

## GENETIC CONTROL OF THE IMMUNE RESPONSE

### In Vitro Stimulation of Lymphocytes by (T,G)-A--L, (H,G)-A--L, and (Phe,G)-A--L\*

BY PETER LONAI‡ AND HUGH O. McDEVITT

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

*Ir-1A* is a dominant autosomal gene(s) which regulates the specific antibody response to several antigens, including three branched, multichain, polypeptide antigens, poly-L-(Tyr,Glu)-poly-D,L-Ala--poly-L-Lys; poly-L-(His,Glu)-poly-D,L-Ala--poly-L-Lys; and poly-L-(Phe,Glu)-poly-D,L-Ala--poly-L-Lys, abbreviated respectively (T,G)-A--L, (H,G)-A--L, and (Phe,G)-A--L (1).<sup>1</sup> The *Ir-1A* gene is located within the *H-2* region of the mouse, between the K end and another immune response gene, *Ir-1B*, which in turn maps between *Ir-1A* and the *Ss-Slp* genes, in the middle of the *H-2* region (2-4).

Adoptive transfer experiments have demonstrated that the *Ir-1A* gene is expressed in lymphoid cells. *Ir-1A*-regulated immune responsiveness was found to be transferable with lymphocytes from high responder animals transferred into lethally irradiated low responder animals (5). The genetic defect in the low responders appears to be expressed in thymus-derived (T) cells, as demonstrated by the following experiments: (a) low responder animals give a normal immune response when immunized with (T,G)-A--L coupled to a highly immunogenic carrier such as methylated bovine serum albumin (6, 7); (b) the primary IgM response elicited by aqueous immunization is equal in high and low responder animals, and this primary response is not dependent on the presence of T cells, since irradiated, thymectomized, bone marrow reconstituted, high and low responder animals are capable of a normal IgM response to (T,G)-A--L while only the intact responder animals make an IgG secondary response (8, 9); (c) tetraparental mice prepared from high and low responder embryos produce high amounts of antibody of the low responder allotype (10). In contrast to these results, limiting dilution experiments of Shearer et al. have suggested that the genetic defect in the response to (Phe,G)-A--L is expressed in both T and B cells (11) and that the genetic defect in the response to (T,G)-A--L is expressed only in B cells (12). The former data, and the antigen-specific nature of the regulation, suggest that the *Ir-1A* gene-product is expressed on T

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<sup>1</sup> Abbreviations used in this paper: CFA, complete Freund's adjuvant; (H,G)-A--L, poly-L-(His,Glu)-poly-D,L-Ala--poly-L-Lys; LPS, *E. coli* 055.35 lipopolysaccharide; PBS, phosphate-buffered saline; PEC, peritoneal exudate cells; PEL, peritoneal exudate lymphocytes; PHA, phytohaemagglutinin-P; (Phe,G)-A--L, poly-L-(Phe,Glu)-poly-D,L-Ala--poly-L-Lys; PWM, pokeweed mitogen; S.I., stimulation index; (T,G)-A--L, poly-L-(Tyr,Glu)-poly-D,L-Ala--poly-L-Lys.

lymphocytes and are compatible with the hypothesis that the *Ir-1A* gene product may be the T-cell antigen receptor, or a part of it, if it is a complex structure (13).

In an effort to obtain more direct evidence on the T-cell expression of *Ir-1A* regulation, and to develop methods for preliminary characterization of the gene product, an in vitro system was established. Lymphocytes of in vivo-sensitized mice were stimulated with the homologous antigen in vitro and lymphocyte transformation was measured by [<sup>3</sup>H]thymidine uptake. The present report describes experiments demonstrating the T-cell dependence of the in vitro stimulation of lymphocytes by (T,G)-A--L, (H,G)-A--L, and (Phe,G)-A--L. The requirements for this stimulation have been compared to the requirements for B-cell stimulation and antigen binding (14), and the blocking activity of different alloantisera has been tested. A preliminary report of some of these experiments has been published elsewhere (15, 16).

## Materials and Methods

*Animals.* Mice of both sexes were used at an age of 2½–4 mo, and were bred at Stanford University. The strains, their origin, *H-2* genotype, and responder status to the three antigens used in this study are given in Table I.

*Antigens.* Two preparations of (T,G)-A--L were used: (T,G)-A--L-509, mol wt 232,000 and (T,G)-A--L-52, mol wt 180,000. One preparation of (H,G)-A--L, was used: (H,G)-A--L-905 mol wt 100,000. (For more details concerning these antigens see refs. 2, 8, 10). Two preparations of (Phe,G)-A--L, were used: (Phe,G)-A--L-929 and (Phe,G)-A--L-1501 (exact details on the molecular weight and residue molar ratio of these antigens are not yet available). The immunogenicity and dependence on *Ir-1A* genetic control of each antigen of the same basic structure was similar (B. D. Deak, in preparation). All antigens were gifts from Dr. Michael Sela, Weizmann Institute, Rehovot, Israel.

*Immunization.* The antigens were injected into the hind footpads of mice in 0.06 ml complete Freund's adjuvant (CFA), or in some experiments, in aqueous solution. The dose of the antigen was, in the case of (T,G)-A--L, 10 µg; in the case of (H,G)-A--L, 10 µg; and of (Phe-G)-A--L, 100 µg. Mice immunized with antigen in CFA were sacrificed 3 wk after immunization, and mice immunized with antigen in aqueous solution were sacrificed 1 wk after immunization.

