GENETIC CONTROL OF IMMUNE RESPONSES IN VITRO*

I. Development of Primary and Secondary Plaque-Forming Cell Responses to the Random Terpolymer L-Glutamic Acid^{60}-L-Alanine^{30}-L-Tyrosine^{10} (GAT) by Mouse Spleen Cells In Vitro*

By JUDITH A. KAPP, CARL W. PIERCE,‡ and BARUJ BENACERRAF

(From the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115)

(Received for publication 19 July 1973)

The immune responses of inbred strains of mice to certain synthetic polypeptide antigens are controlled by dominant immune response (Ir)[1] genes which are closely linked to genes controlling expression of the major histocompatibility antigens (1, 2). The antibody response in mice to one of these antigens, the random terpolymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT), is an "all-or-none" response (3, 4). After injection of GAT in Maalox-pertussis anti-GAT antibody is produced by mice with the *H-2* genotypes *a*, *b*, *d*, or *k* (responder strains), whereas no detectable antibody to GAT is produced by mice with *H-2* genotypes *p*, *q*, or *s* (nonresponder strains). However, both responder and nonresponder strains produce anti-GAT antibodies after immunization with GAT complexed to methylated bovine serum albumin (GAT-MBSA) (5, 6).

We have used the Mishell-Dutton culture system (7, 8) to characterize the primary and secondary plaque-forming cell (PFC) responses developed by mouse spleen cells from both responder and nonresponder strains after stimulation with GAT or GAT-MBSA in vitro. In this communication, we will describe the critical parameters for stimulation of GAT-specific PFC responses in vitro and some of the characteristics of these responses. In subsequent communications, we will describe investigations of the precise nature of the cellular functions regulated by Ir genes.

* This investigation was supported by U.S. Public Health Service Research Grants AI-09897 and AI-09929 from the National Institute of Allergy and Infectious Diseases.

‡ Recipient of U.S. Public Health Service Research Career Development Award 1K4-AI-70173 from the National Institute of Allergy and Infectious Diseases.

[1] *Abbreviations used in this paper*: C, complement; GAT, random terpolymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}; GAT-MBSA, GAT complexed to methylated bovine serum albumin; GAT-SRBC, GAT coupled to sheep red blood cells; HBSS, Hanks' balanced salt solution lacking sodium bicarbonate; Ig, immunoglobulin, IgG is used to refer to IgG_1, IgG_{2a+2b} Ig in aggregate; Ir gene, immune response gene; MEM, completely supplemented Eagle's minimal essential medium; PBS, phosphate-buffered saline; PFC, plaque-forming cell(s); SRBC, sheep red blood cells.

Materials and Methods

Mice.—C57Bl/6J ($H\text{-}2^b$), SJL/J ($H\text{-}2^s$), A/J ($H\text{-}2^a$) mice were purchased from Jackson Laboratories, Bar Harbor, Me. The F_1 hybrid mice of (C57Bl/6 × SJL) matings ($H\text{-}2^{b/s}$) were produced in our animal facilities. A.SW ($H\text{-}2^s$) mice were purchased from Dr. G. Haughton, University of North Carolina; B10.S mice were a gift from Dr. D. Shreffler, University of Michigan. Mice used in these studies were from 2–8-mo old and were maintained on acidified-chlorinated drinking water and laboratory chow ad lib.

Antigens.—One preparation of the random terpolymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT) with an average molecular weight of 55,000 was purchased from Pilot Chemicals Div., New England Nuclear Corporation, Boston, Mass. A second preparation of GAT, average molecular weight of 32,000, was purchased from Miles Laboratory, Kankakee, Ill. The two GAT preparations were identical in their immunological properties. Stock solutions of 10 mg/ml were prepared in normal saline containing 1% Na_2CO_3 at pH 9.0–9.5. The stock solution of GAT was sterilized by filtration and diluted in Hanks' balanced salt solution (HBSS) for addition to spleen cell cultures. A sterile solution of GAT was complexed to a sterile solution of methylated bovine serum albumin (a gift from Dr. Paul Maurer, Jefferson Medical College) according to his technique (6). Sheep red blood cells (SRBC) were prepared as previously described (8) for addition to spleen cell cultures or for use in the hemolytic plaque assay.

Mouse Anti-GAT Serum.—Sera were obtained from several mice undergoing a secondary response to GAT, pooled, decomplemented by heat at 56°C for 30 min and absorbed three times in the cold with packed SRBC equivalent to 20% of the volume of serum.

Rabbit Anti-GAT Serum.—This serum, kindly donated by Dr. E. Dunham, was obtained from rabbits 7 days after the second immunization with 1 mg GAT in Freund's complete adjuvant. The anti-GAT activity of this serum was determined by a modified Farr assay (3) and bound 75 μg GAT/ml of serum. This serum was also heat inactivated and absorbed three times in the cold with packed SRBC equal to 20% of the serum volume.

