THE INVOLVEMENT OF SUPPRESSOR T CELLS IN Ir GENE REGULATION OF SECONDARY ANTIBODY RESPONSES OF PRIMED (RESPONDER × NONRESPONDER)F_1 MICE TO MACROPHAGE-BOUND L-GLUTAMIC ACID^{60}-L-ALANINE^{30}-L-TYROSINE^{10} *

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The precise cellular and molecular mechanisms by which H-2-linked Ir genes regulate immune responses are still poorly understood. We have recently reviewed this topic (1), and suggested three general types of Ir control: (*a*) restrictions on the production and/or effector function of antigen-specific I-region coded T-cell derived helper or suppressor factors; (*b*) restrictions on the ability of Ia antigens, together with the nominal antigen, to trigger T cells, a phenomenon seen as H-2 haplotype restricted macrophage (Mϕ)[1]-T-cell interaction or T helper cell (T_H)-B-cell cooperation; and (*c*) restrictions on the T-cell receptor repertoire presumably induced during thymic differentiation in the presence of one or another set of Ia antigens. This laboratory has studied in detail the Ir gene regulated response to the synthetic polypeptide antigen L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT) as a model for exploring these issues (2–5). The work dealing with soluble factors has been described elsewhere (6–10). Of concern here is the question of Mϕ-T-cell interaction in antibody responses.

Early studies by Kapp et al. (3) revealed that responder (R) or (responder × nonresponder)F_1 ([R × NR]F_1) spleen cells give primary IgG plaque-forming cell (PFC) responses in vitro to either soluble GAT or to GAT bound to Mϕ (3). No differences in the ability of R or NR GAT-Mϕ to induce primary anti-GAT PFC responses in culture could be detected under the experimental conditions employed, suggesting that Ir-gene control of GAT responses was not expressed predominantly at the Mϕ or Mϕ-T-cell level. Further studies demonstrated that R or (R × NR)F_1 mice could be primed in vivo with GAT-Mϕ such that their spleen cells would give secondary in vitro responses only to GAT-Mϕ sharing the I-A subregion with the Mϕ

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[1] *Abbreviations used in this paper:* B6, C57BL/6; (B6D1)F_1, (C57BL/6 female × DBA/1 male)F_1; B10, C57BL/10; CY, cyclophosphamide; D1, DBA/1; FCS, fetal calf serum; GAT, random copolymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}; GT, random copolymer of L-glutamic acid^{50}-L-tyrosine^{50}; HBSS, Hanks' balanced salt solution; Ia^{NR}, Ia antigens from Ir nonresponder haplotype; Ia^{R}, Ia antigens from Ir responder haplotype; Mϕ, macrophage(s); NR, nonresponder; PFC, plaque-forming cell(s); R, responder; SRBC, sheep erythrocytes; (T,G)-A--L, poly-L-(Tyr, Glu)-poly-D,L-Ala-poly-L-Lys; T_H, helper T cell(s); T_s, suppressor T cells(s).

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used for priming, which could be of R or NR origin (5, 11, 12). This restriction in secondary anti-GAT PFC responses was found to be dependent on the T-cell and not the B-cell component of the primed spleen, and was presumably a reflection of H-2 restricted T_H activity. Similar I-A restrictions of responsiveness in mice primed for delayed type hypersensitivity (13) or helper activity (14) by antigen-pulsed Mϕ have been reported subsequently by several groups. More recently, it was found that priming (R × NR)F_1 mice with soluble GAT, rather than Mϕ-bound GAT, resulted in spleen cells which gave secondary responses in vitro only to GAT-R-Mϕ and not to GAT-NR-Mϕ (15). This restriction, which correlates with Ir-gene status in the Mϕ, and not lymphocyte population, indicates in contrast to the earlier data, Ir-gene expression at the level of Mϕ- or Mϕ-T-cell interaction.

Perhaps the most striking feature of both these latter studies on soluble GAT-primed F_1 mice (15) and the former experiments on GAT-Mϕ-primed R mice (5) is the loss of the usual primary PFC response in (R × NR)F_1 mice to GAT-NR-Mϕ or in responder mice to GAT-Mϕ with H-2 haplotypes other than that used for priming. This loss of primary responsiveness, particularly to GAT-NR-Mϕ, taken together with the known ability of soluble GAT to induce strong suppressor T cell (T_s) responses in NR animals (16), suggested to us that in addition to the priming of H-2 restricted T_H, the development of GAT-specific T_s might be important in determining the pattern of secondary responses to Mϕ-associated GAT. The present series of experiments tests this hypothesis, and demonstrates (*a*) the suppressive activity of primed (R × NR)F_1 spleen cells on primary responses to GAT-NR-Mϕ and (*b*) the ability of agents (cyclophosphamide (CY) (17) and antiserum to I-J subregion determinants [18]) known to decrease T_s activity, to permit primed F_1 spleen cells to respond to GAT-NR-Mϕ, concomitant with the loss of suppressor activity in such primed spleen cell populations. Models of T-cell function in primed mice consistent with both the earlier I-A restriction data and the current experiments detailing a role for T_s in Mϕ-T_H restriction phenomena are discussed below.