*Source of Cells.* Experiments were performed either with popliteal and inguinal lymph node cells, or with peritoneal exudate lymphocytes (PEL). The lymph nodes were trimmed of fat and carefully teased with two pairs of sharp forceps into phosphate-buffered saline (PBS). Peritoneal exudate cells (PEC) were obtained 3–4 days after intraperitoneal injection of 3 ml mineral oil by flushing the peritoneal cavity with PBS containing 5 U heparin/ml. The exudate was separated from

TABLE I  
*Inbred Mouse Strains Used, and Their Relevant Characteristics*

Strain	Abbreviation	Background if congenic	<i>H-2</i> haplo-type	Response to:		
				(T,G)-A--L	(H,G)-A--L	(Phe,G)-A--L
C3H.SW	CSW	C3H/DiSn	<i>b</i>	High	Low	High
C3H.SW-Ig-1 <sup>b</sup>	CWB	C3H/DiSn	<i>b</i>	High	Low	High
C3H/DiSn	C3H	—	<i>k</i>	Low	High	High
C3H.Q	—	C3H/HeJ	<i>q</i>	Low	Low	High
B10.S	—	C57BL/10Sn	<i>s</i>	Low	Low	Low

the mineral oil by repeated centrifugation. At this stage it contained approximately 60–75% macrophages, 20–30% lymphocytes, and 5–10% eosinophil leukocytes. The lymphocytes from the peritoneal exudate were purified by filtration through rayon wool according to Rosenstreich et al. (17).

*Cell Cultures.* In the first half of this study a modification of Tyan's technique was used (18). In this modification, 1 million lymph node cells were cultured in no. 2058 Falcon tubes, (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in 1-ml tissue culture medium. The medium was Tyan's modification of MEM-spinner medium, which was supplemented with glutamine, nonessential amino acids, sodium bicarbonate, streptomycin-penicillin, and 5% human serum. In later experiments, Falcon microtest II plates (no. 3040) were used.  $1 \times 10^6$  cells were cultured in 0.2 ml of RPMI-1640, supplemented with glutamine, streptomycin-penicillin, and 5% human serum. All tissue culture media and reagents were purchased from Grand Island Biological Co., Grand Island, N. Y. The cultures were incubated at 37°C, with 7.5% CO<sub>2</sub>-air mixture, in a humidified incubator, and were labeled for approximately 16–18 h before the termination of the experiment with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine (6.7 Ci/mM, New England Nuclear, Boston, Mass.). After labeling, the cells were collected on Whatman glass fiber filters and processed according to Robbins et al. (19). The filters were counted in a toluene, 2-5-diphenyloxazol, 1, 4-bis[2-(5-phenyloxazolyl)]-benzene scintillation mixture, in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). For every experimental point, at least three simultaneous cultures were made. The stimulation index (S.I.) was calculated as follows:

$$\frac{\bar{X} \text{ cpm in the presence of antigen}}{\bar{X} \text{ cpm in the absence of antigen}} = \text{S.I.}$$

In several experiments mitogens other than the specific antigen were used. Phytohaemagglutinin-P (PHA-P), pokeweed mitogen (PWM), and lipopolysaccharide (LPS) were obtained from Difco Laboratories, Detroit, Mich. For further details of antigen dose and incubation time, please see the text.

*Isolation of T Cells.* T cells were isolated from the peritoneal exudates by the nylon wool filtration technique (20). The separation resulted in a population of 80–90% lymphocytes which were 85–95% Thy 1.2 positive by cytotoxic titration. The yield was approximately 25–35% of the initial nucleated cell count.

*Antisera.* A polyvalent rabbit antimouse immunoglobulin was kindly provided by Dr. L. A. Herzenberg, Department of Genetics, Stanford University School of Medicine. The properties of this serum have been described previously (21). The anti-*H-2<sup>g</sup>* serum (AS-348A) was produced in (B10.A-(5R)  $\times$  LP.RIII)F<sub>1</sub> mice against DBA/1 spleen and thymus cells. It contains antibodies to *H-2.17* and *30*, which are the K and D private specificities of *H-2<sup>g</sup>* (9). The anti *H-2<sup>g</sup>* serum (AS 598E) was produced in B10.BR  $\times$  C3H)F<sub>1</sub> mice against C57BL/10Sn cells. Both sera were gifts from Dr. G. D. Snell, Jackson Laboratories, Bar Harbor, Maine. To remove potential autoantibodies and nonspecific toxicity, these sera were adsorbed with BALB/c (*H-2<sup>d</sup>*) lymphocytes. Anti-Thy-1.2 (anti- $\theta^{C3H}$ ) serum was obtained from Dr. U. Hämmerling, Sloan-Kettering Memorial Cancer Institute, New York. The anti-Thy-1.2 serum was produced in (A $\theta^{AKR}$   $\times$  AKRH-2b)F<sub>1</sub> mice against the ASL-1 tumor of A origin. In this congenic combination the main antigenic difference is the  $\theta^{C3H}$ -antigen carried by the tumor cells.

## Results

*Dose-Response Studies.* C3H.Q mice were immunized with (Phe,G)-A--L in CFA. 3 wk later, cultures were set up in the presence of 10, 50, 100, 200, and 400  $\mu$ g antigen per ml. The antigen was given at 0 time, and the cultures were incubated for 4 days. Fig. 1 shows that the optimal dose was 200  $\mu$ g/ml. The optimal antigen dose for (H,G)-A--L in C3H mice and for (T,G)-A--L in C3H.SW mice was determined in a similar manner. Fig. 2 demonstrates that the optimal dose of (H,G)-A--L is 100  $\mu$ g/ml. The same dose was found to be optimal for (T,G)-A--L. These doses were used throughout our studies. The difference in

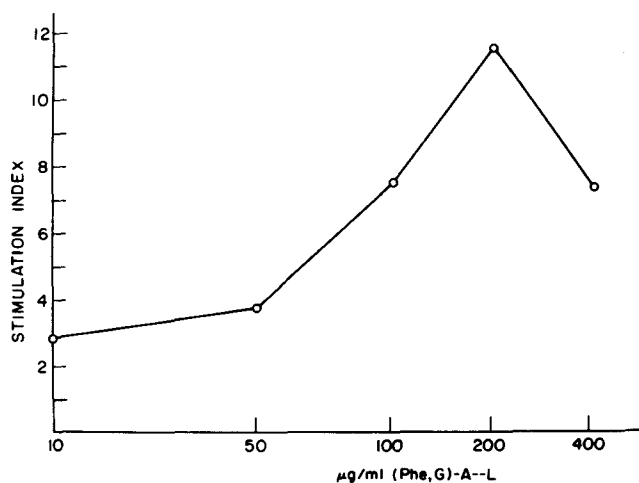


FIG. 1. Antigen dose response curve for in vitro stimulation with (Phe,G)-A--L. C3H.Q lymph node lymphocytes from mice immunized with (Phe,G)-A--L in CFA.

