Macrophages (Mφ) have the critical function of presenting antigens in a highly immunogenic form to responding antigen-specific T and B cells in the initiation of immune responses (1, 2). In primary antibody responses to T-cell-dependent antigens in vitro, genetic restrictions in Mφ-lymphocyte interactions are not apparent (1-4). By contrast, interactions between Mφ and immune T cells in the generation and functional expression of carrier-specific murine helper T cells in vitro (5), DNA-synthetic responses to antigens by immune guinea pig (6, 7) and murine (8) T lymphocytes, and expression of delayed hypersensitivity reactions in mice (9) are controlled by products of the I region (or its equivalent) of the major histocompatibility complex (MHC) of the species. The environment in which these T cells are first exposed to antigens has emerged as a critical factor in the subsequent expression of the genetic restrictions (3, 4, 10). Thus, immune murine lymphocytes develop secondary plaque-forming cell (PFC) responses preferentially when stimulated in vitro with antigen on Mφ syngeneic to the Mφ used to immunize the spleen cells in vivo (3, 4). These genetic restrictions are antigen-specific, they operate at the level of the immune T cell, and they are controlled by products of the I-A subregion of the H-2 complex (3, 4, 11).

The products of MHC-linked immune response (Ir) genes themselves may be involved in mediating genetic restrictions in Mφ-T-cell interactions. For example, in guinea pigs, immune (nonresponder × responder)F₁ T cells develop DNA-synthetic responses to antigens under the control of MHC-linked Ir gene(s) when the antigen is associated with responder, but not nonresponder, parental Mφ (6, 7). These observations led to the hypothesis that the Ir gene product is functionally expressed in Mφ, and nonresponder Mφ lack this product and are therefore unable to present antigens in an immunogenic form to stimulate the
T cells (7). Similar observations have recently been made with an antigen under Ir gene control in delayed hypersensitivity responses in mice (12).

The antibody response of mice to the terpolymer of L-glutamic acid\(^{68}\)-L-alanine\(^{38}\)-L-tyrosine\(^{18}\) (GAT) is controlled by an MHC-linked Ir gene(s) (13). Since allogeneic nonresponder M\(\phi\) stimulate primary PFC responses to GAT by responder lymphoid cells comparable to those stimulated by syngeneic responder M\(\phi\) (3, 4, 14), the defect in nonresponder mice does not appear to be expressed in the M\(\phi\), but rather appears to be due to a preferential development of GAT-specific suppressor T cells (13, 15). In light of the aforementioned studies with guinea pigs indicating M\(\phi\) localized Ir gene defects, the capacity of responder and nonresponder M\(\phi\) to stimulate responses to GAT by spleen cells from virgin and immune (responder \(\times\) nonresponder)F\(1\) mice was investigated and is the subject of this communication.

**Materials and Methods**

**Mice.** In experiments carried out at Harvard Medical School, C57BL/6 (B6) and DBA/1 (D1) mice were purchased from The Jackson Laboratory, Bar Harbor, Maine; (C57BL/6 \(\times\) DBA/1)F\(1\), (B6 \(\times\) D1)F\(1\), mice were bred in the Department of Pathology Animal Facility. In experiments carried out at The Jewish Hospital, C57BL/10 (B10), D1, DBA/2 (D2), C3H/He (C3H), (C57BL/10 \(\times\) DBA/1)F\(1\), [(B10 \(\times\) D1)F\(1\)], and (C57BL/6 \(\times\) DBA/2)F\(1\), [(B6 \(\times\) D2)F\(1\)] mice were bred in the Animal Facility in the Yalem Building. All mice were maintained on laboratory chow and acidified-chlorinated water ad lib. and used at 10-20 wk of age. In a single experiment, animals were sex and age matched. D1 mice are nonresponders to GAT; all other mice are responders.

**Antigens.** GAT (Miles Laboratories Inc., Elkhart, Ind.), mol wt \(\approx\) 45,000, was prepared for use as antigen in culture (16), for preparing GAT-M\(\phi\) (3), and for coupling to sheep erythrocytes for use as indicator cells in the PFC assay (16) as previously described.