Materials and Methods

Mice. C57BL/6 (B6) or C57BL/10 (B10) H-2^b, DBA/1 (D1), H-2^q, and CBA, H-2^k mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. (C57BL/6 female × DBA/1 male)F_1 [(B6D1)F_1] and (CBA female × DBA/1 male)F_1 mice were bred in the Department of Pathology animal facility. B10.G, H-2^q mice were the gift of Dr. Martin E. Dorf, Harvard Medical School. All animals were maintained on standard laboratory chow and chlorinated water ad lib, and used at 8–20 wk old. Mice used in any given experiment were age and sex matched as closely as possible. DBA/1 and B10.G mice are GAT nonresponders. B6, B10, and CBA mice are responders.

Antigens. GAT lot 6 (Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.) mol wt ≅ 38,000, was prepared for use as antigen in culture, for in vivo priming, and for preparation of GAT-sheep erythrocytes (GAT-SRBC) and GAT-Mϕ as previously described (2, 3).

Immunization. F_1 mice were primed by a single i.p. inoculation of 100 μg GAT in a mixture of aluminum-magnesium hydroxide gel (Maalox, William H. Rorer, Inc., Ft. Washingon, Pa.) and pertussis vaccine (Eli Lilly and Co., Indianapolis, Ind.) administered 4–8 wk before removal of spleens for culture. Some mice were pretreated 3 days before immunization with 5 mg/kg cyclophosphamide (Cytoxan, Mead Johnson Pharmaceutical Division, Mead Johnson & Co., Evansville, Ind.) dissolved in saline. Other groups of mice were given four daily i.v. inoculations containing 5 μl of an anti-I-J^b or anti-I-J^k antiserum diluted in saline, beginning on the day of immunization (18). These antisera, provided by Dr. Martin Dorf of this department, were raised and characterized as described previously (19). Anti-I-J^b = B10.A(5R) anti-B10.A(3R); anti-I-J^k = B10.A(3R) anti-B10.A(5R).

Preparation of GAT-Mϕ. Macrophages were pulsed with GAT by either of two techniques. In most experiments, GAT-bearing Mϕ were prepared in vitro by previously described techniques (13). In brief, 2×10^6/ml of viable peptone induced peritoneal exudate cells (PEC) (>75% Mϕ) were incubated at 4°C for 1 h in 100 μg/ml GAT at pH 9–9.5, then washed four times in cold Hanks' balanced salt solution (HBSS) and resuspended in HBSS to the desired concentration for addition to culture. In one series of experiments, mice injected 3 days previously with 1.5 ml of 10% protease peptone (Difco Laboratories, Detroit, Mich.) were inoculated i.p. with 100 μg of GAT in HBSS at neutral pH. 1 h later the peritoneal cavity was lavaged with cold HBSS, and the recovered cells (60–90% Mϕ) washed four times before resuspending in HBSS for addition to cultures (in vivo pulsed Mϕ).

Cell Culture and Hemolytic Plaque Assay. All cell cultures were performed according to a modified Mishell-Dutton protocol described previously (19). 7.5×10^6 viable spleen cells in completely supplemented Eagle's minimal essential medium containing 10% fetal calf serum (FCS) (Reheis Chemical Division of Armour Pharmaceutical Co., Chicago, Ill.) and 10 mM Hepes were cultured with soluble GAT (1–2.5 μg/ml) or GAT-Mϕ (10^4-4×10^4) in a 1-ml final volume in the 16-mm wells of a flat-bottom tissue culture plate (FB-16-24TC, Linbro Chemical Co., Hamden, Conn.). These cultures were incubated with rocking for 5 days at 37°C in a humidified atmosphere of 10% CO_2, 7% O_2, 83% N_2, and fed daily with 70 μl of a 1:1 mixture of nutritional cocktail and FCS. At the end of this culture period, replicate wells were pooled, the cells washed three times, and GAT-specific IgG PFC determined by a slide modification of the Jerne hemolytic plaque assay, using GAT-SRBC as indicator cells. For the experiments involving mixtures of various spleen populations, 3.75×10^6 of each cell type were added in 0.5 ml to each culture well, which were then stimulated and assayed as above. All data are from representative experiments, and are expressed as GAT-specific IgG PFC/culture of 7.5×10^6 spleen cells.

