Responses of Normal B10 and B10.A Spleen Cells to TNP-(T,G)-A–L and TNP-(H,G)-A–L. The primary IgM anti-TNP PFC responses of normal B10 and B10.A spleen cells to TNP-(T,G)-A–L and TNP-(H,G)-A–L were examined (Table I). The response of spleen cells from normal B10 donors to TNP-(T,G)-A–L is dependent upon the concentration of TNP-(T,G)-A–L in the cultures. An optimal response was detected at a final concentration of 250 μg/ml, although there was a broad range in which a significant response above the no antigen control occurred. In contrast, the response of normal B10.A spleen

TABLE I

Response of B10 and B10.A Spleen Cell to Various Concentrations of TNP-(T,G)-A–L and TNP-(H,G)-A–L

TNP-(T,G)-A–L	Direct PFC/10^7 cultured cells*	
	B10	B10.A
μg/ml		
0	34 (1.40)	44 (1.64)
10	218 (1.89)	28 (1.43)
100	413 (1.19)	43 (1.61)
250	1,391 (1.30)	45 (1.66)
500	783 (1.29)	66 (1.84)
TNP-(H,G)-A–L	Direct PFC/10^7 cultured cells‡	
μg/ml	B10	B10.A
0	36 (1.37)	35 (1.36)
5	0	260
50	69 (2.4)	430 (1.34)
250	57 (1.9)	920 (1.41)
500	80 (2.03)	517 (1.92)

* Geometric mean (SE) of TNP-inhibitable PFC of five individual spleens over two experiments.

‡ Geometric mean (SE) of TNP-inhibitable PFC of five consecutive experiments, each consisting of pools of three spleens, except at 5 μg/ml which was performed in one experiment.

cells to TNP-(T,G)-A–L was not different from the no antigen control at any of the doses tested. The reciprocal strain pattern of responsiveness was observed to TNP-(H,G)-A–L. Normal B10.A spleen cells responded to TNP-(H,G)-A–L with the optimal response also detected at a final concentration of 250 μg/ml. Normal B10 spleen cells, however, did not significantly respond above control at any of the doses tested, although there was a suggestion of a small, though not statistically significant, response at the highest dose of 500 μg/ml.

The observations that B10 spleen cells responded to TNP-(T,G)-A–L but not TNP-(H,G)-A–L while B10.A spleen cells responded to TNP-(H,G)-A–L but not TNP-(T,G)-A–L could be interpreted as indicating either that: (*a*) the in vitro primary IgM PFC response is under the control of one or more *H-2*-linked *Ir* genes; or (*b*) the differences are due to other factors introduced by the in vitro culture system. A number of experiments were performed to examine the latter possibility.

One possibility was that the culture conditions required to elicit a response from B10.A spleen cells were different from the conditions necessary for B10 spleen cells. Therefore, the in vitro conditions for obtaining a response of both normal B10 and normal B10.A spleen cells to a third antigen, TNP-KLH were determined. The conditions for obtaining peak responses to TNP-KLH by both strains, indeed by all strains studied, were identical and occurred on day 4 at a dose of 10 μg/ml (Table II and Table V).

Another possibility was that B10.A spleen cells did not survive well under these culture conditions in the presence of TNP-(T,G)-A–L. Therefore, the

TABLE II
Similar Responses of Normal B10 and B10.A Spleen Cells to Various Concentrations of TNP-KLH

TNP-KLH	Direct PFC/10^7 cultured cells*	
	B10	B10.A
μg/ml		
0	0	40
0.1	80	480
1	2,080	1,920
10	4,360	9,240
100	2,000	2,360

* TNP-inhibitable PFC response of a pool of three replicate cultures after 4 days in culture.

TABLE III
*Percent Viable Cell Recovery of B10 and B10.A Spleen Cells**

Antigen	B10	B10.A
O	43.1 ± 3.7	65.4 ± 0.6
TNP-(T,G)-A–L‡	59.6 ± 7.2	62.6 ± 1.9
TNP-KLH§	62.6 ± 1.1	61.0 ± 3.2

* Arithmetic mean ± SE of triplicate cultures harvested on day 4.
‡ 250 μg/ml.
§ 10 μg/ml.

viability of B10 and B10.A spleen cells after 4 days in culture with TNP-(T,G)-A–L, TNP-KLH, and without antigen was examined (Table III). There were no differences in the percent recovery of viable cells, as measured by dye exclusion, between B10 and B10.A spleen cells either with or without antigen.