Goat Antimouse μ-Chain Serum.—This serum was a gift from Dr. R. Asofsky, National Institutes of Health. The specificity of the serum and its effects on the development of immunoglobulin (Ig) class-specific anti-SRBC responses in vitro have been described in detail previously (9). This serum was added to spleen cell cultures with antigen at culture initiation as 50 μl of a 1:25 dilution of serum.

Rabbit Antimouse Immunoglobulin Sera.—A polyvalent rabbit antimouse IgG serum was routinely used to develop indirect PFC. Rabbit antisera specific for mouse γ_1, γ_{2a+b}, or γ_A Ig classes were used in some experiments to develop class-specific indirect PFC. The specificity of these sera has been previously described in detail (8). These sera were also a gift from Dr. R. Asofsky.

Immunization of Mice.—Mice were immunized with 10 μg GAT or with GAT-MBSA containing 10 μg GAT as a suspension in a mixture of magnesium-aluminum hydroxide gel (Maalox, Wm. H. Rorer, Inc., Fort Washington, Pa.) and pertussis vaccine (Eli Lilly and Co., Indianapolis, Ind.) as previously described (3).

Preparation of GAT Coupled SRBC (GAT-SRBC).—GAT was coupled to SRBC with chromium chloride ($CrCl_3$) (J. T. Baker Chemical Co., Phillipsburg, N. J.) by a modification of the method described by Gold and Fudenberg (10). The optimal ratio of reactants was determined empirically and is described below. SRBC in Alsever's solution (Colorado Serum Co., Denver, Col.) were washed three times by centrifugation and resuspended to 10% in 0.85% NaCl. $CrCl_3$ was pulverized and dissolved in 0.85% NaCl (10 mg/ml) immediately before use. At room temperaure, 1.0 ml of 10% SRBC was mixed with 1.0 ml of the GAT solution (10 mg/ml) in a 50 ml centrifuge tube. 1 ml of the freshly prepared solution of $CrCl_3$ was pipetted into the mixture of GAT and SRBC. The reaction was allowed to proceed for 1–2 min without stirring and was terminated by adding 45 ml of PBS (0.15M NaCl containing 0.001 M phos-

phates, pH 7.2). The coupled cells were washed three times by centrifugation with 50 ml PBS and resuspended in medium L-15 (Microbiological Associates, Bethesda, Md.) to 1% for the ^{51}Cr release assay or to 7.5% for the hemolytic plaque assay.

Antibody-Mediated Lysis of GAT-SRBC.—The susceptibility of GAT-SRBC indicator cells to lysis by anti-GAT antibodies and complement (C) was determined. SRBC and GAT-SRBC were labeled with ^{51}Cr by reacting 0.1 mCi $Na_2{}^{51}CrO_4$ (The Radiochemical Centre, Amersham, England) with 3×10^8 cells in 1.0 ml of medium L-15 for 30 min at 37°C. The cells were washed three times by centrifugation with 50 ml volumes of L-15 containing 10% fetal calf serum. The ^{51}Cr-labeled cells, 0.1 ml of 3×10^7 cell/ml, were mixed with 0.1 ml of diluted mouse anti-GAT serum and 0.1 ml of guinea pig serum (BBL Div. of BioQuest, Cockeysville, Md.) as a source of C. After incubation at 37°C for 1 h, the amount of ^{51}Cr released into the supernate was determined using a Packard gamma scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). The percent of ^{51}Cr released by the antiserum was determined using a distilled water-lysed control as 100% release and subtracting the amount of ^{51}Cr released by C alone from the total released by antiserum and C by the following relationship:

$$\%\,^{51}Cr \text{ release} = \frac{(^{51}Cr \text{ released by antiserum} + C) - (^{51}Cr \text{ released by C})}{(^{51}Cr \text{ released by } H_2O \text{ lysis}) - (^{51}Cr \text{ released by C})} \times 100.$$

Reproducibility of Coupling GAT to SRBC.—GAT-SRBC prepared on several different days with GAT were tested with one mouse anti-GAT serum and C in the ^{51}Cr release assay. Comparison of the 50% end points in Fig. 1, shows a difference of less than one twofold dilution indicating that this method of preparation GAT-SRBC provided indicator cells which measure anti-GAT activity in serum in a consistent manner. This anti-GAT serum released no ^{51}Cr when tested on uncoupled SRBC. When GAT-SRBC prepared with GAT from Miles Laboratory or from Pilot Chemicals Co. were compared in a ^{51}Cr release assay there was no significant difference in 50% end points with the standard mouse anti-GAT serum. Furthermore, when soluble GAT was added to a $\frac{1}{40}$ dilution of this serum, 100% of the ^{51}Cr released by the anti-GAT serum was inhibited with GAT at concentrations of 0.1 μg or greater. These concentrations of GAT had no effect on the release of ^{51}Cr from labeled SRBC by an anti-SRBC serum. GAT-