Results

Comparison of In Vitro Stimulatory Activity of Macrophages Pulsed with GAT In Vivo or In Vitro. Several studies have shown that nonresponder Mϕ-bearing GAT stimulate in vitro primary PFC responses by R or (R × NR)F_1 spleen cells (3, 4, 15). These data differ from those obtained in studies on secondary GAT responses of (R × NR)F_1 spleen cells (15) and experiments by others in both the mouse and guinea pig showing the inability of NR-derived antigen-bearing Mϕ to trigger T-cell responses to Ir-controlled antigens (20–23). One possible explanation for these conflicting data is that NR-Mϕ pulsed with GAT under nonphysiologic conditions in vitro might artifactually be able to stimulate primary GAT responses, in comparison to NR-Mϕ bearing GAT acquired in vivo under more physiologic conditions (24). To investigate this point, R (B6) and NR(D1) Mϕ were prepared either by the standard in vitro method or by injecting GAT i.p. into peptone pretreated mice, followed by harvest of the GAT bearing PEC. These in vitro- and in vivo-pulsed Mϕ were then used in culture for stimulating primary or secondary anti-GAT PFC responses using (B6D1)F_1 spleen cells. Table I presents data which reveal that in vivo prepared cells act identically to in vitro pulsed cells in terms of primary stimulation or pattern of secondary response restrictions. Therefore, the mode of GAT-Mϕ preparation is not responsible, per se, for the pattern of activity of GAT-bearing NR-Mϕ in triggering GAT PFC responses in vitro.

GAT-Primed (R × NR)F_1 Spleen Cells Suppress the Primary Anti-GAT PFC Response to GAT-NR-Mϕ. To test the hypothesis that the absence of secondary anti-GAT responses to GAT-NR-Mϕ was at least in part attributable to active suppression, GAT-primed (R × NR)F_1 spleen cells exhibiting this restricted response pattern were mixed with normal unprimed F_1 spleen cells, and the mixture challenged with either

TABLE I

In Vitro Stimulatory Activity of in Vivo vs. in Vitro Pulsed GAT-Mϕ for Primary and Secondary PFC Responses

Responding (B6D1)F_1 spleen cells	Antigen*	IgG GAT-specific PFC/culture
Normal	1 μg GAT	300
	GAT-B6-Mϕ (In vitro)	395
	GAT-B6-Mϕ (In vivo)	728
	GAT-D1-Mϕ (In vitro)	550
	GAT-D1-Mϕ (In vivo)	728
GAT-primed‡	1 μg GAT	390
	GAT-B6-Mϕ (In vitro)	345
	GAT-B6-Mϕ (In vivo)	660
	GAT-D1-Mϕ (In vitro)	<10
	GAT-D1-Mϕ (In vivo)	<10

* 2×10^4 GAT-Mϕ prepared by pulsing with soluble GAT as described in Materials and Methods, were added per culture.

‡ Animals primed i.p. with 100 μg GAT in Maalox-pertussis 5 wk before use.

soluble GAT, GAT-B6 (R)-Mϕ, or GAT-D1 (NR)-Mϕ. If the failure of primed F_1 spleen cells to respond to GAT-D1-Mϕ was due solely to the absence of a GAT-D1-specific cell population, one would predict that the response in the mixture would be ≥½ that of 7.5×10^6 normal spleen cells alone. If suppression were involved in the secondary restrictions, however, the response of the cocultured cells to GAT-D1-Mϕ should be ≪½ that of the normal cells only. Table II shows that the response of such 1:1 mixtures of normal and GAT-primed syngeneic F_1 spleen cells to GAT-D1-Mϕ is essentially at background levels (<15 PFC/culture); i.e., substantially less than one-half the 1977 PFC/culture of normal spleen cells alone stimulated by the same GAT-NR-Mϕ. This finding indicates the presence of suppressor cells in the primed spleen cell population, active on the PFC response to GAT-NR-Mϕ. In contrast, stimulation with GAT-B6-Mϕ of either normal spleen cells, GAT-primed spleen cells, or a 1:1 mixture of the two cell types gave virtually identical anti-GAT PFC (1,267 vs. 1,200 vs. 1,095 PFC/culture, respectively). This demonstrates that the suppressive activity shown by stimulating cocultures of normal and primed cells with GAT-NR-Mϕ cannot be detected with GAT-R-Mϕ, as expected from the responses of the primed cells alone to such GAT-R-Mϕ. As discussed below, however, these data do not exclude the possibility that the primary response of the normal cells to GAT-B6-Mϕ was also suppressed, but that this suppression was masked by the response of the primed cells themselves to these GAT-R-Mϕ. Finally, identical 1:1 mixtures of normal and primed cells exposed to soluble GAT, which would be presented by F_1 Mϕ possessing H-2^b (R) genes, give a significant PFC response, as expected.