It was also possible that the response of normal B10.A spleen cells to TNP-(T,G),A–L would appear later than that of normal B10 spleen cells. As was shown recently,[2] the peak response of normal B10 spleen cells to TNP-(T,G)-A–L occurred on day 4. There was no response of B10.A spleen cells to TNP-(T,G)-A–L as late as day 5. Beyond day 5, the viability of spleen cells under these culture conditions were too poor to allow any meaningful determinations (data not shown).

The apparent lack of responsiveness of B10 spleen cells to TNP-(H,G)-A–L and of B10.A spleen cells to TNP-(T,G)-A–L could conceivably have been due to the generation of IgG PFC which were not detected as direct plaques. However, when this possibility was examined, neither normal B10 nor B10.A spleen cells developed any detectable IgG anti-TNP PFCs to either TNP-(T,G)-A–L or TNP-(H,G)-A–L (data not shown).

It was also possible that altering the number of TNP groups per molecule of (T,G)-A–L would reverse the unresponsiveness of B10.A spleen cells to TNP-(T,G)-A–L. TNP ratios of from one to eight TNP groups per 100,000 daltons of (T,G)-A–L were tested (Table IV). Higher ratios were not used to avoid altering the normal structure of the (T,G)-A–L molecule further. B10.A spleen cells did not significantly respond to any of the preparations tested while B10 spleen cells

TABLE IV
Response of Normal B10 and B10.A Spleen Cells to (T,G)-A–L Conjugated with Various Amounts of TNP

TNP_x-(T,G)-A-L‡	Direct PFC/10^7 cultured cells*	
	B10	B10.A
TNP_1-(T,G)-A-L§	363 (1.3)	40 (2.0)
TNP_5-(T,G)-A-L‖	1,585 (1.06)	28 (1.44)
TNP_8-(T,G)-A-L‖	727 (1.12)	2 (2.42)

* Geometric mean (SE) of net TNP-inhibitable PFC of triplicate culture groups.
‡ Number of TNP groups per 100,000 daltons of (T,G)-A-L.
§ 100 μg/ml (optimal concentration for this preparation).
‖ 250 μg/ml (optimal concentrations for these preparations).

responded to all of them, although the magnitude and dose optimum of the response were somewhat dependent upon the actual substitution ratio used.

Since the response to these antigens has previously been shown to be T-cell dependent,[2] the unresponsiveness of normal B10 spleen cells to TNP-(H,G)-A–L and of normal B10.A spleen cells to TNP-(T,G)-A–L could conceivably have been overcome by altering the ratio of T cells and B cells in culture. Normal spleen cells were separated into a T-cell-depleted population (B cells) by pretreatment with RαMB serum + C and into a T-cell-enriched population (T cells) by passage over a nylon wool column. There was little or no response to TNP-KLH, TNP-(T,G)-A–L, or TNP-(H,G)-A–L by either normal B10 B cells (0% T) or T cells (100% T) cultured alone (Fig. 1). The responses to TNP-KLH and TNP-(T,G)-A–L were reconstituted to at least the level of the unseparated spleen by the addition of graded numbers of syngeneic T cells. With the addition of more T cells and the consequent decline in the number of B cells and probably macrophages (since the total cell number in the cultures were held constant), the response to TNP-(T,G)-A–L declined, as was discussed previously.[2] In contrast, the unresponsiveness of B10 B cells to TNP-(H,G)-A–L persisted regardless of the number of T cells added. In the B10.A, there was also essentially no response to any of the antigens in the B cell (0% T) or T cell (100% T) populations cultured alone. The responses to both TNP-KLH and TNP-(H,G)-A–L were fully reconstituted by the addition of graded numbers of syngeneic T cells. On the other hand, a significant response to TNP-(T,G)-A–L was not detected at any of the T cell/B cell ratios examined.

Thus, the in vitro primary IgM anti-TNP responses to TNP-(T,G)-A–L, TNP-(H,G)-A–L, and TNP-KLH were T-dependent. Furthermore, the unresponsiveness of the appropriate nonresponder strain could not be reversed by altering the ratio of syngeneic T cells and B cells in culture.

It was therefore concluded that culture conditions were very unlikely to account for the different responses of normal B10 and B10.A spleen cells to TNP-(T,G)-A–L and TNP-(H,G)-A–L.