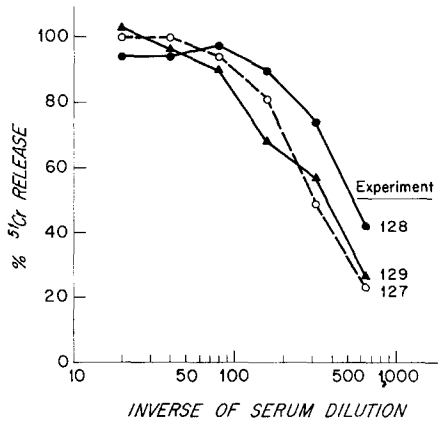

FIG. 1. Percent of ^{51}Cr released specifically from ^{51}Cr-labeled GAT-SRBC by a standard mouse anti-GAT serum and complement. Each line represents a separate experiment with a different preparation of GAT-SRBC.

SRBC indicator cells prepared by this method can also be used in microtiter systems to detect hemagglutinating activity in sera of mice immunized with GAT or GAT-MBSA in vivo.

Spleen Cell Cultures.—Suspensions of single spleen cells containing 10×10^6 nucleated cells/ml of culture medium (MEM) were incubated according to the method of Mishell and Dutton (7) with modifications previously described in detail (8). Experimental groups were incubated as duplicate cultures and each experiment contained cultures unstimulated, stimulated with 10^7 SRBC, and appropriate concentrations of GAT or GAT-MBSA.

Hemolytic Plaque Assay.—PFC in spleen cell cultures or in the spleens of mice immunized in vivo were enumerated by a modification of the Jerne hemolytic plaque technique described in detail previously (8). Suspensions of spleen cells were washed three times before assay to remove any soluble GAT. IgM PFC were enumerated after incubation of indicator red cells, SRBC or GAT-SRBC, and spleen cells in semi-solid agarose using guinea pig serum as a source of C. IgG PFC were developed with polyvalent rabbit antimouse IgG serum and C in assay preparations containing sufficient goat antimouse μ-chain antibody to inhibit the development of all IgM PFC (8). Each cell suspension was examined in agarose mixtures containing SRBC and in mixtures containing GAT-SRBC. The specificity of anti-GAT PFC was verified by adding 10 μg of soluble GAT to the assay mixtures to inhbit development of anti-GAT plaques. This concentration of soluble GAT reproducibly inhibited all anti-GAT PFC, while causing no, nonspecific, inhibition of development of anti-SRBC PFC. GAT-specific PFC were calculated by subtracting the number of PFC uninhibited by soluble GAT on GAT-SRBC from the number of PFC detected on GAT-SRBC in the absence of soluble GAT. Data are expressed as GAT-specific PFC/spleen for in vivo experiments and as GAT-specific PFC/culture for in vitro experiments.

RESULTS

Primary GAT-Specific Responses In Vivo.—In preliminary experiments, the first GAT-specific PFC in C57Bl/6 mice were detected 4 days after immunization with GAT and the maximum number was obtained by day 7. Fig. 2 illustrates the primary GAT-specific PFC responses in spleens of SJL (nonresponders), C57Bl/6 (responders), and the F_1 hybrid of C57Bl/6 $\times$ SJL (responders) mice 7 days after immunization in vivo with 10 μg of GAT as

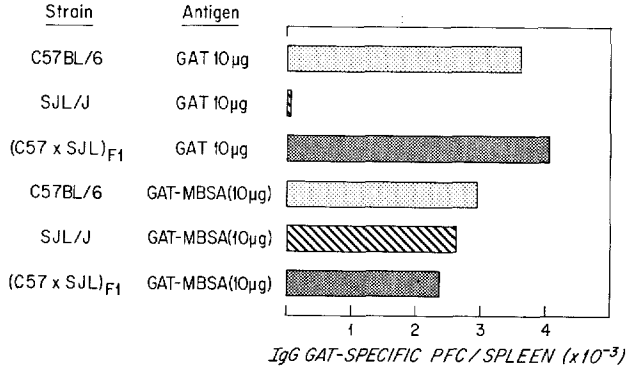

FIG. 2. Primary IgG GAT-specific PFC responses in spleens from C57Bl/6, SJL/J, and F_1 (C57 $\times$ SJL) mice immunized intraperitoneally 7 days before assay with 10 μg GAT as GAT-MBSA in Maalox-pertussis.

GAT-MBSA in Maalox-pertussis. No IgM PFC specific for GAT were found in the spleens of any of these mice at any time from 4 to 15 days after immunization. The C57Bl/6 and the F_1 mice developed GAT-specific IgG PFC responses when immunized with GAT, whereas the SJL mice did not. All three strains developed GAT-specific IgG PFC responses to GAT-MBSA.

