GENETIC CONTROL OF IMMUNE RESPONSES IN VITRO

III. Tolerogenic Properties of the Terpolymer L-Glutamic Acid^{60}-L-Alanine^{30}-L-Tyrosine^{10} (GAT) for Spleen Cells from Nonresponder (H-2^s and H-2^q) Mice*

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In previous reports in this series, we have described experiments investigating the regulation of the antibody response to the synthetic random terpolymer L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT)[1] by an H-2-linked immune response (Ir) gene in vitro (1, 2). As reported for antibody responses elicited in vivo (3–6) spleen cells from both responder (H-$2^{a,b,d,k}$) and nonresponder (H-$2^{p,q,s}$) mice develop IgG plaque-forming cells (PFC) specific for GAT in vitro when stimulated with GAT complexed to the carrier methylated bovine serum albumin (GAT-MBSA), but only spleen cells from responder mice develop GAT-specific IgG PFC responses to GAT.

Using spleen cells fractionated according to their ability to adhere to plastic dishes the following conclusions were reached: (*a*) macrophages (adherent cells) are required for the responses to GAT and GAT-MBSA in vitro; (*b*) macrophages from responder and nonresponder mice bind GAT equally and GAT bound to either type of macrophage can elicit a GAT-specific PFC response by nonadherent responder lymphoid cells; and (*c*) GAT bound to responder or nonresponder macrophages failed to elicit a GAT-specific PFC response by nonresponder lymphoid cells. Treatment of spleen cells with anti-theta (θ) serum and complement (C) before culture initiation abolished responses to GAT and GAT-MBSA, demonstrating the requirement for thymus-derived (T) cells in the responses. Furthermore, spleen cells from GAT-primed X-ir-

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[1] *Abbreviations used in this paper*: B cell, precursor of antibody-producing cell; GAT, random terpolymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}; GAT-MBSA, GAT complexed to methylated bovine serum albumin; IgG, refers to IgG_1 and $IgG_{(2a+2b)}$; Ir gene, specific immune response gene; PFC, plaque-forming cell(s); T cell, thymus-derived helper cell; T_0, time of initiation; θ, surface alloantigen on T cells.

radiated responder mice were shown to possess GAT-specific helper T cells, while no GAT-specific helper function could be demonstrated in spleen cells from GAT-primed nonresponder mice. These data suggest that the defect in genetic nonresponder spleen cells is the failure of their T cells, after interaction with GAT, to provide appropriate helper function for the initiation of the B cell response (2).

In the studies reported in this paper we have investigated: (*a*) the effect of GAT on the GAT-specific immune response to GAT-MBSA in vivo, and (*b*) the effect of soluble GAT on the immune response to GAT-MBSA by spleen cells in vitro. We have found that GAT is tolerogenic for nonresponder spleen cells at concentrations which are not tolerogenic, but rather immunogenic, for responder spleen cells. In addition, we have found that nonresponder B cells are more readily tolerized by GAT than are responder B cells. The significance of these observations with respect to T-B cell collaboration and the mechanism(s) of *Ir* gene control of immune responses (7) will be discussed.

Materials and Methods

Mice.—C57BL/6J (*H-2*^{b}), SJL/J (*H-2*^{s}), and DBA/1 (*H-2*^{q}) mice were purchased from Jackson Laboratories, Bar Harbor, Maine. B10.S (*H-2*^{s}), a congenic resistant strain of mice, were a gift of Dr. D. Shreffler, University of Michigan, Ann Arbor, Mich. and bred in our animal facilities. (C57BL/6 × SJL)F_1 (*H-2*$^{b/s}$) mice were also bred in our animal facilities. These mice have the same *H-2* haplotype (*H-2*$^{b/s}$) as F_1 mice from a C57BL/6 × B10.S mating. Mice used in these experiments were 2- to 8-mo old and were maintained on laboratory chow and acidified chlorinated water ad libitum.

Antigens.—GAT, mol wt 32,000, was purchased from Miles Laboratories, Inc., Miles Research Div., Kankakee, Illinois. Methylated bovine serum albumin (MBSA) was a gift of Dr. P. Maurer, Jefferson Medical College, Philadelphia, Pa. Preparations of SRBC, GAT, and GAT-MBSA for addition to cultures have been described in previous papers (1). 10^7 SRBC, or GAT-MBSA containing 5 μg GAT and/or various amounts of soluble GAT were added to cultures according to the experimental protocol.

