THE INVOLVEMENT OF SUPPRESSOR T CELLS IN Ir GENE REGULATION OF SECONDARY ANTIBODY RESPONSES OF PRIMED (RESPONDER × NONRESPONDER)F1 MICE TO MACROPHAGE-BOUND L-GLUTAMIC ACID⁰⁰-L-ALANINE³⁰-L-TYROSINE¹⁰

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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)→T-cell interaction or T helper cell (Th)→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 acid⁰⁰-L-alanine³⁰-L-tyrosine¹⁰ (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)F1 (R × NR)F1 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)F1 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.
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)F1 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 F1 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)F1 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)F1 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 F1 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

Mouse. C57BL/6 (B6) or C57BL/10 (B10) H-2^b, DBA/1 (D1), H-2^k, and CBA, H-2^k mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. (C57BL/6 female × DBA/1 male)F1 [(B6D1)F1] and (CBA female × DBA/1 male)F1 mice were bred in the Department of Pathology animal facility. B10.G, H-2^k 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. F1 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. Washington, 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^a or anti-I-J^k antiserum diluted in saline, beginning on the day of immunization (18). These antiseras, provided by Dr. Martin Dorf of this department, were raised and characterized as described previously (19). Anti-I-J^a = 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 × 10^6) 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% CO₂, 7% O₂, 83% N₂, 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)F₁ spleen cells (3, 4, 15). These data differ from those obtained in studies on secondary GAT responses of (R × NR)F₁ 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 artifically 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)F₁ 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)F₁ 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)F₁ spleen cells exhibiting this restricted response pattern were mixed with normal unprimed F₁ 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)F₁ 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 × 10⁵ 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 F₁ 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 × 10⁸ 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 F₁ 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 F₁ Mφ possessing H-2b (R) genes, give a significant PFC response, as expected.

Ability of CY or Anti-I-J Antiserum Treatment to Permit Secondary Responses of Primed F₁ 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 T₁ generation to polypeptide antigens in suppressor, nonre-
**Fig. 1.** (B6D1)F1 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 × 10^9 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 F1, or against the I-J^k subregion as a control. This anti-I-J^b antiserum is from the same pool previously shown to prevent 3-glutamic acid-S0-tyrosine-50 (GT)-T8 activity in GT-primed CBA (H-2K) 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 F1 mice to give secondary responses to GAT-D1-Mφ. Thus, two different experimental regimens, known to interfere with antigen specific T8 function, have similar abilities to prevent the appearance of the restricted secondary response typical of untreated GAT-primed F1 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 X NR)F1 mice possessing suppressor cells is an H-2 linked phenomena. In this experiment, congenic mice B10 (H-2b,R) or B10.G (H-2a, 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)F1 Spleen Cells

| Responding (B6D1)F1 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 × 10⁶ spleen cells/culture. Mixtures contain 3.75 × 10⁶ spleen cells of each type.
‡ 2 × 10⁴ 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^d antigens are preferentially induced and that such suppressor cells only function when GAT is represented on a D1 (H-2^d) Mϕ surface. Although the results in Table II showing responses of primed F1 spleen cells to soluble GAT (presumably presented by F1 Mϕ with both H-2^b and H-2^d 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^d 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 F1 spleen cell populations.

Demonstration of Suppressor-Mediated Restrictions of Secondary GAT PFC Responses in Another (R × NR)F1 Strain. To test whether the results obtained in the preceding experiments were of a general nature, or peculiar to the (B6D1)F1 only, a limited number of experiments were performed using (CBA × D1)F1 mice. As shown in Table V, priming such F1 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<sup>b</sup> Antiserum at the Time of GAT Priming Prevents the Suppression Detected in Secondary in Vitro Responses of (B6D1)<sub>F1</sub> to GAT-D1-MΦ*

| Exp. I                  | Responding (B6D1)<sub>F1</sub>* spleen cells | Antigen<sup>‡</sup> | IgG GAT-specific PFC/culture |
|------------------------|---------------------------------------------|---------------------|------------------------------|
| Normal                 | 1 µg GAT                                    | GAT-B10-MΦ          | 265                          |
| GAT-primed             | 1 µg GAT                                    | GAT-B10.G-MΦ        | 200                          |
|                        |                                             | GAT-D1-MΦ           | 10                           |
| Anti-I-J<sup>b</sup> treated, GAT-primed | 1 µg GAT                                    | GAT-B10-MΦ          | 900                          |
|                        |                                             | GAT-B10.G-MΦ        | 10                           |
|                        |                                             | GAT-D1-MΦ           | <10                          |
| Anti-I-J<sup>b</sup> treated, GAT-primed | 1 µg GAT                                    | GAT-B10-MΦ          | 240                          |
|                        |                                             | GAT-B10.G-MΦ        | 260                          |
|                        |                                             | GAT-D1-MΦ           | 255                          |

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

† 1 × 10<sup>4</sup> 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])<sub>F1</sub> Spleen Cells to GAT-B6-Macrophages*

| Exp. II                  | Responding (B6D1)<sub>F1</sub>* spleen cells | Antigen<sup>‡</sup> | IgG GAT-specific PFC/culture |
|-------------------------|---------------------------------------------|---------------------|------------------------------|
| Normal                  | 1 µg GAT                                    | GAT-B6-MΦ           | 275                          |
|                         |                                             | GAT-D1-MΦ           | 186                          |
|                         |                                             | GAT-B6-MΦ + GAT-D1-MΦ | 225                          |
| GAT-primed†             | 1 µg GAT                                    | GAT-B6-MΦ           | 1,250                        |
|                         |                                             | GAT-D1-MΦ           | 855                          |
|                         |                                             | GAT-B6-MΦ + GAT-D1-MΦ | 1,115                        |
| CY-pretreated,§ GAT-primed | 1 µg GAT                                   | GAT-B6-MΦ           | 570                          |
|                         |                                             | GAT-D1-MΦ           | 645                          |
|                         |                                             | GAT-B6-MΦ + GAT-D1-MΦ | 710                          |