Development of Primary and Secondary GAT-Specific PFC Responses In Vitro.—Spleen cells from normal C57Bl/6, SJL, and the F_1 (C57Bl/6 × SJL) mice were stimulated in vitro with either 10^7 SRBC, GAT-MBSA (5 μg GAT/culture), or various concentrations of soluble GAT. After 5 days, the time of the maximum response to SRBC, the cultures were examined for anti-SRBC PFC and anti-GAT PFC. The results in Fig. 3 show that cultures of C57Bl/6

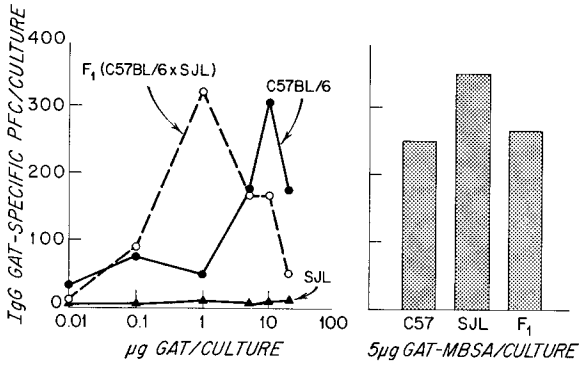

FIG. 3. Primary IgG GAT-specific PFC responses in cultures of spleen cells from C57Bl/6, SJL, and F_1 (C57 × SJL) mice stimulated with various doses of soluble GAT in vitro. The bar graph shows the IgG GAT-specific PFC responses in cultures of the same preparations of spleen cells stimulated with 5.0 μg GAT as GAT-MBSA.

and F_1 spleen cells developed GAT-specific IgG PFC responses to several doses of soluble GAT. Although 10 μg GAT stimulated the maximum response in cultures of C57Bl/6 spleen cells in this experiment, many other experiments have shown that 1 μg and 10 μg GAT stimulated comparable PFC responses; concentrations of soluble GAT higher than 10 μg always elicited a lower PFC response. Cultures of SJL spleen cells developed no GAT-specific IgG response at any concentration of GAT. All three strains developed GAT-specific IgG PFC responses to GAT-MBSA, and, although not shown, all three strains developed IgM and IgG anti-SRBC PFC responses. Again, no IgM PFC specific for GAT were detected in any of these cultures.

Kinetics of Appearance of GAT-Specific IgG PFC.—Cultures of spleen cells from normal C57Bl/6 and SJL mice, and C57Bl/6 mice immunized 6 wk earlier with 10 μg GAT in Maalox-pertussis were examined at various times after initiation for the development of PFC responses to SRBC and GAT.

At the time this experiment was done, 1 μg GAT elicited the maximal GAT-specific PFC responses in cultures of spleen cells from normal C57Bl/6 mice. The GAT-specific IgG PFC response in cultures of C57Bl/6 spleen cells (Fig. 4) reached a peak after 5 days incubation with 1 μg of GAT; no GAT-specific IgG PFC response was detected in cultures incubated with 0.1 μg GAT. Spleen cells from C57Bl/6 mice primed with GAT developed GAT-specific IgG PFC responses to both 0.1 μg and 1.0 μg GAT (Fig. 5), which also reached peaks after 5 days incubation. The GAT-specific PFC responses by spleen cells from

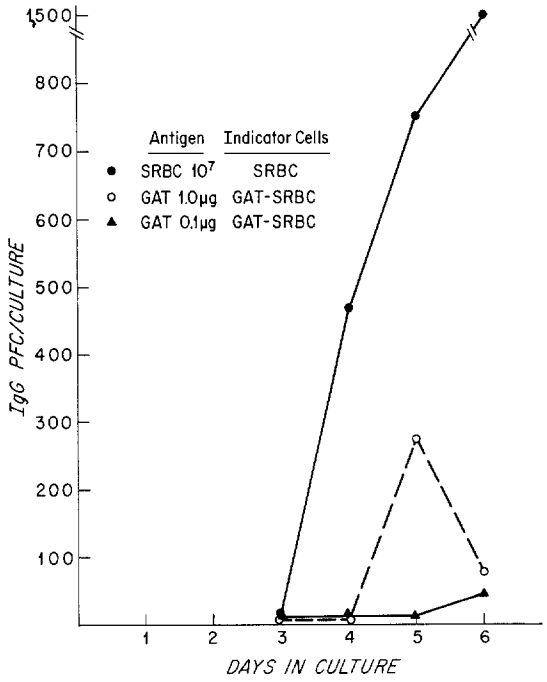

FIG. 4. Kinetics of the primary IgG PFC responses in cultures of spleen cells from C57Bl/6 mice stimulated with 10^7 SRBC, 1.0 μg GAT, or 0.1 μg GAT.