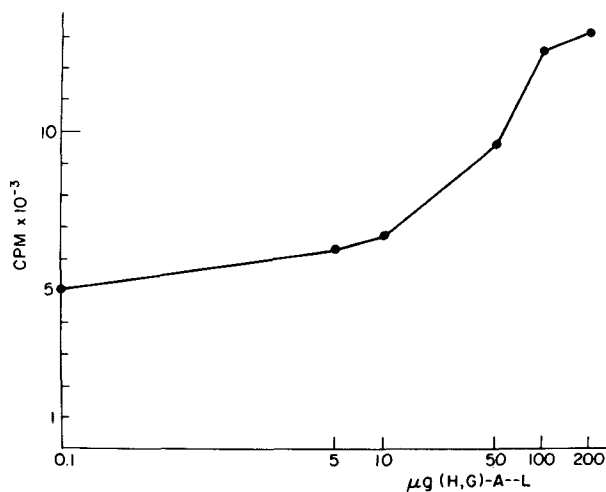


FIG. 2. Antigen dose-response curve for in vitro stimulation with (H,G)-A--L. C3H lymph node lymphocytes from mice immunized with (H,G)-A--L in CFA.

antigen dose between (Phe,G)-A--L and (T,G)-A--L or (H,G)-A--L is perhaps explained by the higher molecular weight of the latter two. The optimal dose determined by us is in good agreement with the data of Tyan (18). However, we did not obtain stimulation values as high as he reports; and in our case no primary stimulation was observed.

*The Kinetics of the In Vitro Response.* 30 cultures were established from lymph node cells of C3H.Q mice immunized with (Phe,G)-A--L in CFA, 15 of them in the presence of antigen, and 15 in the absence of antigen. Triplicate cultures were processed daily for [<sup>3</sup>H]thymidine uptake (Fig. 3). The peak of the response was on the 4th day of incubation. However, it was apparent that DNA

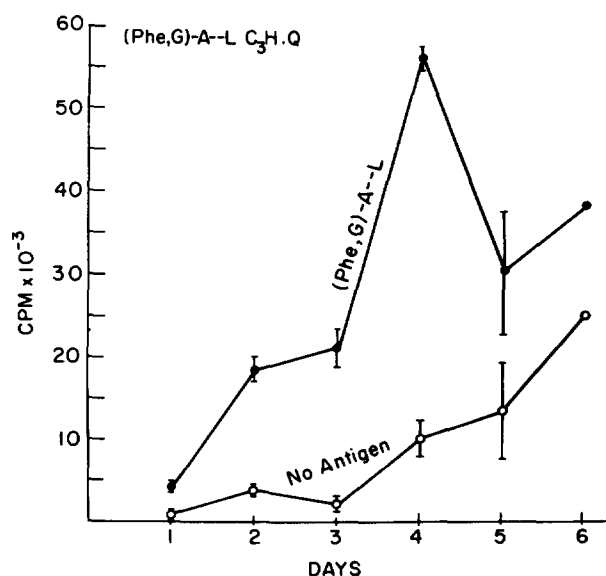


FIG. 3. Kinetics of in vitro antigen stimulation. C3H.Q lymph node cells from mice immunized with (Phe,G)-A-L in CFA.

synthesis in cultures to which no antigen had been added also followed an upward curve, thus decreasing the stimulation indices. In previous experiments (P. Lonai, unpublished data) it had been shown that the tissue culture supernatant of CFA-immunized lymph node cells, in the first 48 h of cultivation, contains a stimulatory substance. Therefore, an experiment was performed in which the cultures were incubated for 24 h in the absence of antigen. After this time the tubes were centrifuged, the medium replaced, and antigen added. In this case, lower nonstimulated control values, and better stimulation indices were obtained (Fig. 4). This experiment was performed with C3H mice immunized with (H,G)-A-L in CFA. In subsequent experiments this method was used, except in the case of aqueous immunization, and with PEL cells, where no preincubation was required.

*Optimal Time for Antigen Exposure.* One of the aims of these experiments was to test different serological reagents which might be specific for hypothetical antigen receptor structures. Ideally, in such blocking experiments, the blocking reagent should be present only for the time required for binding the antigen sufficiently for stimulation, after which, both the antigen and the blocking reagent should be removed from the system. In this system, initially unbound antigen cannot stimulate receptors which may reappear after capping and internalization (22). Another advantage of such an approach is that it avoids the possible toxic effect of the added serum. To achieve this objective, the following experiment was performed: six groups of cultures were established from C3H.Q mice immunized with (Phe,G)-A-L. One group was incubated for 5 days in the continuous presence of antigen. The other groups were washed and the medium was replaced without antigen after different time intervals between 30 min and

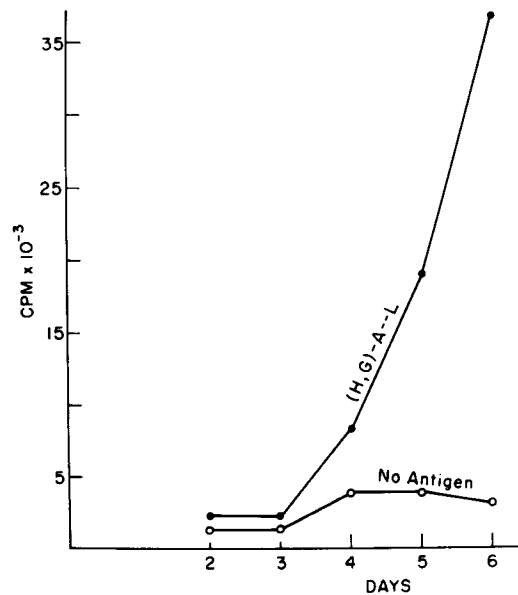


FIG. 4. Kinetics of in vitro antigen stimulation. The effect of preincubation before the addition of (H,G)-A--L. C3H lymph node cells from mice immunized with (H,G)-A--L in CFA.