Immunization of Mice.—Mice were injected intraperitoneally with 10 μg GAT as GAT-MBSA or 50 μg MBSA in a mixture of Maalox (Wm. H. Rorer, Inc., Fort Washington, Pa.) and pertussis vaccine (Eli Lilly & Co., Indianapolis, Ind.) as previously described (3). Mice were immunized with GAT according to the experimental protocol.

Spleen Cell Cultures and PFC Assay.—Suspensions of single spleen cells containing 10×10^6 nucleated cells in culture medium (MEM) were incubated according to the method of Mishell and Dutton (8) with modifications previously described (9). The hemolytic plaque assay using GAT-SRBC as indicator cells has been described previously (1).

Preparation of B Cells.—An attempt was made to eliminate helper T cells from normal spleen cell suspensions by treatment with AKR anti-θ C3H serum and C before culture initiation (2). As a control, a fraction of the spleen cell suspension was treated with C alone. After treatment, 10×10^6 cells in MEM were added to cultures with or without additional cells and GAT and/or GAT-MBSA according to the experimental protocol.

Source of T Cells.—The helper cell function of antigen-primed T cells has been demonstrated to be radioresistant, whereas B cells and normal T cells are radiosensitive (10). Therefore, mice primed with GAT-MBSA or MBSA 1–4 mo earlier were X irradiated with 700–800 R as previously described (2). Within 3 h after irradiation, the mice were sacrificed and single cell suspensions were prepared from their spleens. 10×10^6 cells in MEM were added to

cultures with or without additional cells and GAT and/or GAT-MBSA according to the experimental protocol.

RESULTS

Effect of GAT on the Immune Response to GAT-MBSA In Vivo.—On day 1, C57BL/6 ($H\text{-}2^b$) and DBA/1 ($H\text{-}2^q$) mice were injected i.p. with 10 μg GAT in Maalox-pertussis or with Maalox-pertussis alone. On day 4, both normal control mice and the primed mice were injected i.p. with GAT-MBSA, containing 10 μg GAT, in Maalox-pertussis. All mice were sacrified 7 days later and their spleens examined for GAT-specific IgG PFC (Fig. 1). Preinjection of responder C57BL/6 mice with GAT caused

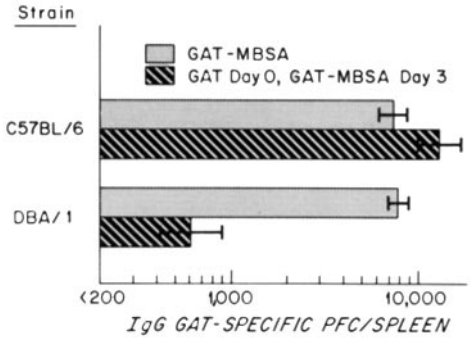

FIG. 1. Effect of GAT on responses to GAT-MBSA by responder and nonresponder mice in vivo. Normal C57BL/6 and DBA/1 mice were injected i.p. with 10 μg GAT in Maalox-pertussis on day 1. Three days later these mice and normal C57BL/6 and DBA/1 mice were injected i.p. with GAT-MBSA containing 10 μg GAT in Maalox-pertussis. 7 days later the mice were sacrificed and their spleens examined for IgG GAT-specific PFC. The graph shows the geometric mean of the PFC responses of 8–12 mice, the brackets indicate the standard error of the mean.

no decrease in their immune responses to GAT-MBSA. However, 10 μg GAT caused a decrease in the GAT-specific immune response of DBA/1 nonresponder mice from a geometric mean of 7,800 PFC/spleen to a geometric mean of 620 PFC/spleen. (200 PFC/spleen is the lowest number of PFC detectable in this assay.) Although not shown, injection of Maalox-pertussis alone on day 1 caused no decrease in GAT-specific PFC responses to GAT-MBSA in either the responder or nonresponder strains of mice. Furthermore, injection of 10 μg GAT in Maalox-pertussis on day 1 did not decrease the primary immune response to SRBC injected i.p. on day 4 in either of the strains of mice (not shown). Thus, we conclude that the failure of nonresponders to develop a GAT-specific PFC response to GAT is not merely a failure to recognize GAT determinants. Quite the contrary, nonresponder mice react to these determinants and after an encounter with GAT, nonresponder mice are less able to produce GAT-specific antibody upon immunization with GAT-

MBSA. Furthermore, nonresponder DBA/1 mice were unable to develop GAT-specific PFC responses when injected with GAT-MBSA as long as 5 wk after receiving 10 μg GAT in Maalox-pertussis.