H-2 Linkage of the Primary IgM Anti-TNP Response to TNP-(T,G)-A–L and TNP-(H,G)-A–L. The responsiveness of normal B10 spleen cells to TNP-(T,G)-A–L but not TNP-(H,G)-A–L, and the reciprocal responsiveness of

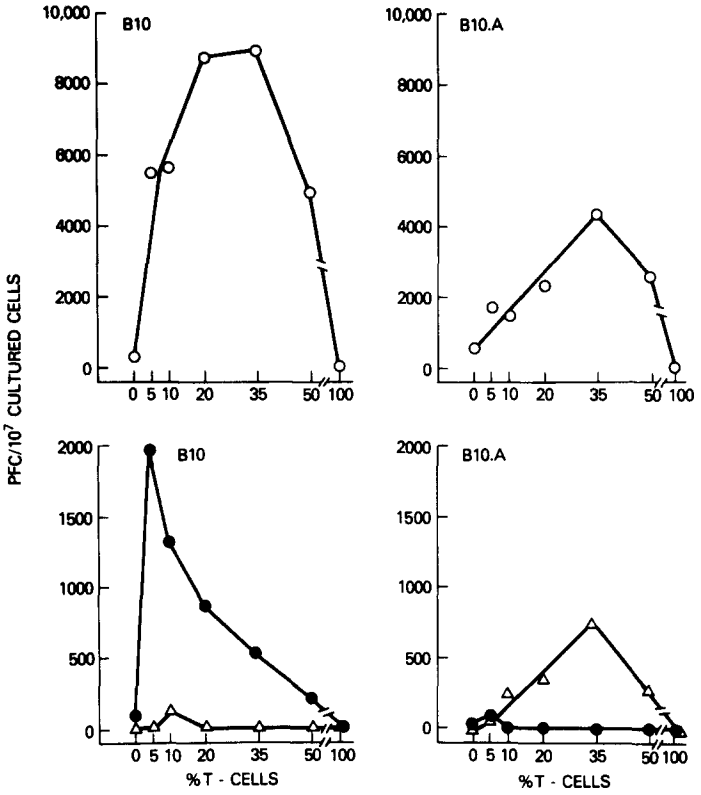

FIG. 1. Effect of various numbers of syngeneic splenic T cells on the primary IgM response of normal B10 and B10.A splenic B cells to TNP-KLH (○——○), TNP-(T,G)-A–L (●——●), and TNP-(H,G)-A–L (△——△). All responses are expressed as TNP-inhibitable PFC/10^7-cultured cells. Total cell number was held constant at 5×10^5 cells/culture and consisted of varying mixtures of syngeneic T cells (nylon nonadherent spleen cells) and B cells (RαMB + C-treated spleen cells). Percent T cells was calculated as ([number of nylon nonadherent spleen cells]/5×10^5) × 100. Since the total cell number per culture was held constant, increases in T-cell number were accompanied by reciprocal decreases in the number of B cells (and probably macrophages) present in each culture. Immunofluorescent studies revealed the following number of T and B cells in each population:

	Percent RαMB$^+$	Percent sIg$^+$
B10 whole spleen	22	42
RαMB + C-treated (B cells)	0	69
Nylon nonadherent (T cells)	86	1.0
B10.A whole spleen	29	59
RαMB + C-treated (B cells)	0	80
Nylon nonadherent (T cells)	93	1.5

normal B10.A spleen cells to TNP-(H,G)-A–L but not TNP-(T,G)-A–L strongly suggested that the responses to these two antigens were genetically controlled and linked to the *H-2* complex. To further explore this point, the responses to TNP-KLH, TNP-(T,G)-A–L, and TNP-(H,G)-A–L by a variety of strains with different *H-2* haplotypes and different backgrounds were determined (Table V).

TABLE V

The In Vitro Primary Anti-TNP IgM Response to TNP-(T,G)-A-L and TNP-(H,G)-A-L, but Not TNP-KLH, is H-2 Linked