24 h. It was found (Fig. 5), that 3–4 h presence of the antigen in the cultures is sufficient for stimulation. This protocol was used in all the blocking experiments.

*The Nature of the Responsive Cell.* In guinea pigs, antigen induced secondary in vitro lymphocyte transformation appears to be primarily a T-cell dependent function (23). In the mouse, this reaction is highly sensitive to anti-Thy-1.2 plus complement. However, Elfenbein and co-workers have demonstrated a small, but significant, contribution of B cells in the guinea pig lymphocyte proliferation reaction (24). To obtain an estimate of T-cell dependency in this system, the following experiments were performed: first, in several experiments the cells were pretreated with anti-Thy-1.2 and complement before antigenic stimulation, in which case, very low or no in vitro response was observed; second, to further assess T-cell dependency, PEC from (T,G)-A--L immunized C3H.SW mice were filtered through a nylon wool column according to Julius et al. (20). The resulting cell suspension was 91% Thy-1.2 positive. These cells gave a significant stimulation index in the presence of (T,G)-A--L, and the stimulation index could be increased if  $5 \times 10^4$  unfractionated, nonimmune PEC were added as a macrophage source (Fig. 6). These results suggest that the reaction requires no, or only a very small number of B cells; that the reaction is macrophage dependent; and that the majority of responding cells are thymus derived.

*The Genetic Regulation of the In Vitro Response.* The genetic regulation of immune responsiveness to the three synthetic antigens studied has been characterized mainly by measuring serum antibody levels. In vitro lymphocyte stimulation by antigen offers a method for determining whether *Ir-1A* regulated immune responsiveness is expressed in a T-cell response. Several experiments

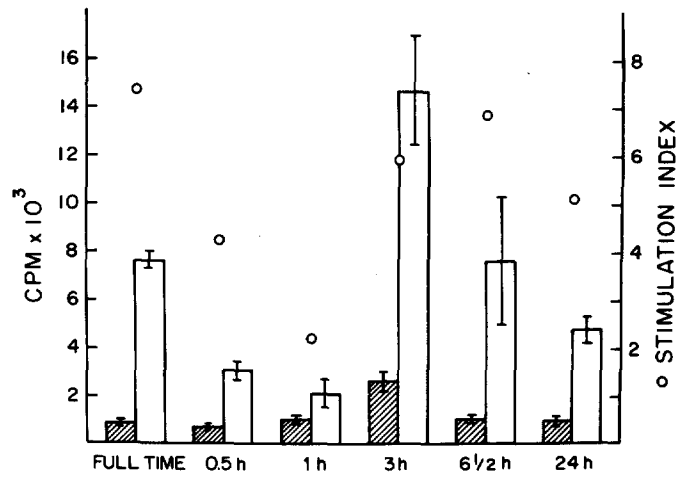


FIG. 5. The time required for the presence of antigen in vitro. C3H.Q lymph node cells from mice immunized with (Phe,G)-A-L in CFA. Hatched columns, no antigen added. Open columns, antigen added. Open circles, stimulation index.

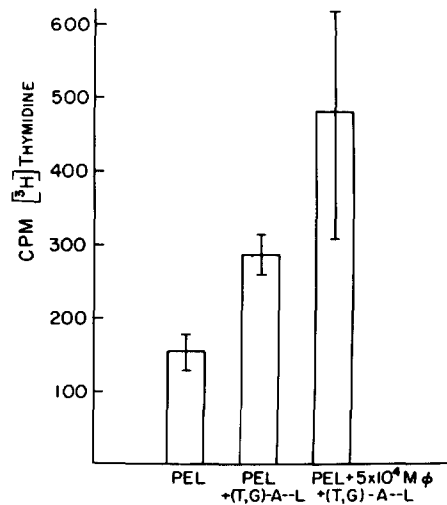


FIG. 6. In vitro antigen stimulation of purified T cells. C3H.SW mice were immunized with (T,G)-A-L in CFA. The peritoneal exudate cells were removed and T cells separated on a nylon wool column. Mφ: 5 × 10<sup>4</sup> peritoneal exudate cells, unpurified.

were performed in which antigen-induced stimulation of lymphocytes from primed high responder and low responder mice was measured in the presence of the homologous antigen. A summary of these experiments is shown in Table II. The experiments demonstrated that the in vitro system differentiates between high and low responder genotypes in the same manner as does in vivo antibody production, but apparently to a greater extent, since the cells from low responder animals gave no detectable stimulation.

*The Requirement for Metabolic Activity for Effective In Vitro Stimulation.*

TABLE II  
*H-2-Linked Immune Responsiveness to Synthetic Polypeptides  
 As Measured by Secondary In Vitro Stimulation\**

Strain	<i>H-2</i>	S.I. after primary immunization in vivo and stimulation in vitro with:		
		(T,G)-A--L	(H,G)-A--L	(Phe,G)-A--L
CWB	<i>b</i>	12.03	0.98	5.31‡
C3H	<i>k</i>	0.99	7.79 12.06	2.5‡
C3H.Q	<i>q</i>	ND	1.15	4.47 2.57‡
B10.S	<i>s</i>	1.12‡	ND	0.56‡

\* Data collected from three experiments. In every experiment more than one strain, or more than one antigen was used, so that high and low response could be measured within one experiment.