Effect of GAT Presented in Various Forms on the Immune Response to GAT-MBSA In Vivo.—We investigated the effect of adjuvants on the induction of tolerance in nonresponder mice by GAT in vivo. 100 or 10 μg GAT was injected i.p. in saline, in Maalox, or in Maalox-pertussis. Three days later, these mice, as well as untreated control mice, were immunized with GAT-MBSA in Maalox-pertussis. The results of a typical experiment are shown in Table I. Regardless of the method of presentation, GAT did not decrease the anti-GAT response of C57BL/6 mice to GAT-MBSA. In nonresponder DBA/1 mice, GAT in saline did not decrease the GAT-specific immune response to GAT-MBSA, whereas 100 and 10 μg GAT in Maalox with or without pertussis did inhibit this response.

Dose of GAT in Maalox Required to Induce Tolerance in Nonresponder Mice in Vivo.—The results of an experiment in which DBA/1 mice were injected with various amounts of GAT in Maalox and subsequently challenged with GAT-MBSA in Maalox-pertussis are shown in Table II. The degree of suppression of the GAT-specific immune response to GAT-MBSA is directly related to the dose of GAT administered. It is significant that the doses of GAT (100, 10, and 1.0 μg) which are tolerogenic in nonresponder mice are pre-

TABLE I

Effect of Adjuvants on the Induction of Tolerance by GAT In Vivo

GAT	IgG GAT-specific PFC/spleen*	
	C57BL/6	DBA/1
None	5,000	6,150
	5,400	7,000
100 μg in saline	5,250	3,000
	6,250	8,550
10 μg in saline	4,800	7,200
	4,500	4,750
100 μg in Maalox	9,500	<200
	10,400	300
10 μg in Maalox	9,400	<200
	4,050	<200
10 μg in Maalox + pertussis	11,500	600
	8,800	250

* Mice were injected with GAT on day 1, GAT-MBSA in Maalox-pertussis on day 4, and were sacrificed 7 days after injection of GAT-MBSA; values are from individual mice.

TABLE II

Dose of GAT Required to Induce GAT-Specific Tolerance to GAT-MBSA in DBA/1 Mice in Vivo

Dose of GAT in Maalox at day 1	IgG GAT-specific PFC/Spleen*	
None	13,300	13,700
100 μg	1,600	400
10 μg	<200	800
1.0 μg	2,000	400
0.1 μg	9,400	13,700

* All mice received GAT-MBSA in Maalox on day 4 and were sacrificed 7 days later. Values represent PFC responses of individual mice.

cisely those which are immunogenic in responder mice (4). Doses of GAT of 0.1 μg or less are not immunogenic in responder mice (4), and, as shown here, are not tolerogenic in nonresponder mice.

Effect of GAT on GAT-Specific Immune Responses In Vitro.—Next, we examined the effect of GAT on GAT-specific immune responses to GAT-MBSA in vitro. Spleen cells from normal C57BL/6 and DBA/1 mice were prepared as usual and cultured at 10×10^6 cells/dish. Cultures were stimulated with SRBC, GAT, or GAT-MBSA. Soluble GAT in various concentrations was added to other cultures with SRBC or GAT-MBSA at culture initiation. The results of one experiment are presented in Table III. Soluble GAT at the highest dose, 10 μg/culture, had no effect on the anti-SRBC PFC responses by spleen cells from either of the strains of mice. As usual, there was no detectable response of DBA/1 spleen cells to any dose of soluble GAT. In this experiment, the peak response to soluble GAT by cultures of C57BL/6 spleen cells occurred at 1.0 μg GAT/culture, whereas the higher dose (10 μg GAT) was somewhat inhibitory. Both strains of mice responded to GAT-MBSA. Soluble GAT added to cultures of C57BL/6 spleen cells, along with GAT-MBSA, caused a decrease in the GAT-specific PFC response only at the highest dose (10 μg). However, this inhibition is correlated with the observation that 10 μg GAT alone depressed the optimal response to GAT and would likewise be expected to inhibit the response to GAT-MBSA. In contrast, the response to GAT-MBSA of nonresponder spleen cells was suppressed by 10, 1.0, and 0.1 μg doses of GAT. These data demonstrate that tolerance induction by GAT in nonresponder mice originally observed in vivo can be studied in vitro.