GAT-primed mice were always approximately two times greater in magnitude than the responses in cultures of spleen cells from normal C57Bl/6 mice. No IgM PFC specific for GAT were detected at any time in cultures of normal or primed spleen cells from C57Bl/6 mice. Spleen cells from normal SJL mice (Fig. 6) did not develop GAT-specific IgM or IgG PFC responses to either dose of soluble GAT at any time, however, they did develop an anti-SRBC IgM and IgG PFC response reaching a peak after 4 days incubation with SRBC. In subsequent in vitro studies, the PFC responses were assayed after 5 days incubation.

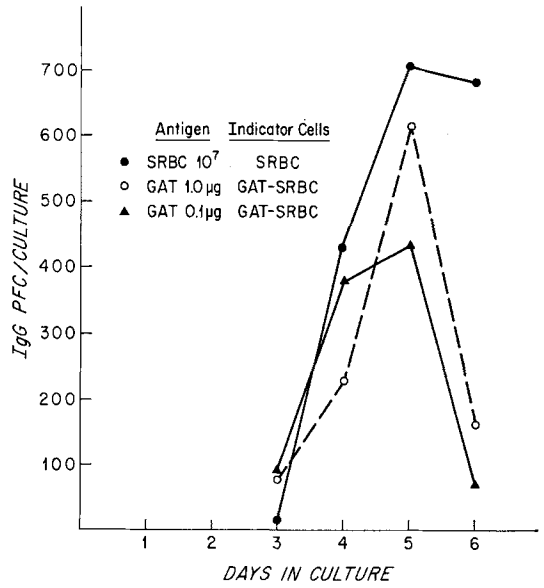

FIG. 5. Kinetics of the secondary IgG PFC responses in cultures of spleen cells from C57Bl/6 mice immunized with 10 μg GAT in Maalox-pertussis 4 wk before culture initiation and stimulated in vitro with 10^7 SRBC, 1.0 μg GAT, or 0.1 μg GAT.

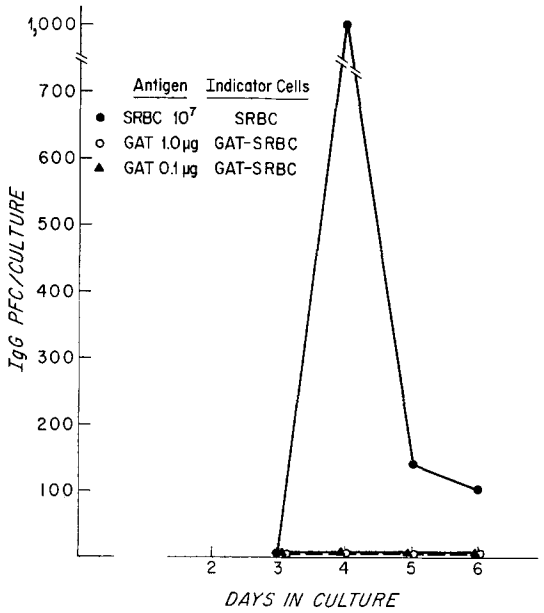

FIG. 6. Kinetics of the primary IgG PFC responses in cultures of spleen cells from SJL mice stimulated with 10^7 SRBC, 1.0 μg GAT, or 0.1 μg GAT.

Specificity of Stimulation by GAT In Vitro.—The experiment shown in Fig. 7 demonstrates that the primary GAT-specific PFC response in cultures of C57Bl/6 spleen cells was the result of specific antigenic stimulation by GAT. Rabbit anti-GAT serum, extensively absorbed with SRBC in the cold, was added to cultures stimulated with SRBC or GAT at culture initiation. 50 μl of a $\frac{1}{125}$ dilution of this serum had no effect on the anti-SRBC IgG PFC response, but, completely inhibited the GAT-specific IgG PFC response stimulated by 10 μg GAT. 50 μl of a $\frac{1}{625}$ dilution of anti-GAT serum had no effect on either the anti-SRBC or GAT-specific PFC responses.

Immunoglobulin Class of GAT-Specific PFC.—The Ig class of the antibody synthesized by GAT-specific PFC was examined by developing the PFC with class-specific rabbit antimouse γ_1, γ_{2a+b}, and γ_A sera. For reference, the Ig

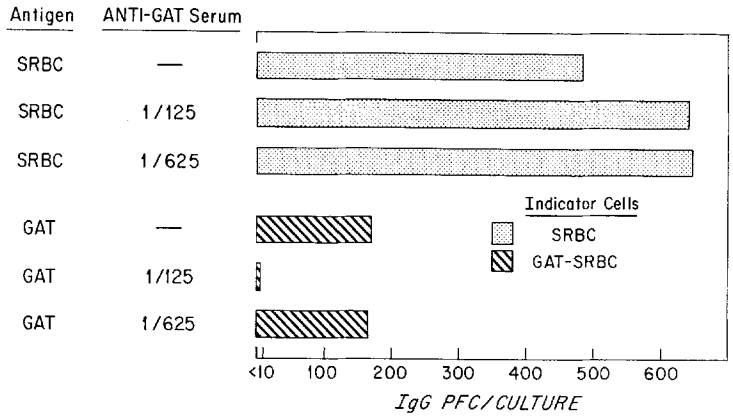

FIG. 7. The effect of rabbit anti-GAT serum on the IgG PFC responses in cultures of spleen cells from C57Bl/6 mice stimulated with 10^7 SRBC or 10 μg GAT. 50 μl of a 1/125 or 1/625 dilution of the antiserum was added at culture initiation.