Ability of CY or Anti-I-J Antiserum Treatment to Permit Secondary Responses of Primed F_1 Spleen Cells to GAT-NR-Mϕ. As a first approach to determining the nature of the suppressive activity in primed spleen cells, two in vivo treatments known to reduce GAT-specific suppressor T-cell activity were employed, in an effort to prevent restriction of secondary GAT responses. Several groups of mice were pretreated with 5 mg/kg freshly prepared CY i.p., 3 days before GAT priming. This protocol has been shown to prevent T_s generation to polypeptide antigens in suppressor, nonre-

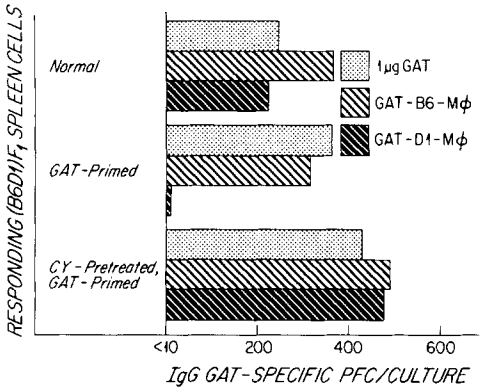

FIG. 1. (B6D1)F_1 mice were divided into three groups: (*a*) untreated; (*b*) primed with 100 μg GAT in adjuvant; or (*c*) pretreated with 5 mg/kg CY i.p., then primed with 100 μg GAT in adjuvant 3 days later. 4 wk later, the spleen cells of mice from each group were used as responding cells in modified Mishell-Dutton cultures stimulated with either 1 μg GAT, or 10^4 GAT-pulsed B6-Mϕ, or GAT-pulsed D1-Mϕ. These cultures were assayed on day 5 for GAT-specific IgG PFC using GAT-SRBC as indicator cells. Results are expressed as specific PFC/(7.5 × 10^6 cell) culture.

sponder mouse strains (17) (R. N. Germain et al., unpublished observations). As shown in Fig. 1, such pretreatment permits GAT-primed spleen cells to give secondary responses not only to GAT-B6-Mϕ, as in the untreated group, but also to GAT-D1-Mϕ. Similar data is presented in Tables II, IV, and V. In addition, Table II shows that mixtures of normal spleen cells together with spleen cells from CY-pretreated GAT primed mice do not show the suppression of responses to GAT-D1-Mϕ found in similar cocultures of normal plus untreated primed cells.

In a second type of experiment, mice were given 5 μl per day for 4 days of an antiserum directed against either the I-J^b subregion possessed by the F_1, or against the I-J^k subregion as a control. This anti-I-J^k antiserum is from the same pool previously shown to prevent L-glutamic acid^{50}-L-tyrosine^{50} (GT)-T_s activity in GT-primed CBA (H-2^k) mice (18). Such anti-I-J antiserum-treated mice were primed with GAT on the same day as the first anti-I-J antiserum injection and their spleen cells tested in vitro several weeks later. The data in Table III document that appropriate treatment with anti-I-J^b antiserum and not the control anti-I-J^k antiserum permits primed F_1 mice to give secondary responses to GAT-D1-Mϕ. Thus, two different experimental regimens, known to interfere with antigen specific T_s function, have similar abilities to prevent the appearance of the restricted secondary response typical of untreated GAT-primed F_1 mice. This provides further support for the basic hypothesis that such restricted responses are in part a consequence of suppressor cell generation.

Finally, the data in Table III (and in part, Table V) also indicate that the ability to trigger secondary anti-GAT PFC responses in primed (R × NR)F_1 mice possessing suppressor cells is an H-2 linked phenomena. In this experiment, congenic mice B10 (H-2^b,R) or B10.G (H-2^q, NR) were used as Mϕ sources. The results clearly show that GAT-B10.G (NR)-Mϕ behave identically to GAT-D1(NR)-Mϕ and differently from GAT-B10 (R)-Mϕ which differ only at the H-2 region. These data are (*a*) consistent with earlier studies mapping secondary GAT response restrictions to the I-A subregion of H-2 (11) and(*b*) in accord with the notion that H-2-linked Ir-gene status is important in the suppressor related phenomenon being studied here.

TABLE II

Suppression of GAT-D1-Mϕ Induced Primary GAT-PFC Responses by GAT-Primed (B6D1)F_1 Spleen Cells

Responding (B6D1)F_1* spleen cells	Antigen‡	IgG GAT-specific PFC/culture
Normal	1 μg GAT	1,027
GAT-primed§	1 μg GAT	945
CY-pretreated, GAT-primed§	1 μg GAT	645
Normal + GAT-primed	1 μg GAT	353
Normal + CY-pretreated, GAT-primed	1 μg GAT	713
Normal	GAT-B6-Mϕ	1,267
GAT-primed	GAT-B6-Mϕ	1,200
CY-pretreated, GAT-primed	GAT-B6-Mϕ	1,223
Normal + GAT-primed	GAT-B6-Mϕ	1,095
Normal + CY-pretreated, GAT-primed	GAT-B6-Mϕ	1,058
Normal	GAT-D1-Mϕ	1,977
GAT-primed	GAT-D1-Mϕ	<15
CY-pretreated, GAT-primed	GAT-D1-Mϕ	1,118
Normal + GAT-primed	GAT-D1-Mϕ	<15
Normal + CY-pretreated GAT-primed	GAT-D1-Mϕ	1,043

* 7.5×10^6 spleen cells/culture. Mixtures contain 3.75×10^6 spleen cells of each type.

‡ 2×10^4 in vitro prepared GAT-Mϕ/culture.

§ Mice primed with or without CY pretreatment as described in Materials and Methods.