Effect of GAT on the Development of GAT-Specific Responses to GAT-MBSA by Various Strains of Mice.—The ability of soluble GAT to suppress the GAT-specific PFC response developed by spleen cells from various strains of mice stimulated with GAT-MBSA in vitro was examined. The results (Fig. 2) demonstrate that the GAT-specific response to GAT-MBSA by spleen cells from other nonresponder strains of mice, SJL and B10.S mice ($H\text{-}2^s$), like those from DBA/1 ($H\text{-}2^q$) mice, are inhibited by 10 μg soluble GAT. Spleen cells from the

TABLE III

Effect of GAT on GAT-Specific Immune Responses In Vitro

Strain	Antigen	IgG PFC/culture*	
		SRBC	GAT
C57BL/6	10 μg GAT	—	235
	1.0 μg GAT	—	360
	0.1 μg GAT	—	225
	10^7 SRBC	130	—
	10^7 SRBC + 10 μg GAT	175	—
	GAT-MBSA	—	235
	GAT-MBSA + 10 μg GAT	—	145
	GAT-MBSA + 1.0 μg GAT	—	370
	GAT-MBSA + 0.1 μg GAT	—	255
DBA/1	10 μg GAT	—	<20
	1.0 μg GAT	—	<20
	0.1 μg GAT	—	<20
	10^7 SRBC	395	—
	10^7 SRBC + 10 μg GAT	385	—
	GAT-MBSA	—	235
	GAT-MBSA + 10 μg GAT	—	50
	GAT-MBSA + 1.0 μg GAT	—	<20
	GAT-MBSA + 0.1 μg GAT	—	105

* 10^7 C57BL/6 or DBA/1 spleen cells were cultured.

responder (C57BL/6 × SJL)F_1 mice, like those from C57BL/6 mice, are not rendered tolerant by 10 μg soluble GAT.

Sensitivity of Spleen Cells to Tolerance Induction as a Function of Time after Culture Initiation.—Cultures of spleen cells from normal C57BL/6 and DBA/1 mice were prepared and GAT-MBSA was added to all cultures at the time of initiation (T_0). At T_0 or at 24-h intervals thereafter, soluble GAT (5 μg) was added to these cultures. After 5 days, cultures were harvested and examined for GAT-specific PFC responses; a typical experiment is shown in Table IV. Addition of soluble GAT at any time did not significantly inhibit the anti-GAT response to GAT-MBSA by responder spleen cells. Soluble GAT added at T_0, 24, or 48 h significantly inhibited the development of the GAT-specific PFC responses to GAT-MBSA by spleen cells from nonresponder mice, although the inhibition at 48 h was not as profound as that caused by addition of GAT at T_0 or 24 h. Addition of GAT at 72 h or later had no effect on the GAT-specific PFC response developed by these cultures, indicating that GAT interferes with an early event in the initiation of the GAT-specific antibody response to GAT-MBSA.

Other cultures of these spleen cells were stimulated with GAT-MBSA or GAT-MBSA plus soluble GAT (5 μg) at culture initiation, harvested at 24 or 48 h, washed three times in sterile MEM, and collected by centrifugation.

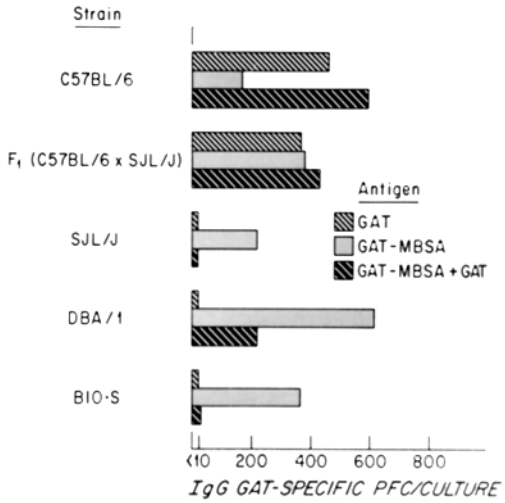

FIG. 2. Effect of GAT on responses to GAT-MBSA by spleen cells from responder and nonresponder mice in vitro. 10^7 spleen cells from normal-responder or nonresponder mice were cultured with 10 μg GAT or GAT-MBSA containing 5 μg GAT or both and PFC responses determined on day 5.