**Culture System and Hemolytic Plaque Assay.** At Harvard Medical School, spleen cells at 7.5 \(\times\) \(10^6\) cells/ml in completely supplemented Eagle's minimal essential medium containing 10% fetal calf serum (Reheis Co., Inc., Kankakee, Ill.) and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (Hepes) (Hepes-MEM) were incubated with GAT-M\(\phi\) or GAT as 1-ml cultures in 16-mm wells of a 24-sample multiwell dish (FB-16-24TC; Linbro Chemical Co., New Haven, Conn.). At the Jewish Hospital, spleen cells at 10\(7\) cells/ml in completely supplemented MEM lacking Hepes were incubated and GAT-M\(\phi\) or GAT as 1-ml cultures in 35-mm dishes (3001; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). After incubation for 5 days under Mishell-Dutton conditions, IgG GAT-specific PFC responses were assayed using the slide modification of the Jerne hemolytic plaque assay (16).

**Preparation of GAT-M\(\phi\) and Immunization of Mice.** The preparation of GAT-M\(\phi\) using peptone-induced peritoneal exudate has been described in detail previously (3). F\(1\) mice were immunized by i.p. injection of 4 \(\times\) \(10^6\) of the appropriate GAT-M\(\phi\) (bearing \(\approx\) 25 ng GAT/10\(6\) cells) or 10 or 100 \(\mu\)g GAT in a mixture of magnesium-aluminum hydroxide gel (Maalox; Wm. H. Rorer, Inc., Ft. Washington, Pa.) and pertussis vaccine (Eli Lilly & Co., Indianapolis, Ind.) (16).

**Results**

Responder \(\times\) nonresponder (B6 \(\times\) D1)F\(1\), mice were immunized i.p. with 100 \(\mu\)g GAT in Maalox-pertussis; 79 days later, spleen cells from these and virgin mice were assayed for development of IgG GAT-specific PFC responses to responder or nonresponder parental GAT-M\(\phi\) and soluble GAT (Table I). Virgin (B6 \(\times\) D1)F\(1\), spleen cells developed comparable primary PFC responses to responder (B6) and nonresponder (D1) GAT-M\(\phi\). A mixture of B6 and D1 GAT-M\(\phi\), equal to twice the number of parental GAT-M\(\phi\) alone, stimulated responses comparable to those stimulated by either parental GAT-M\(\phi\). Immune
TABLE I
Secondary PFC Responses to GAT by Responder × Nonresponder (B6 × D1)F1, Spleen Cells Stimulated by Parental GAT-Mφ

| (B6 × D1)F1, spleen cells* | GAT-Mφ/culture | Day 5 IgG GAT-specific PFC/culture† |
|---------------------------|----------------|-----------------------------------|
|                           | B6-Mφ          | D1-Mφ                             | B6-Mφ + D1-Mφ | GAT† |
| Virgin                    |                |                                   |               |
| 2 × 10⁴                    | 593            | 365                               | 518           | 619  |
| 4 × 10⁴                    | 255            | 69                               | 383           | 383  |
| 2 × 10⁴                    | 338            | <10                               | 270           |      |
| 1 × 10⁴                    | 510            | <10                               | 548           |      |
| 5 × 10³                    | <10            | 30                               | <10           |      |

* (B6 × D1)F1 mice were immunized 79 days previously by i.p. injection of 100 μg GAT in Maalox-pertussis.
† Virgin or GAT-primed (B6 × D1)F1, spleen cells at 7.5 × 10⁶ cells/culture were incubated with the indicated GAT-Mφ or soluble GAT in HEPES-MEM as described in Materials and Methods. GAT-Mφ had ~2 ng GAT/10⁶ cells.
§ Equal numbers of B6 and D1 GAT-Mφ were added to these cultures making the total number of GAT-Mφ/culture twice the number indicated.
|| The optimal dose of soluble GAT was 1 μg/culture for virgin spleen cells and 0.3 μg/culture for primed spleen cells.