GAT-D1-Mϕ Do Not Trigger a Nonspecific Suppression Able to Prevent Secondary Responses to GAT-B6-Mϕ. One possible interpretation of the data presented above is that during antigen priming, suppressor cells specific for GAT in association with H-2^q antigens are preferentially induced and that such suppressor cells only function when GAT is represented on a D1 (H-2^q) Mϕ surface. Although the results in Table II showing responses of primed F_1 spleen cells to soluble GAT (presumably presented by F_1 Mϕ with both H-2^b and H-2^q antigens) appear to contradict this hypothesis, an experiment with mixtures of antigen-pulsed Mϕ was undertaken to test this point more directly. As can be seen in Table IV, the addition of GAT-D1-Mϕ to primed cells together with GAT-B6-Mϕ, failed to cause suppression of the usual GAT-B6-Mϕ-induced response. The remainder of the table demonstrates that all the cells used in this experiment had the response patterns expected of them from the data given above. Thus, GAT-D1-Mϕ do not trigger an (H-2^q plus antigen) specific suppressor population to cause nonspecific suppression of GAT responses. This implies that the secondary response to GAT-B6-Mϕ is not simply a result of the failure to trigger suppressor activity, but reflects, at a minimum, a certain H-2 regulated resistance to the influence of the suppressor cells clearly existing in primed F_1 spleen cell populations.

Demonstration of Suppressor-Mediated Restrictions of Secondary GAT PFC Responses in Another (R × NR)F_1 Strain. To test whether the results obtained in the preceding experiments were of a general nature, or peculiar to the (B6D1)F_1 only, a limited number of experiments were performed using (CBA × D1)F_1 mice. As shown in Table V, priming such F_1 mice with GAT yields spleen cells giving secondary responses in vitro only to R (CBA) and not NR (D1 or B10.G) GAT-Mϕ. Further, the

TABLE III

Treatment with Anti-I-J^b Antiserum at the Time of GAT Priming Prevents the Suppression Detected in Secondary in Vitro Responses of (B6D1)F_1 to GAT-D1-Mϕ

Responding (B6D1)F_1* spleen cells	Antigen‡	IgG GAT-specific PFC/culture
Exp. I		
Normal	1 μg GAT	405
	GAT-D1-Mϕ	235
GAT-primed	1 μg GAT	435
	GAT-D1-Mϕ	<10
Anti-I-J^k treated, GAT-primed	1 μg GAT	490
	GAT-D1-Mϕ	<10
Anti-I-J^b treated, GAT-primed	1 μg GAT	260
	GAT-D1-Mϕ	350
Exp. II		
Normal	1 μg GAT	240
	GAT-B10-Mϕ	460
	GAT-B10.G-Mϕ	290
	GAT-D1-Mϕ	315
Anti-I-J^k treated, GAT-primed	1 μg GAT	710
	GAT-B10-Mϕ	905
	GAT-B10.G-Mϕ	10
	GAT-D1-Mϕ	<10
Anti-I-J^b treated, GAT-primed	1 μg GAT	240
	GAT-B10-Mϕ	260
	GAT-B10.G-Mϕ	265
	GAT-D1-Mϕ	255

* Mice primed with GAT and treated with anti-I-J^k [B10.A(3R) anti-B10.A(5R)] or anti-I-J^b [B10.A(5R) anti-B10.A(3R)] as described in Materials and Methods.

‡ 1×10^4 in vitro prepared GAT-Mϕ per culture.

TABLE IV

GAT-DBA/1-Macrophages Do Not Inhibit the Response of GAT-Primed (Responder[B6] × Nonresponder [D1])F_1 Spleen Cells to GAT-B6-Macrophages

Responding (B6D1)F_1 spleen cells	Antigen*	IgG GAT-specific PFC/culture
Normal	1 μg GAT	275
	GAT-B6-Mϕ	265
	GAT-D1-Mϕ	186
	GAT-B6-Mϕ + GAT-D1-Mϕ	225
GAT-primed‡	1 μg GAT	1,250
	GAT-B6-Mϕ	855
	GAT-D1-Mϕ	<10
	GAT-B6-Mϕ + GAT-D1-Mϕ	1,115
CY-pretreated,§ GAT-primed	1 μg GAT	570
	GAT-B6-Mϕ	645
	GAT-D1-Mϕ	520
	GAT-B6-Mϕ + GAT-D1-Mϕ	710

* GAT-Mϕ prepared in vitro as described in Materials and Methods. 2×10^4 GAT-Mϕ of the indicated type were added to each culture.

‡ Animals were primed i.p. with 100 μg GAT in Maalox-pertussis 5 wk before use.

§ Mice were pretreated with 5 mg/kg CY i.p. 3 days before GAT priming.