Cells harvested from three cultures were plated out in two dishes. These cultures received no further antigen. However, it is important to note that soluble GAT is removed from the cultured cells by several washes, whereas the particulate GAT-MBSA is not removed from washed spleen cells. The results (Table IV) show that if GAT is removed from culture after 24 h, the anti-GAT response to GAT-MBSA is only partially inhibited in cultures of nonresponder spleen cells. By comparison, the GAT-specific response to GAT-MBSA by cultures of nonresponder spleen cells incubated with GAT for 48 h was profoundly depressed. Since contact with GAT for 24 h does not abrogate the anti-GAT response to GAT-MBSA, we feel that the effects of GAT are not irreversible at that early time. Whether the depressed response which occurs after incubation with GAT for 48 h means that the inhibition is irreversible after more prolonged contact with GAT or that the response to GAT-MBSA is delayed in these cultures, and occurs later than the usual 5-day peak response, cannot be determined from these data. Detailed studies of the kinetics of such responses during the 5 days after termination of exposure to GAT should clarify this point.

Greater Susceptibility of Nonresponder B Cells than of Responder B Cells to Tolerance Induction by GAT.—In previous reports from this laboratory (11), Katz et al. have shown, using a double adoptive cell transfer system in vivo, that T cells from F_1 hybrid mice cooperate with B cells from either parental strain in the development of immune responses to conventional antigens such as keyhole limpet hemocyanin. In contrast, if the response to the carrier moiety of the antigen is under *Ir* gene control, carrier-primed T cells from (responder $\times$

TABLE IV

Effect of Addition of GAT at Various Times to Cultures Stimulated by GAT-MBSA

Time 5 μg GAT added	IgG GAT-specific PFC/culture*	
	C57BL/6	DBA/1
No GAT added	765	1,685
T_0	1,050	<10
24 h	650	80
48 h	895	810
72 h	880	1,150
96 h	665	1,725
Cells washed at 24 h		
No GAT	810	2,435
GAT at T_0	1,285	1,770
Cells washed at 48 h		
No GAT	585	1,630
GAT at T_0	1,175	100

* 10^7 C57BL/6 or DBA/1 spleen cells were cultured.

nonresponder)F_1 mice provide effective helper function only to the responder parental B cells (12). As shown in Fig. 3 and in the accompanying paper (13), irradiated GAT-MBSA-primed (C57BL/6 × SJL)F_1 T cells cooperate with both responder and nonresponder parental B cells in cultures stimulated with GAT-MBSA. However, when incubated with soluble GAT, these F_1 T cells provide effective helper function only to the responder parental B cells. Addition of soluble GAT to cultures containing F_1 T cells, parental B cells, and GAT-MBSA (Fig. 3) suppresses the GAT-specific responses of the nonresponder parental B cells but not those of the responder parental B cells.

It has been demonstrated that B cells are more readily rendered unresponsive by antigens in the absence of specific helper T cells (14, 15). Thus, it is possible to argue that the nonresponder B cells in the previous experiment were rendered tolerant to GAT while responder B cells were not, since the responder B cells were influenced by GAT-specific helper cell activity from the F_1 T cells, whereas nonresponder B cells may be influenced only by MBSA helper T-cell activity. In an attempt to determine if a real difference in the ability of responder and nonresponder B cells to be tolerized exists, the following experiment was performed. Anti-θ and C-treated responder B cells, were cultured with GAT-MBSA-primed or MBSA-primed X-irradiated C57BL/6 cells. It can be seen in Table V that GAT-MBSA-primed T cells provided the necessary helper function to B cells for the development of GAT-specific responses to GAT-MBSA and GAT. Furthermore, soluble GAT did not inhibit the response to GAT-MBSA or to GAT, but in fact, increased the response when 10 μg of GAT was added. Cultures of MBSA-primed T cells and C57BL/6 B cells also developed