H-2	Strain	Direct PFC/10^7 cultured cells						
		No antigen*	TNP-KLH‡	*P* value§	TNP-(T,G)-A-L‡	*P* value	TNP-(H,G)-A-L‡	*P* value
b	B10	57 (1.68)	2,675 (1.19)	<0.01	675 (1.13)	<0.01	100 (2.0)	NS
b	A.BY	36 (1.81)	2,100 (1.16)	<0.01	560 (1.17)	<0.01	20 (1.25)	NS
b	C3H.SW	0	1,500 (1.25)	<0.01	660 (1.03)	<0.001	20 (1.44)	NS
a	B10.A	0	2,575 (1.09)	<0.001	0	NS	525 (1.42)	<0.02
a	A/J	25 (1.25)	3,920 (1.04)	<0.001	40 (1.68)	NS	760 (1.10)	<0.001
b/a	(B10 × B10.A)F_1	0	1,400 (1.09)	<0.001	440 (1.07)	<0.001	520 (1.37)	<0.01
a/b	(A/J × A.BY)F_1	60 (1.78)	4,640 (1.10)	<0.001	680 (1.09)	<0.001	560 (1.32)	<0.01
k	B10.BR	54 (1.44)	2,440 (1.07)	<0.001	80 (2.3)	NS	480 (1.45)	<0.02
k	C3H	50 (1.58)	860 (1.20)	<0.01	40 (1.49)	NS	440 (1.04)	<0.001
d	B10.D2	0	2,060 (1.06)	<0.001	240 (1.12)	<0.01	160 (1.10)	<0.01

* Geometric mean (SE) of TNP-inhibitable PFC of parallel triplicate culture groups from a pool of three spleens.

‡ Geometric mean (SE) of net (above no antigen background) TNP-inhibitable PFC of parallel triplicate culture groups. TNP-KLH = 10 μg/ml, TNP-(T,G)-A-L = 250 μg/ml, and TNP-(H,G)-A-L = 250 μg/ml.

§ Significance above 0 as determined by two-tailed Student's *t* test (NS = not significant $P > 0.05$).

Strains with the *H-2*^{b} haplotype all responded to TNP-(T,G)-A–L but not to TNP-(H,G)-A–L, while the *H-2*^{a} and *H-2*^{k} strains responded to TNP-(H,G)-A–L but not to TNP-(T,G)-A–L. *H-2*^{d} spleen cells significantly responded to both antigens, although the response in each case was lower in magnitude than those of the other responder haplotypes. The response was, therefore, *H-2*-linked on at least the three backgrounds studied, with the *H-2*^{b} and *H-2*^{d} haplotypes responding to TNP-(T,G)-A–L and the *H-2*^{a}, *H-2*^{k}, and *H-2*^{d} haplotypes responding to TNP-(H,G)-A–L.

F_1 hybrids between *H-2*^{a} and *H-2*^{b} mice on both the *a* and *b* backgrounds responded to both TNP-(T,G)-A–L and TNP-(H,G)-A–L, which indicated the autosomal dominance of these *H-2*-linked responses.

In addition, the fact that all the strains responded to TNP-KLH but responded variably to TNP-(T,G)-A–L and TNP-(H,G)-A–L showed that the genetic control exhibited was not simply for TNP.

Mapping of the Ir Genes Controlling the Primary IgM Anti-TNP Responses to TNP-(T,G)-A–L and TNP-(H,G)-A–L. By determining the responses to TNP-(T,G)-A–L and TNP-(H,G)-A–L in the B10.A(5R) and the B10.A(4R), two recombinant strains between B10 and B10.A, the genes controlling responsiveness to TNP-(T,G)-A–L and TNP-(H,G)-A–L could be further localized within the *H-2* region to the *H-2*^{a} and *H-2*^{b} haplotypes.

The B10.A(5R) recombination occurred between the *I-B* and *I-J* subregions, while the B10.A(4R) recombination occurred between the *I-A* and *I-B* subregions (9). All strains tested again responded to TNP-KLH (Table VI). Both the B10 and the B10.A(5R) responded to TNP-(T,G)-A–L showing that the gene(s) in the *H-2*^{b} halotype controlling responsiveness to TNP-(T,G)-A–L lies to the left of the *I-J* subregion. Since neither the B10.A nor the B10.A(4R) responded to TNP-(T,G)-A–L, the gene(s) could be further localized to the left of the *I-B* subregion, that is, within the *K* or *I-A* regions of the *H-2*^{b} responder haplotype.