TABLE V

Effect of Cyclophosphamide on Mϕ-Restricted Secondary in Vitro Responses of (Responder [CBA] × Nonresponder [D1])F_1 Mice

Responding (CBA × D1)F_1* spleen cells	Antigen‡	IgG GAT-specific PFC/culture
Exp. I		
Normal	1 μg GAT	310
	GAT-CBA-Mϕ	625
	GAT-D1-Mϕ	520
GAT-primed	1 μg GAT	520
	GAT-CBA-Mϕ	555
	GAT-D1-Mϕ	115
Exp. II		
Normal	GAT-CBA-Mϕ	505
	GAT-D1-Mϕ	495
	GAT-B10.G-Mϕ	760
GAT-primed	GAT-CBA-Mϕ	1,130
	GAT-D1-Mϕ	40
	GAT-B10.G-Mϕ	290
CY-pretreated, GAT-primed	GAT-CBA-Mϕ	615
	GAT-D1-Mϕ	635
	GAT-B10.G-Mϕ	530

* Mice primed with or without CY pretreatment as described in Materials and Methods.
‡ 1 × 10^4 GAT-Mϕ prepared in vitro per culture.

restricted response pattern of such primed mice is prevented, as before, by pretreatment with CY. Suppressive activity of primed (CBA × D1)F_1 spleen cells mixed 1:1 with normal F_1 cells for responses to GAT-D1-Mϕ was also found (data not shown). Therefore, the basic observation of a role for suppressor cells in H-2 restricted secondary PFC responses in vitro appears to be a general phenomenon in the GAT model.

Discussion

The experiments described above support the conclusion that for the Ir-gene-regulated antigen GAT, the lack of secondary PFC responses by GAT-primed (R × NR)F_1 to antigen presented on NR Mϕ is in large measure a reflection of active suppression selectively limiting lymphocyte stimulation by GAT-NR-Mϕ in comparison to GAT-R-Mϕ. In addition, the data strongly suggest that the suppressor cells active in this model are T cells. The evidence for these conclusions derives from (*a*) the direct demonstration of the suppressive activity of GAT-primed (R × NR)F_1 spleen cells on primary PFC responses of normal F_1 spleen cells to GAT-NR-Mϕ (Table II) and (*b*) the ability of CY or anti-I-J^b antiserum treatments (known to remove GAT-specific suppressor T-cell activity in vivo) to prevent GAT priming from leading to restricted secondary responses to GAT-NR-Mϕ (Tables II–V and Fig. 1). Furthermore, susceptibility to restriction of secondary responses by suppressor cells is controlled by the H-2 complex, as shown by the difference in secondary responsiveness of (B6D1)F_1 spleen cells to GAT-Mϕ from the H-2 congenic pair B10 (H-2^b) and B10.G (H-2^q) (Table IV). The data are consistent with similar results recently obtained by Pierce (25).

The major unresolved issue raised by these experiments is the relationship between the generation of suppressor cells and the selective loss of responsiveness to GAT-NR-Mϕ in GAT primed (R × NR)F_1 mice. Several features of GAT as an antigen are relevant to this question. First, mice of nonresponder H-2 haplotype will give primary in vivo GAT PFC responses after maneuvers to reduce T_s activity, i.e. CY pretreatment, anti-I-J antiserum treatment, or adult thymectomy. This implies that such NR mice are capable of some detectable GAT specific Mϕ antigen presentation and, albeit weak, T_H function, if the obscuring action of T_s is first removed. This interpretation is strengthened by previous direct demonstrations that NR Mϕ-bearing GAT can prime R and NR mice for radioresistant (NR-Mϕ restricted) T_H function (4). Second, GAT readily induces specific T_s in NR mice in vivo (16) or in vitro (19), and can activate similar T_s in vitro in responder spleen cell populations depleted of Mϕ by adherence methods.[2] These various observations taken together indicate that NR Mϕ are not absolutely deficient in their ability to present GAT to mouse lymphocytes of R or NR origin, thus explaining the primary in vitro response of (R × NR)F_1 to GAT-D1-Mϕ. Furthermore, the relative absence of appropriate Mϕ GAT presentation seems to favor predominance of T_s over T_H. Finally, the detection of T_H responses elicited by NR Mϕ requires either pretreatment to reduce T_s activity or the presentation of GAT solely on appropriately pulsed Mϕ, which apparently achieves the same end.

These conclusions provide a framework for the analysis of the Ir related restrictions in secondary responses of GAT primed (R × NR)F_1 mice. We propose that injection of soluble GAT into such F_1 mice triggers at least three populations of T lymphocytes: one set of T_H specific for GAT presented in the context of responder Ia (Ia^R), one set of T_H triggered in the absence of Ia^R-GAT presentation, and a minimum of one set of GAT specific T_s. From previous data showing that even in the absence of T_s activity, NR mice make a much smaller GAT PFC response than R to the same stimulus, it can be postulated that the T_H set specific for GAT-Ia^R is much larger or more active than the T_H set not associated with Ia^R. The T_s response to the GAT used for priming is likely to be sufficient to overwhelm this weaker T_H response but not the stronger GAT-Ia^R-associated T_H response. Thus, only GAT-R-Mϕ responsive primed T_H will exist in sufficient number to trigger secondary responses in the face of the T_s present in the splenic population. It should be noted that this hypothesis makes no distinction between T_s arising as a result of direct antigen activation of T_s precursors, or those T_s stimulated through feedback induction of Lyt1,2,3^+Qa1^+ cells by Lyt1^+ cells (26, 27). Furthermore, it is probable that what we discussed above in purely quantitative terms may also be a reflection of a qualitative change in activated T_H, which reduces their susceptibility to suppression.[3] This theory also relies on the notion that suppression is a normal accompaniment of antigen priming (28–30), and that there is a distinct difference in the efficiency with which GAT triggers T_H precursors when associated with Ia^R vs. Ia^{NR}. Attempts to reassess R vs. NR Mϕ presentation of GAT under more