‡ Experiment with PEL cells.

There are strong indications that antigen binding by T cells is a metabolically active process. It has been shown that T cells do not bind specifically to cellular immunosorbents (target cell monolayers) (25) at low temperatures or in the presence of sodium azide (26). On the other hand, it is well known that B cells bind to antigen columns almost equally well at room temperature or in the cold (27). In order to investigate this qualitative difference between antigen binding by T and B cells at the level of actual stimulation, in the case of a genetically controlled response, the following experiment was performed. Lymphocytes from C3H.Q mice immunized with (Phe,G)-A--L were stimulated in vitro with the homologous antigen. Four groups of cultures were established (Fig. 7). In the first group, the cells were exposed to the antigen for 3 h at 37°C and then the cultures were washed as described previously. In the second and third group, the cells were exposed to the antigen at 4°C for 3 h, after which time half of the tubes (group 3) were transferred to 37°C for an additional 3 h of incubation, and the antigen was removed only after this step. The fourth group was incubated with the antigen for the full 5 days of the experiment. Practically no stimulation occurred at 4°C (Fig. 7) and incubation in the cold had no deleterious effect on the cultures, since an additional 3 h incubation with antigen at 37°C after incubation in the cold still resulted in significant stimulation. Similar results were obtained for the temperature dependence of (T,G)-A--L binding by T cells by G. J. Hämmerling et al. (14). This study showed that antigen binding by T cells is significantly increased at 37°C, as compared to T-cell antigen binding at 4°C. However, B-cell binding was not influenced by temperature changes. These experiments suggest some type of physiological difference between the antigen binding and specific stimulatory processes of T and B cells.

*The Absence of Cross-Stimulation between (Phe,G)-A--L and (H,G)-A--*



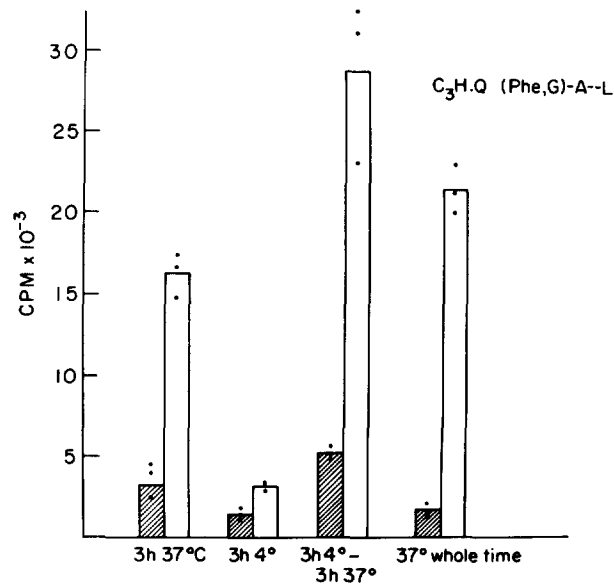


FIG. 7. The temperature dependence of in vitro antigen stimulation. Hatched columns, no antigen present. Open columns, antigen added to cultures.

L. (T,G)-A--L, (H,G)-A--L, and (Phe,G)-A--L are very similar molecules. The antibodies produced against each of them cross-reacts serologically to a high degree with the other two, but they show no in vivo cross-immunogenicity (28). It has been proposed that the B-cell antigen receptor is identical to the immunoglobulin produced by the cell (29). If T cells utilize the same antigen receptors, it would be expected that T cells from animals sensitized with one of these antigens could be stimulated in vitro with the other two. Our experiments show that this is not the case (Fig. 8). C3H.Q mice are responders only to (Phe,G)-A--L. After sensitization in vivo with (Phe,G)-A--L, their cells did not respond to (H,G)-A--L in vitro. C3H mice are responders to both (H,G)-A--L and (Phe,G)-A--L. If the animals were sensitized with (H,G)-A--L, the cells responded in vitro only to (H,G)-A--L and not to (Phe,G)-A--L (Fig. 8). This experiment is also in agreement with those of Hämmerling et al. (4). They have shown that antigen binding by (T,G)-A--L-binding T cells cannot be blocked by the addition of a 10-fold molar excess of (H,G)-A--L or (Phe,G)-A--L, while B-cell binding of (T,G)-A--L was completely blocked by the addition of a 10-fold molar excess of (H,G)-A--L or (Phe,G)-A--L. These results again indicate fundamental differences between the antigen-binding properties of T and B lymphocytes.

*Inhibition of In Vitro Stimulation by Alloantisera.* Because of the genetic linkage between *H-2* and *Ir-1A* and the lack of linkage between *H-2* and the known immunoglobulin structural genes (2, 7), the effect of anti-*H-2* sera and anti-immunoglobulin sera, was studied. The amount of antisera to be used was determined in the following way: for anti-*H-2* sera, a dose was selected for a culture containing  $1 \times 10^6$  cells which was enough to lyse  $5 \times 10^6$  lymphocytes of the appropriate *H-2* genotype in the presence of complement. The polyvalent

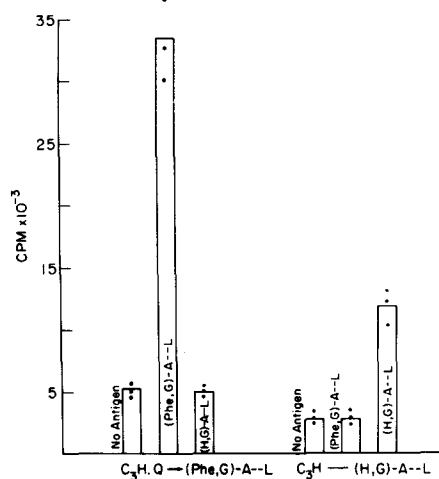


FIG. 8. The absence of "cross stimulation" in the in vitro reaction. C<sub>3</sub>H.Q → (Phe,G)-A--L: C<sub>3</sub>H.Q lymph node cells from mice immunized with (Phe,G)-A--L in CFA. C<sub>3</sub>H → (H,G)-A--L: C<sub>3</sub>H lymph node cells from mice immunized with (H,G)-A--L in CFA.

anti-immunoglobulin serum was used at a dose which was sufficient for optimal staining in indirect immunofluorescence assay of  $5 \times 10^6$  lymphocytes.