* GAT-MΦ prepared in vitro as described in Materials and Methods. 2 × 10<sup>4</sup> 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])F1 Mice

| Responding (CBA × D1)F1* 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 × 10⁴ 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)F1 spleen cells mixed 1:1 with normal F1 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)F1 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)F1 spleen cells on primary PFC responses of normal F1 spleen cells to GAT-NR-MΦ (Table II) and (b) the ability of CY or anti-I-J⁰ 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)F1 spleen cells to GAT-MΦ from the H-2 congenic pair B10 (H-2b) and B10.G (H-2⁰) (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)F₁ 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 Tₜ 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, Tₜ function, if the obscuring action of Tₜ 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) Tₜ function (4). Second, GAT readily induces specific Tₜ in NR mice in vivo (16) or in vitro (19), and can activate similar Tₜ 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)F₁ to GAT-D₁-Mφ. Furthermore, the relative absence of appropriate Mφ GAT presentation seems to favor predominance of Tₜ over Tₜ. Finally, the detection of Tₜ responses elicited by NR Mφ requires either pretreatment to reduce Tₜ 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)F₁ mice. We propose that injection of soluble GAT into such F₁ mice triggers at least three populations of T lymphocytes: one set of Tₜ specific for GAT presented in the context of responder Ia (Ia⁸), one set of Tₜ triggered in the absence of Ia⁸-GAT presentation, and a minimum of one set of GAT specific Tₜ. From previous data showing that even in the absence of Tₜ activity, NR mice make a much smaller GAT PFC response than R to the same stimulus, it can be postulated that the Tₜ set specific for GAT-Ia⁸ is much larger or more active than the Tₜ set not associated with Ia⁸. The Tₜ response to the GAT used for priming is likely to be sufficient to overwhelm this weaker Tₜ response but not the stronger GAT-Ia⁸-associated Tₜ response. Thus, only GAT-R-Mφ responsive primed Tₜ will exist in sufficient number to trigger secondary responses in the face of the Tₜ present in the splenic population. It should be noted that this hypothesis makes no distinction between Tₜ arising as a result of direct antigen activation of Tₜ precursors, or those Tₜ 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 Tₜ, 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 Tₜ precursors when associated with Ia⁸ vs. Ia⁹. 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 acid⁰-L-alanine⁰-L-tyrosine⁰ (GAT)-specific suppressor T-cells in responder mouse strains. J. Immunol. In press.

³ In this regard, the Tₜ present in the GAT primed F₁ 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⁺ Tₜ from primed F₁ mice. Such experiments are currently in progress, as are studies on the Mφ restriction of radioresistant memory Tₜ from untreated or CY pretreated GAT primed (R × NR)F₁ 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 Tₜ are specific for (H-2 + GAT), and that such H-2 restricted Tₜ are preferentially stimulated by H-2⁰NR vs. H-2⁰R. 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)F₁ spleen cells, because (H-2³ + GAT)-restricted Tₜ should be triggered under these circumstances to act on the F₁ TH bearing both H-2⁰b and H-2⁰d. Allelic exclusion of the relevant H-2 region on the Tn 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 Tₜ after priming which would recognize the idiotype of the antigen receptors of TH specific for GAT-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)F₁-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 Tₜ in limiting secondary proliferative responses to GAT-NR-Mφ. Neither pretreatment with CY or adult thymectomy before immunization, nor removal of Lyt2+ Tₜ 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 TH function may proceed without a cell division step that requires GAT-IaR 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 IaNR + (T,G)-A--L to trigger (R × NR)F₁ 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 F₁ Mφ would lead to responses due to GAT-H-2⁰b 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 TH activity for (T,G)-A--L vs. GAT, i.e. that the GAT Ir gene, as expressed in the NR at the Mφ-TH 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 TH 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 TH activity might in fact be mediated by a distinct class of Lyt1^+ I-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 TH and Ts 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 TH and Ts responses concomitantly.

The present study on the respective roles of haplotype restricted TH priming and of Ts 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 TH 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 TH vs. Ts triggered by an antigen as a single entity, but rather the outcome of a series of distinct TH-Ts 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 TH and Ts subsets will soon be available from studies on sequenced polypeptides and their fragments.

Summary

(Responder [R] X nonresponder [NR])F₁ 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 acidβ-L-alanineα-L-tyrosineβ (GAT). However, such (R X NR)F₁ 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 X NR)F₁ mice to GAT-NR-Mφ. This latter interpretation was tested in the current study. [Responder C57BL/6 (H-2b) X nonresponder DBA/1 (H-2q)]F₁ 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 F₁ 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 F₁ 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-Jb 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 F1 cells in response to GAT-NR-Mφ. Similar data
were obtained using [CBA (H-2k) × DBA/1 (H-2q)]F1. 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 con-
trolled differences in Mφ antigen presentation are related to both suppressor cell
generation and overall responsiveness in the GAT model.

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