classes of anti-SRBC PFC were also determined. The results in Table I show that anti-SRBC PFC in cultures of spleen cells from C57Bl/6 and SJL mice are approximately 50% γ_1, 30% γ_2, and 12–20% γ_A. The GAT-specific PFC in cultures of spleen cells from normal SJL mice stimulated in vitro with GAT-MBSA, and from normal C57Bl/6 mice stimulated in vitro with GAT or GAT-MBSA were 80–100% γ_1, 0–3% γ_2, and 6–18% γ_A. Whereas, primary GAT-specific PFC responses by C57Bl/6 and SJL spleen cells were predominately of the γ_1 class, with few or no γ_2 PFC, the secondary GAT-specific responses by SJL spleen cells stimulated with GAT-MBSA and C57Bl/6 spleen cells stimulated with GAT contained significant numbers of GAT-specific PFC of the γ_2 Ig class. The total number of PFC developed by the class-specific antisera does not equal the number of PFC developed by the polyvalent rabbit anti-IgG serum, nor is it expected to, since the polyvalent serum is not a pool of the class-specific sera.

TABLE I

Immunoglobulin Class of GAT-Specific PFC

Mouse strain	Antigen	Indicator cells	PFC/culture			
			IgG*	IgG_1	IgG_{2a+2b}	IgA
C57Bl/6‡	SRBC	SRBC	4,200	2,635	1,480	590
	GAT	GAT-SRBC	1,375	1,155	25	80
	GAT	GAT-SRBC	880	160	5	20
	GAT-MBSA	GAT-SRBC	300	90	0	20
C57Bl/6§	GAT	GAT-SRBC	745	370	160	120
SJL/J‡	SRBC	SRBC	1,740	2,060	1,020	585
	GAT-MBSA	GAT-SRBC	575	365	0	0
SJL/J‖	GAT-MBSA	GAT-SRBC	450	285	40	25

* These PFC were developed with a polyvalent rabbit antimouse IgG serum.

‡ Normal spleen cells.

§ Spleen cells from mice primed with 10 μg GAT in Maalox-pertussis 6 wk previously.

‖ Spleen cells from mice primed with 10 μg GAT as GAT-MBSA in Maalox-pertussis 6 wk previously.

Effect of Antimouse μ-Chain Serum on the Development of GAT-Specific PFC Responses In Vitro.—We have never detected IgM PFC specific for GAT in these experiments. This is unusual since IgM antibodies are usually found in responses to antigens which also stimulate synthesis of IgG antibodies. Furthermore, as previously reported, both primary IgM and IgG PFC responses to SRBC in vitro can be inhibited by incubating spleen cells in the presence of goat antibody to mouse μ-chain. This suppression by anti-μ-chain serum was shown to be mediated through the precursors of antibody-producing cells presumably by combining with the IgM surface receptors for antigen and thus preventing effective stimulation by antigen (9). If the GAT-specific IgG PFC response by spleen cells in vitro could also be inhibited with anti-μ-chain serum, this would be presumptive evidence that precursors of GAT-specific PFC had IgM receptors for antigen on their cell surface. 50 μl of a $\frac{1}{25}$ or $\frac{1}{100}$ dilution of goat anti-μ-chain serum was added to cultures of C57Bl/6 spleen cells stimulated with SRBC, GAT, or GAT-MBSA and to cultures of SJL spleen cells stimulated with SRBC and GAT-MBSA at culture initiation. The results (Table II) demonstrate that GAT-specific IgG PFC responses in both strains were inhibited 90% or more by incubation with the $\frac{1}{25}$ dilution of anti-μ-chain serum; the $\frac{1}{100}$ dilution suppressed the responses by 60–100%.

Responses of Various Strains of Mice to GAT and GAT-MBSA In Vitro.—To rule out any possibility that the failure of spleen cells from SJL mice to respond to soluble GAT in vitro is a peculiarity of this particular strain of mice rather than being attributable to the *H-2*^{s} genotype, we tested two congenic *H-2*^{s} nonresponder strains of mice in our system. We chose the B10.S strain, a congenic resistant strain of the *H-2*^{s} histocompatibility genotype on a C57Bl/10 back-

TABLE II

Effect of Anti Mouse μ-Chain Serum on Development of Primary PFC Responses In Vitro