In the first experiment, the effect of normal BALB/c mouse serum, anti-*H-2<sup>a</sup>* serum and rabbit anti-immunoglobulin serum was tested in different doses on C<sub>3</sub>H.Q lymph node cells sensitized with (Phe,G)-A--L. The antigen and the blocking sera were present in the first 4 h of the experiment, after which the cultures were washed and incubated for 5 days as described in Materials and Methods. As can be seen from Fig. 9, normal mouse serum had no blocking effect on the reaction. The anti-*H-2* serum significantly inhibited stimulation, and the anti-immunoglobulin in serum had no inhibitory effect.

This finding can be interpreted in several ways. It is known that, if T cells have surface immunoglobulin (30) receptors, they may be present in a significantly lower concentration than on B cells (31, 32). Therefore, it could be postulated that the lack of inhibition of stimulation by anti-immunoglobulin might be due to the lack of these rare specificities in our preparation. In the second half of the experiment, the effect of anti-*H-2* sera could be interpreted as being due to a nonspecific effect. Thus, the inhibition observed might be the result of the perturbation of the cell membrane by the binding of large amounts of alloantisera. To test these possibilities, the effect of antibodies to other T-cell surface components was studied, and the inhibitory effect of the anti-immunoglobulin serum was tested on lymphocyte transformation reactions induced by T- or B-cell-specific mitogens.

Thy-1.2 is present in high density on T cells. If the blocking effect of anti-*H-2* sera was due only to the perturbation of the membrane by bound antibody, then it might be expected that anti-Thy-1.2 sera would have a similar effect. Fig. 10 demonstrates a representative experiment. (Phe,G)-A--L-sensitized C<sub>3</sub>H.SW cells were stimulated by (Phe,G)-A--L. The reaction was completely inhibited by anti-*H-2<sup>b</sup>*-serum, while the addition of anti-Thy-1.2 serum in the absence of

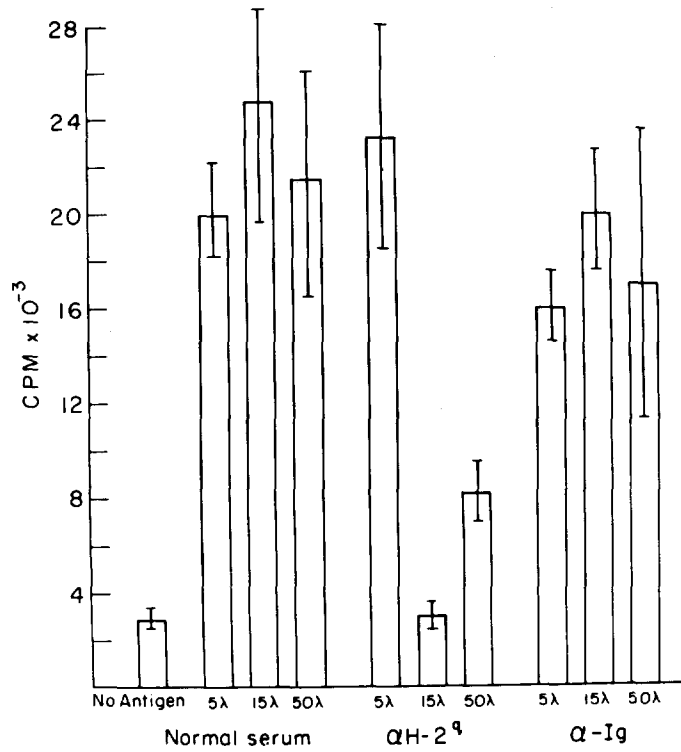


FIG. 9. The effect of alloantisera on in vitro antigen stimulation. C3H.Q lymph node cells from mice immunized with (Phe,G)-A--L.

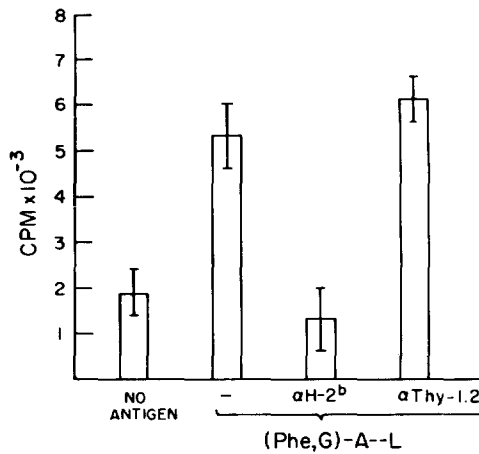


FIG. 10. The effect of anti-Thy-1,2 serum on the in vitro antigen stimulation. C3H.SW lymph node cells from mice immunized with (Phe,G)-A--L in CFA.

complement, had no effect, suggesting that the inhibitory effect of the anti-*H-2* sera is not merely a nonspecific effect and may be the result of the blocking of some structure, identical with, cross-reactive with, or situated near the specific antigen receptor.