TABLE II
Secondary PFC Responses to GAT by Responder × Nonresponder (B10 × D1)F1, Spleen Cells Stimulated by Parental GAT-Mφ

| (B10 × D1)F1, spleen cells* | GAT-Mφ/culture | Day 5 IgG GAT-specific PFC/culture† |
|-----------------------------|----------------|-----------------------------------|
|                            | B10-Mφ         | D1-Mφ                             | C3H-Mφ        | 5 μG GAT |
| Virgin                     | 7 × 10⁴        | 190                               | 180           | 210     |
|                            | 5 × 10⁴        | 250                               | 250           | 250     |
| GAT-primed                 | 7 × 10⁴        | 380                               | 50            | <10     |
|                            | 5 × 10⁴        | 300                               | 80            | 50      |
| B10 GAT-Mφ primed          | 7 × 10⁴        | 270                               | <10           | 270     |
|                            | 5 × 10⁴        | 370                               | <10           | 300     |
| D1 GAT-Mφ primed           | 7 × 10⁴        | 40                                | <10           |        |
|                            | 5 × 10³        | 70                                | <10           |        |

* (B10 × D1)F1 mice were immunized 28 days previously by i.p. injection of 10 μg GAT in Maalox-pertussis (GAT-primed), or 4 × 10⁹ of the indicated Mφ bearing ~25 ng of GAT/10⁶ cells. At culture initiation these spleen cells had <25 IgG GAT-specific PFC/10⁶ cells.
† Virgin or GAT-primed (B10 × D1)F1, spleen cells at 10⁷ cells/culture were incubated with the indicated GAT-Mφ or soluble GAT in MEM. GAT-Mφ had ~2 ng GAT/10⁶ cell.

(B6 × D1)F1 spleen cells, by contrast, developed secondary PFC responses to responder (B6) GAT-Mφ and soluble GAT, but not to nonresponder (D1) GAT-Mφ. Mixtures of equal numbers of B6 and D1 GAT-Mφ-stimulated responses comparable to those stimulated by the same number of B6 GAT-Mφ alone, except at the lowest numbers tested, where 5 × 10⁶ B6 GAT-Mφ also failed to stimulate a response. These data with mixtures of B6 and D1 Mφ indicate that the failure of immune F1 spleen cells to respond to the nonresponder D1 GAT-Mφ alone is not due to a nonspecific suppressive mechanism initiated by these Mφ.

In the next experiments, responder × nonresponder (B10 × D1)F1 mice were immunized i.p. with 10 μg GAT in Maalox-pertussis, or responder or nonresponder parental GAT-Mφ; 28 days later, spleen cells from these and virgin mice were assayed for development of IgG GAT-specific PFC responses to parental B10 and D1 GAT-Mφ, third party C3H GAT-Mφ, and soluble GAT (Table II). Virgin F1 spleen cells developed comparable primary responses to...
all GAT-Mφ and soluble GAT. The GAT-primed F₁ spleen cells developed secondary responses to responder B10 GAT-Mφ and soluble GAT, but failed to respond to nonresponder D1 or third party C3H GAT-Mφ. Identical results were observed in responses of F₁ spleen cells primed with responder B10 GAT-Mφ. By contrast, F₁ spleen cells primed with nonresponder D1 GAT-Mφ developed secondary responses with D1 GAT-Mφ and soluble GAT, but failed to respond to third party C3H or responder B10 GAT-Mφ. A preliminary study using responder × responder (B6 × D2)F₁ mice immunized with GAT indicates that these spleen cells develop secondary PFC responses after stimulation with either B10 or D2 GAT-Mφ, but fail to respond to third party C3H GAT-Mφ.