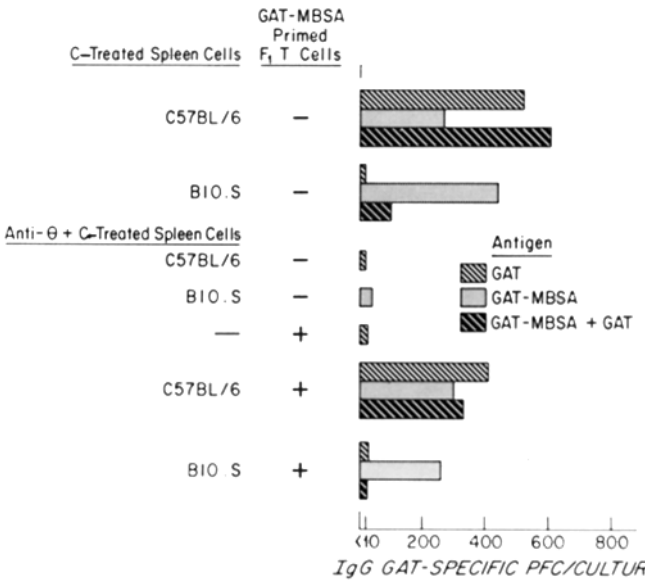

FIG. 3. Effect of GAT on responses to GAT-MBSA in cultures of responder and nonresponder B cells containing GAT-MBSA-primed irradiated F_1 spleen cells. Soluble GAT (10 μg) or GAT-MBSA containing 5 μg of GAT or both were added to cultures containing 10×10^6 C-treated spleen cells from normal C57BL/6 or B10.S mice and to cultures containing 10×10^6 B cells (anti-θ + C-treated spleen cells) plus 10×10^6 GAT MBSA-primed irradiated (C57BL/6 × SJL)F_1 spleen (T) cells. Controls demonstrate that cultures of 10×10^6 B cells or 10×10^6 GAT-MBSA-primed irradiated T cells alone were unable to develop GAT-specific PFC responses.

a GAT-specific PFC response to GAT-MBSA. However, no GAT-specific PFC response was detected in these cultures when incubated with GAT. Furthermore, addition of soluble GAT to cultures stimulated by GAT-MBSA did not interfere with the GAT-specific PFC response. Thus, in the absence of detectable GAT-specific helper T-cell function, B cells from responder mice were not rendered unresponsive by soluble GAT.

DISCUSSION

Nonresponder *H-2*^{s} and *H-2*^{q}-mice fail to develop immune responses to GAT but nevertheless may be stimulated to form anti-GAT antibodies by immunization with GAT bound to an immunogenic carrier such as MBSA for which helper T-cell function can be generated (1–5). Furthermore, as shown in the experiments presented in this paper, administration of GAT renders nonresponder mice unable to develop GAT-specific PFC responses to a subsequent challenge with GAT-MBSA. The addition of soluble GAT, together with GAT-MBSA, to spleen cell cultures from nonresponder strains of mice abolishes anti-GAT responses. GAT is, therefore, tolerogenic in vivo and in vitro for nonre-

TABLE V

Failure of GAT to Suppress GAT-Specific PFC Responses to GAT-MBSA by Responder B Cells in the Absence of GAT-Specific Helper T Cells

10^7 Anti-θ and C-treated C57BL/6 spleen cells	10^7 X-irradiated C57BL/6 cells primed to:	Antigen	IgG GAT-Specific PFC/Culture
+	—	1.0 μg GAT	0
+	—	GAT-MBSA	0
−	GAT-MBSA	GAT-MBSA	0
+	GAT-MBSA	10 μg GAT	440
+	GAT-MBSA	1.0 μg GAT	375
+	GAT-MBSA	0.1 μg GAT	95
+	GAT-MBSA	GAT-MBSA	435
+	GAT-MBSA	GAT-MBSA + 10 μg GAT	590
+	GAT-MBSA	GAT-MBSA + 1.0 μg GAT	440
+	GAT-MBSA	GAT-MBSA + 0.1 μg GAT	380
−	MBSA	GAT-MBSA	0
+	MBSA	10 μg GAT	0
+	MBSA	1.0 μg GAT	0
+	MBSA	0.1 μg GAT	0
+	MBSA	GAT-MBSA	410
+	MBSA	GAT-MBSA + 10 μg GAT	395
+	MBSA	GAT-MBSA + 1.0 μg GAT	355
+	MBSA	GAT-MBSA + 0.1 μg GAT	320

sponder cells under conditions where immunity is elicited in responder cells. Certain critical aspects of the tolerogenic properties of GAT for nonresponder mice are particularly informative: (*a*) The minimal dose of GAT required to induce tolerance in nonresponder mice is precisely the dose required to stimulate immunity in responder mice. (*b*) GAT behaves as a tolerogen in vivo for nonresponder mice, even when administered with a strong adjuvant such as pertussis vaccine. (*c*) Unresponsiveness to GAT is a long-lasting phenomenon; spleen cells from GAT-treated nonresponder mice were shown to remain unresponsive for as long as 5 wk after a dose of 10 μg of GAT, although this is a readily metabolized polypeptide. (*d*) The tolerogenic effect in vitro is induced between 24 and 72 h after culture initiation which is also the critical time when immunocompetent cells become irreversibly committed to respond to antigen (16).