Mouse strain	Dilution of antiserum*	Antigen	Indicator cells	PFC/culture	
				IgM	IgG
C57Bl/6	None	SRBC	SRBC	750	2,135
	1/25	SRBC	SRBC	5	140
	1/100	SRBC	SRBC	180	475
	None	GAT	GAT-SRBC	0	410
	1/25	GAT	GAT-SRBC	0	5
	1/100	GAT	GAT-SRBC	0	75
	None	GAT-MBSA	GAT-SRBC	0	300
	1/25	GAT-MBSA	GAT-SRBC	0	0
	1/100	GAT-MBSA	GAT-SRBC	0	125
SJL	None	SRBC	SRBC	1,920	1,110
	1/25	SRBC	SRBC	0	60
	1/100	SRBC	SRBC	95	135
	None	GAT-MBSA	GAT-SRBC	0	245
	1/25	GAT-MBSA	GAT-SRBC	0	0
	1/100	GAT-MBSA	GAT-SRBC	0	0

* Anti-μ serum was added to 1.0 ml cultures as 50 μl of the indicated dilution.

ground. This strain has the same *H-2* type as the SJL, and is otherwise essentially identical with the C57Bl/6 responder mice used in these studies. We also tested the A.SW strain of mice, a congenic resistant strain also of the $H\text{-}2^s$ histocompatibility type, but on an A strain background. A/J mice were included in this study since they possess the same non-*H-2* genotype as the A.SW, but they have the $H\text{-}2^a$ histocompatibility type and are thus responders to GAT. Spleen cells from all five strains were incubated with SRBC, or 10 μg GAT, or 5 μg GAT as GAT-MBSA. All strains developed IgM and IgG anti-SRBC PFC responses. The GAT-specific IgG PFC responses to GAT and GAT-MBSA are shown in Fig. 8. No IgM PFC specific for GAT were detected in cultures of any of these mice. Spleen cells from all five strains of mice developed GAT-specific IgG PFC responses to GAT-MBSA; but only the C57Bl/6 and A/J strains developed responses to soluble GAT. None of the three strains of mice bearing the $H\text{-}2^s$ genotype developed a response to soluble GAT in culture, proving that this failure is not a peculiarity of the SJL strain of mice. Furthermore, the linkage of responsiveness to GAT and *H-2* histocompatibility type previously reported using in vivo serological techniques (3) has now been demonstrated for PFC responses using an in vitro system.

DISCUSSION

These experiments demonstrate that mouse antibody to the random terpolymer GAT can be detected by complement-dependent lysis of SRBC which have GAT complexed to their surfaces. This hemolysis can be quantitated by

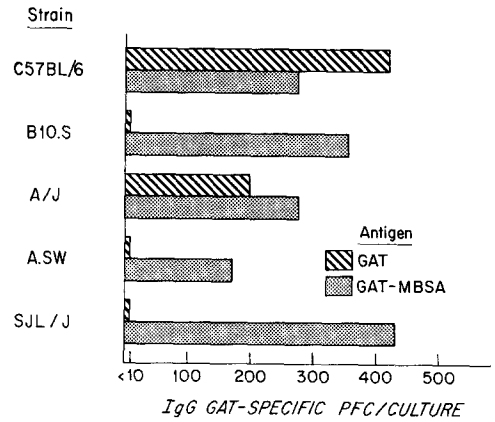

FIG. 8. Comparison of the IgG GAT-specific PFC responses in cultures of spleen cells from responder and nonresponder strains of mice stimulated with 10 μg GAT or 5 μg GAT as GAT-MBSA.

measuring the percent of ^{51}Cr released from ^{51}Cr-labeled GAT-SRBC by serum antibodies or by enumerating localized areas of hemolysis formed by antibodies released from lymphoid cells in a hemolytic plaque assay using GAT-SRBC as the indicator cells. Using GAT-SRBC as indicator cells in the hemolytic plaque assay, we have shown that primary and secondary PFC responses specific for GAT can be stimulated in cultures of spleen cells from responder mice incubated with soluble GAT or with the particulate GAT-MBSA. Furthermore, GAT-specific PFC can be stimulated in cultures of spleen cells from nonresponder mice incubated with GAT-MBSA, but not in cultures incubated with GAT.

The maximal GAT-specific PFC responses were detected 5 days after culture initiation; the optimal concentration of soluble GAT varied between 1 to 10 μg GAT/culture. In the primary response in vitro, all detected GAT-specific PFC elicited by GAT or GAT-MBSA have been indirect PFC predominantly of the γ_1 class with few or no PFC of the γ_2 class. The majority of GAT-specific PFC in the secondary response in vitro are also of the γ_1 class, but a significant number of GAT-specific PFC of γ_2 class are also detected. No direct, IgM, GAT-specific PFC have been detected in vivo or in vitro. There are three possible explanations for this observation: (*a*) IgM antibodies specific for GAT are produced but GAT-SRBC are not lysed by these antibodies in the presence of complement; (*b*) some IgM GAT-specific PFC are present but are too few in number or too transient in their secretory phase to be detected; or (*c*) no IgM antibodies to GAT are synthesized. There is no direct evidence to support any one of these possibilities; however, some circumstantial evidence suggests that the first explanation is unlikely. That is, we have found that equal numbers of IgM anti-SRBC PFC can be detected using SRBC or GAT-SRBC as indicator cells. Therefore, the coupling of GAT to the SRBC has not rendered the red cells insensitive to lysis by IgM antibody and complement. Unless mouse IgM anti-GAT antibody is unable to fix complement, the first explanation is an unlikely