The effect of the anti-*H-2* and anti-immunoglobulin sera was also tested in blocking the action of mitogens of known cellular specificity. PHA in soluble form is a T-cell stimulant, while PWM primarily stimulates B cells (33, 34). Bacterial LPS appears to be an exclusive B-cell mitogen (35). To evaluate the effect of our alloantisera on stimulation by these mitogens, the effect of anti-*H-2<sup>a</sup>* and of polyvalent anti-Ig was studied on (Phe,G)-A--L-sensitized C3H.Q cells stimulated by (Phe,G)-A--L, PHA and PWM. Fig. 11 demonstrates the inhibitory effect of the anti-*H-2* serum, and the lack of effect of the anti-Ig serum on specific antigen stimulation. In the same experiment, neither of the sera inhibited PHA stimulation while the anti-immunoglobulin serum had strong, and the anti-*H-2* serum a weak effect on PWM stimulation of the same cells. The experiment demonstrates that the anti-Ig serum does inhibit B-cell proliferation. Subsequently, we studied the effect of the alloantisera on B-cell stimulation by LPS. C3H.SW spleen cells were stimulated with LPS or PHA. Anti-Ig or anti-*H-2<sup>a</sup>* sera were added to the cultures. It can be seen from Table III that anti-immunoglobulin completely blocks LPS stimulation, while anti-*H-2* has only a slight effect. In agreement with former experiments, the two sera had no, or only slight, effect on PHA stimulation.

These experiments suggest that the antibody binding to specific surface

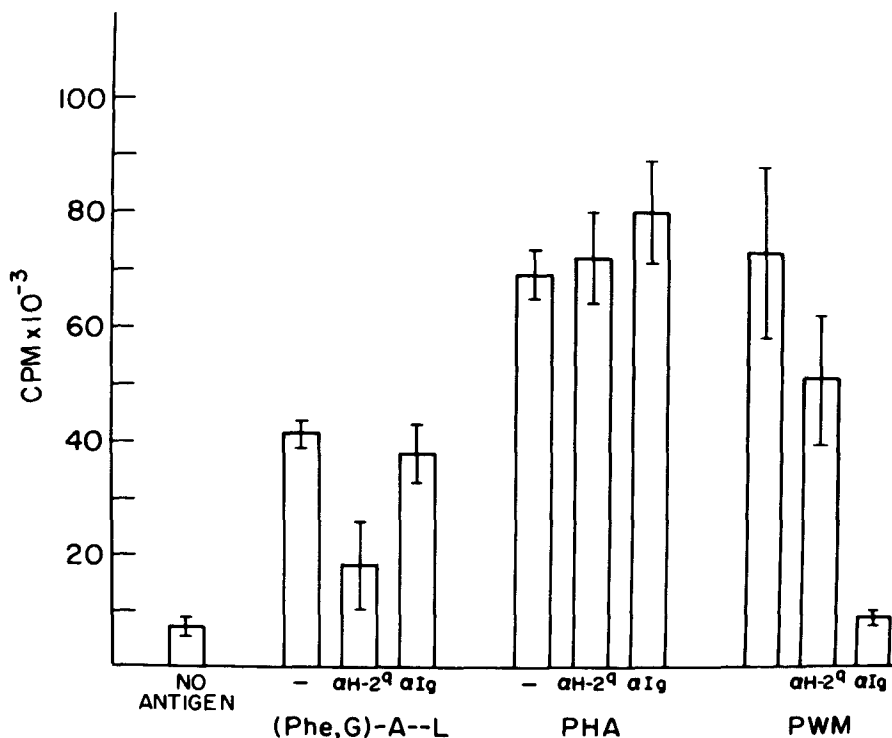


FIG. 11. The effect of alloantisera on stimulation by homologous antigen, PHA and PWM of C3H.Q mice sensitized with (Phe,G)-A--L.

TABLE III  
*The Effect of Polyvalent Anti-Ig Serum and of Anti-H-2 Serum on PHA- and LPS-Induced Stimulation of C3H.Q Spleen Cells*

	PHA	LPS	Anti-H-2	Anti-Ig	NMS*	cpm ± SD		S.I.	Inhibition‡
									%
Exp. 1§	-	-	-	-	-	395	74		
	-	-	+	-	-	524	218		
	-	-	-	+	-	528	181		
	-	-	-	-	+	553	69		
	+	-	-	-	-	7,861	3,073	19.9	—
	+	-	+	-	-	7,766	4,626	19.6	1.2
	+	-	-	+	-	6,894	2,324	17.5	12.3
	+	-	-	-	+	7,801	1,572	19.8	0.8
	-	+	-	-	-	1,104	37	2.8	—
	-	+	+	-	-	779	74	2.0	29.4
Exp. 2	-	-	-	-	-	1,482	380		
	-	-	+	-	-	884	128		
	-	-	-	+	-	1,982	446		
	-	-	-	-	+	2,142	639		
	+	-	-	-	-	32,765	5,236	22.1	—
	+	-	+	-	-	37,693	3,220	25.4	0.0
	+	-	-	+	-	26,164	3,806	17.7	20.1
	+	-	-	-	+	31,523	3,134	21.3	3.8
	-	+	-	-	-	11,835	3,841	8.0	—
	-	+	+	-	-	9,831	2,043	6.6	16.9
-	+	-	+	-	3,875	685	2.6	67.3	
-	+	-	-	+	10,635	1,057	7.2	10.1	

\* Normal mouse serum.

‡ Calculated from the values of untreated cultures respectively for each group.

§ In this experiment 5  $\mu$ l of 1:5 diluted PHA-P, 50  $\mu$ g/ml PS, 15  $\mu$ l of anti-H-2<sup>a</sup>, and 15  $\mu$ l of anti-Ig serum was used.

|| In this experiment 10  $\mu$ l of 1:5 diluted PHA-P, 100  $\mu$ g/ml LPS and the same amount of alloantisera as in Exp. 1 were used.

components or cross-reactive or closely situated structures can inhibit lymphocyte transformation. *H-2* seems to be involved in antigen-specific T-cell stimulation. Immunoglobulins appear to be involved in some way in the stimulation of B cells by LPS. This latter finding demonstrates that the anti-immunoglobulin serum employed does have specificities towards lymphocyte surface immunoglobulin involved in some way in LPS stimulation, and cannot be considered inactive.