How can the tolerogenic properties of GAT for nonresponder spleen cells be explained when all the parameters of this phenomenon listed above are taken into account? Several possible mechanisms can be evoked which are not all necessarily mutually exclusive: (*a*) The remarkable susceptibility of nonresponder animals to tolerance induction by GAT reflects fundamental differences in B cells of responder and nonresponder animals in their ability to be rendered unresponsive by this polypeptide. The finding that responder, in contrast to nonresponder, B cells when cultured with MBSA-primed irradiated

(responder × nonresponder)F_1 helper T cells develop a response to GAT-MBSA which is not inhibited by soluble GAT favors this interpretation. This hypothesis, however, would imply the functional expression of the *Ir* gene in B cells as well as in T cells and would also lead to the conclusion that *Ir* genes are not clonally expressed in these cells. The nonclonal expression of *Ir* gene products in immunocompetent cells is difficult to reconcile with the remarkable specificity of the recognition controlled by these genes (7).

(*b*) There are no differences in responder and nonresponder B cells in their ability to bind or respond to GAT. The increased susceptibility of nonresponder spleen cells to tolerance induction could be explained by the absence of GAT-specific helper T-cell function. It has indeed been shown that spleen cells in the absence of activated helper T cells are easily tolerized (14, 15). We have also shown that GAT-specific helper function cannot be demonstrated in nonresponder mice immunized with GAT. In the absence of helper function for GAT, GAT-specific B cells would be expected to become specifically tolerant. This interpretation postulates the expression of the *Ir* gene in T cells to the exclusion of B cells and implies that the *Ir* gene product functions as a receptor molecule of T cells as proposed earlier (7).

The first two hypotheses are not easily reconciled, however, with the long-lasting tolerance observed after relatively low doses (10 μg) of the readily metabolizable GAT. They would require that nonresponder GAT-specific B cells remain unresponsive to GAT-MBSA in the presence of MBSA-primed syngeneic T cells several weeks after tolerance induction. This possibility is currently being investigated.

(*c*) Our data could also be explained by the hypothesis suggested earlier by Gershon et al. (6) that genetic nonresponders do not develop GAT-specific helper T cells, but develop only suppressor T cells in response to GAT. This would imply that the *Ir* gene product could be restricted to a certain class of T cells concerned with helper function where they could behave as antigen receptors. These T cells would be lacking in nonresponder animals. According to this interpretation, the function of the *Ir* gene product would be crucial in the development of tolerance vs. immunity. The system we have developed to analyze the anti-GAT response of fractionated spleen cells from nonresponder animals to GAT-MBSA at various times after tolerance induction by GAT should permit a choice between these various possibilities presented and thus a better understanding of the precise function of the *Ir* gene product in immunocompetent cells.

SUMMARY

Although nonresponder, *H-2*^{s} and *H-2*^{q}, mice fail to develop GAT-specific PFC responses to GAT, they do develop GAT-specific PFC responses when stimulated by GAT complexed to an immunogenic carrier such as methylated bovine serum albumin. The studies described in this paper show that injection of non-

responder mice with GAT specifically decreases their ability to develop anti-GAT PFC responses to a subsequent challenge with GAT-MBSA. Addition of GAT to cultures of spleen cells from nonresponder mice also prevents development of the GAT-specific PFC responses stimulated by GAT-MBSA. Thus, interaction of nonresponder spleen cells with GAT leads to the induction of unresponsiveness in vivo and in vitro.

Various parameters of the tolerance induction have been investigated and described. A comparison of the effects of GAT on B cells indicates that nonresponder B cells are more readily rendered unresponsive by soluble GAT than are responder B cells. The significance of these data for our understanding of *Ir* gene regulation of the immune response is discussed.

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