**Discussion**

These experiments illustrate three major points concerning the ability of spleen cells from virgin and immune (responder × nonresponder)F₁ mice to develop PFC responses after stimulation with GAT or parental GAT-Mφ in vitro. First, virgin F₁ spleen cells develop comparable primary IgG GAT-specific PFC responses after stimulation with either parental or unrelated third party GAT-Mφ. Second, spleen cells from F₁ mice immunized with either parental GAT-Mφ develop secondary responses in vitro to the GAT-Mφ used for immunization in vivo. These results are consistent with previous observations that spleen cells from mice immunized with syngeneic or allogeneic GAT-Mφ develop secondary responses in vitro to GAT-Mφ that are syngeneic at the I-A subregion of the H-2 complex with the Mφ used for priming (3, 4, 11). In many of these experiments, Mφ from nonresponder mice induced these genetic restrictions in spleen cells of homozygous responder mice (3, 4, 11). These observations and others (14), led to the earlier conclusion that the defect in nonresponder mice, in the case of GAT, was not in the Mφ.

The third observation is that spleen cells from F₁ mice immunized with GAT develop secondary responses in vitro only when stimulated with responder parental GAT-Mφ. This indicates that in virgin (responder × nonresponder)F₁ mice immunized with GAT, which would of necessity be presented by F₁ Mφ, the responder component of the F₁ Mφ predominates and selectively primes a population of F₁ T cells capable of being activated subsequently only by responder GAT-Mφ in culture. These results imply that Ir-gene function may be expressed in murine Mφ, and they are in agreement with observations in proliferative responses with F₁ guinea pig lymphocytes (6, 7) and delayed hypersensitivity responses in F₁ mice (12).

We must now resolve the contradiction between; (a) the ability of nonresponder GAT-Mφ to stimulate primary responses in both homozygous responder and heterozygous (responder × nonresponder)F₁ mice, and to prime these mice such that secondary responses are restricted to the nonresponder GAT-Mφ, and (b) the failure of GAT-primed F₁ mice to develop secondary responses to nonresponder GAT-Mφ. It is possible that Ir-gene function is expressed in murine Mφ and that nonresponder Mφ express some, but not all, of these functions with regard to GAT. Thus, only in certain circumstances (e.g. the F₁ animal), would these differences be revealed. The expression of only one set of responder Ir gene(s) in the immunocompetent cells of the heterozygous, (re-
spender × nonresponder)F₁ may impose restrictions on the interactions among F₁ Mφ and different subsets of T cells during priming with GAT. Alternatively, if, as demonstrated in guinea pigs (17), F₁ mice possess distinct subsets of T cells, each capable of interacting with one but not the other parental Mφ, it is possible that helper T cells specific for responder GAT-Mφ are primed by injection of GAT, whereas helper T cells capable of responding to nonresponder GAT-Mφ are not primed. This hypothesis is supported by these previous observations in nonresponder mice: (a) GAT induces suppressor, but not radioresistant helper T cells (13, 14, 18); (b) radioresistant GAT-specific helper T cells can be induced by injection of nonresponder mice with GAT-Mφ or GAT-MBSA (13, 18); and, (c) injection of GAT not only induces suppressor T cells, but also prevents the development of GAT-specific helper T cells after injection of GAT-MBSA (13).

Summary

The development of IgG L-glutamic acid⁶°-L-alanine³°-L-tyrosine¹⁰ (GAT)-specific plaque-forming cell responses in vitro by virgin and immune (responder × nonresponder)F₁ spleen cells after stimulation with responder and nonresponder parental GAT-macrophages (Mφ) was investigated. Virgin F₁ spleen cells developed comparable primary responses to both parental GAT-Mφ. By contrast, F₁ spleen cells from mice immunized with GAT or responder parental GAT-Mφ developed secondary responses after stimulation with only responder parental GAT-Mφ. Spleen cells from F₁ mice immunized with nonresponder parental GAT-Mφ developed secondary responses to these GAT-Mφ, but failed to respond to responder parental GAT-Mφ. These results are discussed in the context of genetic restrictions regulating Mφ-T-cell interactions in secondary antibody responses and the possible expression of Ir-gene function in Mφ.

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