Neither the B10 nor the B10.A(5R) responded to TNP-(H,G)-A–L, localizing the gene(s) in the *H-2*^{a} responder haplotype for TNP-(H,G)-A–L to the left of the *I-J* subregion. Both the B10.A and B10.A (4R) responded to TNP-(H,G)-A–

TABLE VI

Gene(s) within the K or I-A Regions of H-2 Regulate the Anti-TNP Primary IgM Responses to TNP-(T,G)-A-L and TNP-(H,G)-A-L

Strain (KABJECSD)	No. of exp.	0 Antigen*	No. of exp.	Direct PFC/10^7 cultured cells: TNP-KLH‡	*P* value§	No. of exp.	TNP-(T,G)-A-L‡	*P* value	No. of exp.	TNP-(H,G)-A-L‡	*P* value
B10 (bbbbbbbb)	13	32 (1.18)	13	4,797 (1.14)	<0.001	13	538 (1.30)	<0.001	5	51 (1.64)	NS
B10.A (kkkkkddd)	12	37 (1.31)	12	1,610 (1.32)	<0.001	12	37 (1.34)	NS	5	359 (1.90)	<0.02
B10.A(5R) (bbbkkddd)	7	46 (1.30)	7	2,523 (1.38)	<0.001	7	397 (1.67)	<0.01	3	31 (1.25)	NS
B10.A(4R) (kkbbbbbb)	7	33 (1.50)	7	926 (1.73)	<0.001	7	34 (1.24)	NS	3	301 (1.20)	<0.01

* Geometric mean (SE) of TNP-inhibitable PFC in a series of consecutive experiments. Each experiment generally consists of parallel triplicate culture groups from a pool of three spleens (each culture group is a pool of two parallel cultures).

‡ Geometric mean (SE) of net (above 0 antigen background) TNP-inhibitable PFC in a series of consecutive experiments. TNP-KLH = 10 μg/ml, TNP-(T,G)-A-L = 250 μg/ml, and TNP-(H,G)-A-L = 250 μg/ml.

§ Significance above 0 as determined by two-tailed Student's *t* test (NS = not significant $P > 0.05$).

L, localizing the gene(s) controlling responsiveness to this antigen within the *K* or *I-A* regions of the $H\text{-}2^a$ responder haplotype.

Unfortunately, no recombinant strains exist between B10 and B10.A which allow further localization of these genes to either the *K* or *I-A* regions of the $H\text{-}2^a$ and $H\text{-}2^b$ haplotypes.

Discussion

The *Ir-1* locus was described as controlling the secondary IgG responses to the random-branched chain polypeptides (T,G)-A–L, (H,G)-A–L, and (Phe,G)-A–L (3). This locus was autosomal dominant and mapped within the *H-2* complex. In contrast, the primary IgM response to these antigens is generally thought to be neither T-dependent nor under genetic control (4). The work presented here with TNP conjugates of (T,G)-A–L, (H,G)-A–L, and KLH in an in vitro microculture system and assaying the primary IgM anti-hapten PFC responses of normal spleen cells from a variety of strains, demonstrated that the primary IgM response to these antigens was indeed T-dependent and, for TNP-(T,G)-A–L and TNP-(H,G)-A–L but not TNP-KLH, under autosomal dominant *H-2*-linked *Ir* control which mapped within the *K* or *I-A* regions of the *H-2* complex.

The different responses to TNP-(T,G)-A–L and TNP-(H,G)-A–L among the strains tested were due to differences in *Ir* genes and not due to factors introduced by the in vitro culture system since: (*a*) each strain had the capability of responding in this culture system as shown by the fact that each responded to one or the other synthetic polypeptide and that all responded similarly to a third antigen, TNP-KLH; (*b*) the antigens were not selectively toxic since there were no differences in viabilities during culture among the strains, either in the presence or absence of antigen; (*c*) nonresponders did not respond even if left a longer time in culture, nor did they produce IgG antibody which would not have been detected as direct PFC, and (*d*) the unresponsiveness of nonresponders could not be reversed despite variation of either the TNP/carrier substitution ratio or of the relative ratio of T cells and B cells put into culture.

The results of adoptive transfer experiments with thymectomized high and low responder strains immunized with (T,G)-A–L in saline conflict with the present results in that the primary IgM response was T-independent and not genetically controlled (4). A possible explanation for this discrepancy may relate to the structure of (T,G)-A–L. The T-dependence of the primary IgM response to the defined synthetic-branched chain copolymers (T-T-G-G)-A–L and (T-G-T-G)-A–L has been studied (10). The primary IgM response to (T-T-G-G)-A–L is T-dependent while the response to (T-G-T-G)-A–L is T-independent. It seems possible that different batches of the random copolymer (T,G)-A–L may vary in the relative dominance of the two sequences, resulting in differences in the T-dependence of the primary IgM responses observed. In support of this concept, it has also been recently reported that the in vivo primary IgM response to certain preparations of (T,G)-A–L may be under genetic control while others are not (11). The relative T-dependence of these different preparations was not examined.