## Discussion

The in vitro lymphocyte transformation system used in these experiments possesses the antigenic specificity characteristic of the *Ir-1A* gene(s) (cf. Table II). In this system, antigen specific tritiated thymidine uptake appears to be B-cell independent, since purified T cells exhibit a degree of responsiveness comparable to nonpurified lymph node or PEL cells. The fact that purified T cells exhibit an in vitro responsiveness which is identical in its specificity to the in vivo specificity of the *Ir-1A* gene indicates that this gene is expressed in thymus-derived immunocompetent cells. This conclusion is supported by several ancillary findings. First, the specificity of antigen recognition in this in vitro system is more precise than the binding specificity of humoral antibodies directed against the three serologically cross-reactive synthetic antigens (T,G)-A--L, (H,G)-A--L, and (Phe,G)-A--L (Fig. 8). Since T-cell antigen binding also differentiates between these three antigens, while B-cell antigen binding does not (14), and since in vivo immunogenicity demonstrates the same specificity as this in vitro system (28), these three observations together indicate that the specificity characteristics of the *Ir-1A* gene(s) are expressed in thymus-derived cells. Additional support for this conclusion comes from numerous experiments demonstrating no deficit in specific B-cell function or responsiveness in low responder animals when immunized with the antigen coupled to a recognizable carrier molecule (6-10, 16).

However, the results presented in these studies do not exclude the concomitant expression of *Ir-1A* gene products in B cells for the following reasons: (a) primary sensitization may require the presence of B cells for adequate T-cell priming; since the response studied is a secondary response, the contribution of B cells in the primary event leading to T-cell sensitization remains unknown. (b) Shearer, Mozes, and Sela have demonstrated by limiting dilution cell transfer experiments that the genetic defect in immune response to (T,G)-A--L is a B-cell defect, while that for immune response to (Phe,G)-A--L is expressed in both B and T cells (11, 12). The former of these two findings is in disagreement with the present study. Because of the different experimental design in these two systems, it is difficult to determine what might have caused the difference in experimental results. However, the demonstration that isolated T cells show the same pattern of responsiveness and specificity as is shown by in vivo immunization seems to us to support the conclusion that the *Ir-1A* gene(s) is expressed in T cells.

The requirement that the cells must be in a state of active metabolism for successful in vitro stimulation, which was observed in these experiments (Fig. 7), may be a general feature of T-cell interaction with antigens. Specific adherence of T cells to allogeneic cellular immunoabsorbents (26), (T,G)-A--L binding to T cells (14), and in vitro stimulation of T cells by antigen are either decreased or do not occur at low temperatures, and are inhibited by sodium azide and sodium nitrite, while antigen binding to B cells is not influenced by any of these manipulations. This requirement of T cells for an active metabolic state for optimal interaction with antigen may reflect a fundamental difference in the nature of the T-cell membrane, or in the nature of the T- and B-cell antigen receptor, or in the way in which the receptor is incorporated into the cell membrane of the respective cell types.

The studies on inhibition of in vitro antigen stimulation by alloantisera reported here suggest either serological or topographical relationship between antigens coded by the *H-2* complex and the T-cell antigen receptor. A direct correlation between the genetic regulation of the immune response and the specificity of blocking of antihistocompatibility sera on in vitro antigen stimulation was demonstrated by Shevach and colleagues (36, 37). These authors showed that in vitro stimulation of lymphocytes from  $F_1$  hybrid guinea pigs derived from strains which are responders to two different antigens could be selectively blocked only by antihistocompatibility sera directed against the histocompatibility antigens of the strain which is a responder to the antigen used for in vitro lymphocyte stimulation (39). Bluestein (40) showed that intact Ig and (Fab)<sub>2</sub> fragments were effective in blocking in an identical system, while active Fab monomer was ineffective, indicating that the relationship between histocompatibility antigens and T-cell antigen recognition in the guinea pig system is topographical, rather than due to serological cross-reaction or identity.

The lack of inhibition of in vitro antigen stimulation by anti-immunoglobulin sera (this report, 39) suggests either that the receptor involved is not an immunoglobulin, or that it is an immunoglobulin of a hitherto unknown class which does not react with the specificities included in our polyvalent anti-immunoglobulin sera. Further experiments are required for a more precise definition of the relationship between components of the major histocompatibility complex and specific antigen recognition. The use of alloantisera directed against specific regions of the *H-2* complex, including sera directed against *H-2K*, *Ia* (41), and *H-2D*, should permit further definition of the relationship of these cell surface antigens to T-cell-mediated antigen recognition.

### Summary

In vitro antigen-induced tritiated thymidine uptake has been used to study the response of sensitized lymphocytes to (T,G)-A--L, (H,G)-A--L, and (Phe,G)-A--L in responder and nonresponder strains of mice. The reaction is T-cell and macrophage dependent. Highly purified T cells (91% Thy 1.2 positive) are also responsive, suggesting that this in vitro lymphocyte transformation system is not B-cell dependent. Lymphocytes from high and low responder mice stimulated in vitro react as responders and nonresponders in a pattern identical to that seen with in vivo immunization. Stimulation occurs only if soluble antigen is added at physiological temperatures; antigen exposure at 4°C followed by washing and incubation at 37°C fails to induce lymphocyte transformation. Stimulation is specific for the immunizing antigen and does not exhibit the serologic cross-reactivity which is characteristic of these three antigens and their respective antisera. The reaction can be inhibited by anti-*H-2* sera but not by anti-immunoglobulin sera. The anti-immunoglobulin sera did, however, inhibit lipopolysaccharide or pokeweed mitogen stimulation. These results suggest that the *Ir-1A* gene(s) are expressed in T cells, and that there are fundamental physiologic differences between T- and B-cell antigen recognition.

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