[2] M. Pierres and R. N. Germain. 1978. Antigen-specific T-cell mediated suppression. IV. Role of macrophages in generation of L-glutamic $acid^{60}$-L-$alanine^{30}$-L-$tyrosine^{10}$ (GAT)-specific suppressor T-cells in responder mouse strains. *J. Immunol.* In press.

[3] In this regard, the T_s present in the GAT primed F_1 spleen cells are probably able to suppress primary responses to GAT-B6-Mϕ as well. This could be examined by evaluating the responses of normal spleen cells to GAT-B6-Mϕ in the presence of purified Lyt2,3^+ T_s from primed F_1 mice. Such experiments are currently in progress, as are studies on the Mϕ restriction of radioresistant memory T_H from untreated or CY pretreated GAT primed (R × NR)F_1 mice.

limiting (Mϕ or T cell) conditions than formerly employed are in progress to establish the validity of this latter assumption.

A second hypothesis to explain the current results would postulate that T_s are specific for (H-2 + GAT), and that such H-2 restricted T_s are preferentially stimulated by $H\text{-}2^{NR}$ vs. $H\text{-}2^{R}$. As indicated above, this hypothesis is made unlikely by the ability of mixtures of GAT-B6-Mϕ and GAT-D1-Mϕ, or soluble GAT, to give substantial GAT responses with primed $(B6D1)F_1$ spleen cells, because $(H\text{-}2^{q}$ + GAT)-restricted T_s should be triggered under these circumstances to act on the F_1 T_H bearing both $H\text{-}2^{b}$ and $H\text{-}2^{q}$. Allelic exclusion of the relevant H-2 region on the T_H would explain this result, but no evidence in favor of this possibility yet exists. Furthermore, Pierce et al. (5) have shown that GAT-Mϕ primed mice cannot give secondary responses to GAT-Mϕ of H-2 haplotypes not present during the priming, a finding which is also inconsistent with this interpretation. Similarly, a third explanation postulating the occurrence of T_s after priming which would recognize the idiotype of the antigen receptors of T_H specific for $GAT\text{-}Ia^{NR}$ is compatible with the requirement for allelic exclusion just discussed, but would not explain the third party nonresponse (suppression) seen by Pierce et al. Therefore, the first hypothesis appears to be the most attractive working model.

Several other investigators have studied secondary responses to Ir-controlled antigens presented by R or NR Mϕ. In both guinea pig and murine systems, (R × NR)F_1-primed T-cell proliferation to such antigens occurs only when these molecules are presented on Mϕ-bearing responder Ia antigens (20, 21, 24). Recent studies by Yano et al. (31) have utilized a similar approach to that employed in the present study to evaluate the role of T_s in limiting secondary proliferative responses to GAT-NR-Mϕ. Neither pretreatment with CY or adult thymectomy before immunization, nor removal of $Lyt2^{+}$ T_s at the time of assay revealed a latent response to GAT-NR-Mϕ. The reason for this difference between proliferative and PFC assays for assessment of GAT-NR-Mϕ function is unclear at present. It may be a reflection of the involvement of distinct T-cell subsets in the two types of responses, or an indication that T_H function may proceed without a cell division step that requires $GAT\text{-}Ia^{R}$ triggering. Alternatively, differences in assay sensitivity and other technical considerations may be the determining factors. It is clear that further study is needed to resolve this issue.

In a primary in vitro model, Singer et al. (22) have demonstrated that for TNP-poly-L-(Tyr, Glu)-poly-D,L-Ala-poly-L-Lys [(T,G)-A--L], only R and not NR Mϕ can trigger PFC responses, and have suggested that this reflects the inability of $[Ia^{NR}$ + (T,G)-A--L] to trigger (R × NR)F_1 lymphocytes. These data are quite distinct from those obtained using GAT, which regularly stimulates primary PFC responses when presented on NR-Mϕ. It is unlikely that the results in the GAT model are solely a reflection of antigen transfer to R Mϕ since (1) in the secondary cultures, such transfer to the F_1 Mϕ would lead to responses due to $GAT\text{-}H\text{-}2^{b}$ antigen presentation, and such responses are not seen and (2) NR mice can be primed with and can respond to GAT under the appropriate circumstances in the absence of any R Mϕ (see above). It is therefore more probable that the difference between the (T,G)-A--L and GAT systems reflects a more profound inability on the part of NR-Mϕ to trigger any T_H activity for (T,G)-A--L vs. GAT, i.e. that the GAT Ir gene, as expressed in the NR at the Mϕ-T_H level, is leaky. Although the simplest explanation for this minimal helper