Whether or not the *Ir* genes controlling the in vitro primary IgM anti-hapten response are the same *Ir* genes controlling the in vivo secondary IgG anti-(T,G)-

A – L and anti-(H,G)-A – L responses is unclear. The genes controlling the in vitro primary responses must have at least some carrier specificity since some strains respond to TNP-(T,G)-A – L, some to TNP-(H,G)-A – L, and all respond to TNP-KLH. The genes resemble one another in that they are autosomal dominant, they map identically within the *H-2* complex, and they have identical responder and nonresponder haplotypes, so far as has been tested.

The in vitro studies described in this present report clearly demonstrated that the primary IgM response can be T-dependent and under *Ir* gene control. Thus, it is concluded that while *Ir* genes may function in the switch from specific IgM to IgG production as has been proposed (12), the same or other *Ir* genes can also govern the ability to generate an IgM response upon initial exposure to antigen. Similarly, genetic control of the in vivo primary IgM response to Glu^{56} Lys^{35} Phe^{9} has recently been reported (13). The present results further suggest that the primary IgM responses to antigens such as TNP-(T,G)-A – L and TNP-(H,G)-A – L are under *Ir* gene control when such responses are T-dependent.

Two other in vitro systems in which responses to soluble synthetic polypeptides are observed have been described. Both the primary and secondary in vitro responses to the linear copolymer Glu^{60} Ala^{30} Tyr^{10} are genetically controlled, but both result in only IgG production (14). The other system involves the use of a two stage Marbrook-Diener culture system, the first to educate T cells to (T,G)-A – L and the second to assay the ability of these (T,G)-A – L-educated T cells to help in the anti-DNP response to DNP-(T,G)-A – L (15). In contast to the results presented in this report, the anti-DNP response in this system was not under genetic control (16). The possible explanations for this discrepancy include the requirement for educated rather han normal T cells as helper cells, the use of a different preparation of (T,G)-A – L, or differences in the method of hapten conjugation. This last possibility arises since 2,4-dinitofluorobenzene which was the haptenating reagent used in the Marbrook-Diener culture system studies, can modify the tyrosine residues of (T,G)-A – L more extensively than TNBS, which was used in the present studies (17, 18). Experiments are under way to examine these points.

The genetically controlled in vitro responses described in this report require the interaction of subpopulations of normal spleen cells which are relatively well characterized and which can be distinguished and separated by established methods.[2] Thus, the potential exists for careful determination of the cellular levels (among macrophages, T cells, and B cells) at which *Ir* defects are expressed in nonresponder haplotypes. It is also possible that this system can be used as an in vitro assay for various antigen-specific and nonspecific helper and suppressor factors which may be involved in regulation of the immune response. Experiments in these areas are now in progess.

Summary

The in vitro primary IgM anti-hapten responses to trinitrophenyl (TNP) conjugates of poly-L-(Tyr,Glu)-poly-D,L-Ala – poly-L-Lys (T,G)-A – L and poly-L(His,Glu)-poly-D,L-Ala – poly-L-Lys (H,G)-A – L were shown to be T-cell dependent and under autosomal dominant *H-2*-linked *Ir* gene control which mapped within the *K* or *I-A* regions of the *H-2* complex. The in vitro response to

TNP-keyhole limpet hemocyanin, while T-dependent, was not under demonstrable genetic control. The genes governing the in vitro primary IgM antihapten responses to TNP-(T,G)-A–L and TNP-(H,G)-A–L resemble the *Ir* genes controlling the in vivo secondary IgG responses to (T,G)-A–L and (H,G)-A–L in that they are autosomal dominant, map identically within the *H-2* complex, and have identical responder and nonresponder haplotypes. It is concluded that *Ir* genes can govern the ability to generate an IgM response upon initial exposure to antigen.

The authors wish to thank Doctors William Terry, Gene Shearer, David Sachs, and Donald Mosier for many helpful suggestions throughout the course of this work. We wish to than Dr. Edna Mozes for her generous gift of (H,G)-A–L and Dr. Richard Asofsky for kindly providing specific anti-mouse Ig sera for use in the plaque-forming cell assay. We are grateful to Ms. Karen Hathcock for her excellent technical assistance, and to Mr. Walter Lyles and Mr. Frances Jones for their expert animal handling. We wish to thank Ms. Judy Steckel for help in the preparation of this manuscript.

Received for publication 12 May 1977.

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