possibility. Although the finding that development of GAT-specific IgG PFC responses can be inhibited by incubation of the spleen cell cultures with goat antimouse μ-chain serum tells us that anti-GAT PFC precursors have IgM receptors for antigen on their cell surfaces, it does not tell us whether or not IgM antibodies specific for GAT are ever synthesized. At this time, we cannot choose between explanations 2 and 3. The lack of detectable IgM antibody specific for GAT is nonetheless significant. GAT elicits an all-or-none response in mice and thus differs from some other synthetic polypeptide antigens the response to which is under the control of Ir genes. (T, G)-A-L, for example, elicits an IgM antibody response in all strains of mice, but an IgG response only in those strains possessing the appropriate Ir gene (11, 12). If little or no IgM anti-GAT antibody is synthesized in mice after stimulation with GAT, then the all-or-none character of this immune response is the same as the all-or-none character of the IgG response to (T, G)-A-L.

The GAT-specific IgG PFC response in cultures of spleen cells from various inbred strains of mice stimulated with GAT or GAT-MBSA in vitro was investigated. Cultures of spleen cells from mice of the *H-2*^{s} histocompatibility type (SJL, B10.S, A.SW), which were previously classified by serological techniques as nonresponder strains (3), produced no GAT-specific PFC when incubated with GAT in vitro. Cultures of spleen cells from C57Bl/6 (*H-2*^{b}) and A/J (*H-2*^{a}) mice, previously classified as responder strains, produced IgG PFC specific for GAT when stimulated with soluble GAT in vitro. GAT-specific IgG PFC responses developed in cultures of spleen cells from both responder and nonresponder strains which were stimulated with GAT-MBSA in vitro. The development of GAT-specific PFC responses in cultures of spleen cells from *H-2*^{s} nonresponder mice stimulated with GAT-MBSA demonstrates that nonresponder mice have precursors of antibody-producing cells specific for GAT and that those cells have the capacity to synthesize GAT-specific antibodies when stimulated under the appropriate circumstances. The failure of spleen cell cultures of nonresponder mice to synthesize antibodies specific for GAT when stimulated with soluble GAT suggests that these mice lack GAT-specific thymus-derived "helper" cells.

The immune response genes controlling the response to GAT were again demonstrated to be linked to the genes controlling the *H-2* antigens. The dominance of this Ir gene is shown by the ability of the F_1 (C57 $\times$ SJL) mice (*H-2*$^{b/s}$) to develop GAT-specific PFC responses when stimulated with soluble GAT in vitro.

These data therefore show that genetic control of GAT-specific PFC responses in vitro correlates precisely with previous data obtained by measuring serum antibody responses to GAT. The in vitro system described in this study will be used as a model to study the cellular events regulated by the *H-2*-linked Ir genes. The cellular requirements for the development of antibody responses to GAT and GAT-MBSA in vitro are the subject of the accompanying paper (13). Subsequent papers in this series will deal with the cellular defect(s) in non-

responder mice which are responsible for their failure to develop antibody responses to soluble GAT both in vivo and in vitro.

SUMMARY

In vivo, the antibody response in mice to the random terpolymer L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT) is controlled by a histocompatibility-linked immune response gene(s). We have studied antibody responses by spleen cells from responder and nonresponder mice to GAT and GAT complexed to methylated bovine serum albumin (GAT-MBSA) in vitro. Cells producing antibodies specific for GAT were enumerated in a modified Jerne plaque assay using GAT coupled to sheep erythrocytes as indicator cells. Soluble GAT stimulated development of IgG GAT-specific plaque-forming cell (PFC) responses in cultures of spleen cells from responder mice, C57Bl/6 (H-2^b), F_1 (C57 × SJL) (H-$2^{b/s}$), and A/J (H-2^a). Soluble GAT did not stimulate development of GAT-specific PFC responses in cultures of spleen cells from nonresponder mice, SJL (H-2^s), B10.S (H-2^s), and A.SW (H-2^s). GAT-MBSA stimulated development of IgG GAT-specific PFC responses in cultures of spleen cells from both responder and nonresponder strains of mice. These data correlate precisely with data obtained by measuring the in vivo responses of responder and nonresponder strains of mice to GAT and GAT-MBSA by serological techniques. Therefore, this in vitro system can effectively be used as a model to study the cellular events regulated by histocompatibility-linked immune response genes.

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