function in GAT-NR is that (Ia^{NR} + GAT) can produce a weak immunogen capable of stimulating cells from the same Ia restricted T_H subset as GAT-Ia^R, it is also possible that help and suppression are intimately linked in the GAT system through the feedback loop described by Eardley et al. (26). In this case, the NR T_H activity might in fact be mediated by a distinct class of $Lyt1^+$ $I\text{-}J^+$ cells responsible for feedback suppressor induction and also helper function (2). In either case, we no longer perceive any major difficulties in applying to the GAT model a general theory of Ir-gene function based (*a*) on the identity of some Ir-gene products with Ia molecules on macrophages and B cells and (*b*) on the importance of antigen presentation to T cells in the context of the macrophages' Ia molecules (1). The GAT system, however, with its unequal balance between T_H and T_s induction in R vs. NR haplotypes, may reflect more accurately and informatively the complexities encountered in studies of immune responses to native antigens capable of stimulating both T_s and T_H responses concommitantly.

The present study on the respective roles of haplotype restricted T_H priming and of T_s activity in regulating secondary immune responses also has implications for antigens not under unigenic Ir control. It is becoming clear that responses to distinct determinants of a complex antigen molecule must be considered separately to fully understand the overall response to that antigen (33–35). Intramolecular antigen competition may reveal itself as a low overall response to a given molecule, due to Ts which act preferentially on the T_H to one vs. another epitope. This in turn may reflect an imbalance in (Ia + determinant 1) vs. (Ia + determinant 2) immunogenicity in analogy to the difference postulated above for (Ia^R + GAT) vs. (Ia^{NR} + GAT). Thus, what we generally view as an immune response may not reflect the balance of T_H vs. T_s triggered by an antigen as a single entity, but rather the outcome of a series of distinct T_H-T_s competitions whose overall balance reflects the sum of action at the Mϕ-T-cell level of Ir genes specific for each determinant of the entire molecule. It is likely that direct demonstration of such Ir control of epitope specific T_H and T_s subsets will soon be available from studies on sequenced polypeptides and their fragments.

Summary

(Responder [R] × nonresponder [NR])F_1 mice give indistinguishable primary in vitro plaque-forming cell (PFC) responses to either R or NR parental macrophages (Mϕ) pulsed with the Ir-gene controlled antigen L-glutamic $acid^{60}$-L-$alanine^{30}$-L-$tyrosine^{10}$ (GAT). However, such (R × NR)F_1 mice, if primed to GAT, retained in vitro responsiveness to GAT-R-Mϕ, but no longer responded to GAT-NR-Mϕ. This suggested (*a*) a possible Mϕ-related locus for Ir gene activity in this model, and (*b*) the occurrence of active suppression after priming with GAT leading to a selective loss of the usual primary responsiveness of (R × NR)F_1 mice to GAT-NR-Mϕ. This latter interpretation was tested in the current study. [Responder C57BL/6 ($H\text{-}2^b$) × nonresponder DBA/1 ($H\text{-}2^q$)]F_1 mice were primed with 100 μg GAT in pertussis adjuvant. 4–8 wk later, spleen cells from such mice were tested alone or mixed with normal unprimed F_1 spleen cells for PFC responses to GAT-R-Mϕ and GAT-NR-Mϕ. The primed cells failed to respond to GAT-NR-Mϕ, and moreover, actively suppressed the normal response of unprimed F_1 cells to GAT-NR-Mϕ. If the primed spleen cell donor had been treated with 5 mg/kg cyclophosphamide 3 days before

priming or with 5–10 μl/day of an antiserum to the I-J^b subregion [B10.A(5R) anti B10.A(3R)] during the first 4 days postpriming (both procedures known to inhibit suppressor T-cell activity), cells from such mice responded in secondary culture to both GAT-R-Mϕ and also GAT-NR-Mϕ. In addition, such spleen cells no longer were capable of suppressing normal F_1 cells in response to GAT-NR-Mϕ. Similar data were obtained using [CBA (H-2^k) × DBA/1 (H-2^q)]F_1. Further, it was shown that (*a*) primary responsiveness to GAT-NR-Mϕ was not an artifact of in vitro Mϕ pulsing, because in vivo GAT-pulsed Mϕ showed the same activity and (*b*) the secondary restriction for Mϕ-antigen presentation was controlled by H-2 linked genes. These data suggest an important role for suppressor T cells in H-2 restricted secondary PFC responses, and also provide additional support for the hypothesis that Ir-gene controlled differences in Mϕ antigen presentation are related to both suppressor cell generation and overall responsiveness in the GAT model.

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