# INHIBITION OF ANTIGEN-SPECIFIC T LYMPHOCYTE ACTIVATION BY STRUCTURALLY RELATED Ir GENE-CONTROLLED POLYMERS

## Evidence of Specific Competition for Accessory Cell Antigen Presentation*

By KENNETH L. ROCK AND BARUJ BENACERRAF

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

Most T lymphocytes are not activated by antigen (Ag)[1] alone but recognize Ag in association with major histocompatibility complex (MHC) gene products (1–4). This recognition occurs through a clonally distributed receptor(s) whose presence is inferred from the specific activation requirements of the T lymphocyte. For the Ia-restricted T cell this recognition requires an antigen-presenting cell (APC) (4). Additionally, the ability to generate T cell-dependent responses to protein and polypeptide Ag is determined by MHC-encoded Ir genes, which are intimately involved in this process of MHC-restricted recognition (5, 6). There is conclusive evidence that Ia molecules, which restrict the recognition of nominal AG, are Ir gene products (7–12).

While the phenomenology of H-2-restricted recognition of Ag is well established, the underlying mechanisms are not understood. Specifically, the precise nature of both the T cell receptor(s) and its Ag and MHC ligand(s) as well as the basis of Ir gene unresponsiveness is not known. Numerous theories have been advanced that raise most of the possible permutations, from a single receptor with linked ligands to dual receptors with unlinked ligands, but experimental verification is largely lacking (13–21). Elegant somatic cell hybridization experiments by Kappler et ál. (22) have strongly suggested that restricted recognition is not mediated by independent dual receptors in conjunction with unlinked antigen and Ia molecules. There is increasing evidence for some form of interaction between Ag and Ia molecules. Thus, Ir gene products appear to select the antigenic determinants to which a response is generated (23–25). Heber-Katz et al. (26) have clearly shown that the fine specificity of a single T cell clone's activation is determined by the accessory cells' MHC genotype, which strongly suggests an interaction between nominal Ag and MHC molecules. However, the nature of such an interaction is unknown. One possibility is that the interaction between nominal Ag and the accessory cell is specific. It is clear that Ir gene control discriminates antigenic differences as small as a single amino acid (25). In addition, structurally related Ag that elicit a clonally distinct T cell response appear to follow identical Ir gene control (27, 28). Accordingly we proposed a model where Ia molecules

* Supported by grant AI-14732 and training grant 2T32-CA-09130-06 from the National Institutes of Health.

[1] *Abbreviations used in this paper*: Ag, antigen; APC, antigen-presenting cell; GA, L-glutamic acid$^{60}$-L-alanine$^{40}$; GAT, L-glutamic acid$^{60}$-L-alanine$^{40}$-L-tyrosine$^{10}$; GL, L-glutamic acid$^{60}$-L-lysine$^{40}$; GT, L-glutamic acid$^{50}$-L-tyrosine$^{50}$; IL-2, interleukin 2; OVA ovalbumin, KLH, keyhole limpet hemocyanin; MHC, major histocompatibility complex; TNP, trinitrophenyl.



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Volume 157 May 1983 1618–1634

TABLE I
*Origin and Specificity of T Cell Hybridomas*

| Hybrid | Origin | I region restriction | Antigen specificity | Reference |
|---|---|---|---|---|
| | T cell blast × tumor* | | | |
| RF7.24.3 | BALB/c × BW5147 | I-A$^d$ | GAT | 30 |
| RF9.140 | BALB/c × BW5147 | I-A$^d$ | GAT | This paper |
| RF10.14 | BALB/c × BW5147 | I-A$^d$ | GAT | 30 |
| RF15.7 | BALB/c × BW5147 | I-A$^d$ | TNP | This paper |
| RF13.64 | B10 × BW5147 | I-A$^b$ | GAT | 30 |
| DO11.10 | BALB/c × BW5147 | I-A$^d$ | OVA | 47 |
| AODK10.4 | B10.D2 × AO40.10AG1 | I-A$^d$ | KLH | 22 |

* AO40.10AG1 is a double T cell hybridoma produced by the fusion of B10.A T cells with FS614.13, a B6D2F$_1$ × BW5147 hybrid.

can specifically associate with Ag via a finite number of binding sites that recognize short amino acid sequences (29). Alternatively, the interaction could be general, e.g., via protein-protein interactions between nominal Ag and Ia molecules perhaps upon apposition by the T cell receptor(s). The available evidence however, does not discriminate between these possibilities.

A unique prediction of the specific association model is that distinct Ag, which use the same interaction site, should compete for association and subsequent presentation under limiting conditions. During the course of studies characterizing a series of Ag-specific Ia-restricted T cell hybridomas we observed a specific competition between structurally related copolymers under Ir gene control. The analysis of this phenomenon is the subject of this report.

## Materials and Methods

*Mice.* BALB/cAnN mice,[2] ages 6–10 wk, were purchased from Charles River Breeding Laboratories, Inc., Kingston, NY. C57BL/10, C57BL/6, CBF$_1$, and BDF$_1$ mice, ages 5–7 wk, were purchased from The Jackson Laboratory, Bar Harbor, ME. D2GD mice were kindly provided by Dr. M. Dorf, Harvard Medical School.

*Antigens.* The random copolymers L-glutamic acid$^{60}$-L-alanine$^{30}$-L-tyrosine$^{10}$ (GAT) (Vega Biochemicals, Tucson, AZ) mol wt 30–50,000; L-glutamic acid$^{50}$-L-tyrosine$^{50}$ (GT) mol wt ~130,000 (Lot 9) or 55,300 (Lot 10); L-glutamic acid$^{60}$-L-alanine$^{40}$ (GA) mol wt ~360,000; L-glutamic acid$^{60}$-L-lysine$^{40}$ (GL) mol wt ~40,000 (Miles Laboratories Inc., Elkhart, IN) were dissolved in 1% bicarbonate-buffered saline and subsequently adjusted to pH 7.0–7.4. Ovalbumin (OVA) (Miles Laboratories Inc.) and keyhole limpet hemocyanin (KLH) (Calbiochem-Behring Corp., San Diego, CA) were dissolved in phosphate-buffered saline, pH 7.2–7.4.

*T Cell Hybridomas.* T cell hybridomas were derived as previously described (30). Briefly, in vivo primed, in vitro restimulated T cell blasts were fused via polyethylene glycol to the azaguanine-resistant AKR thymoma BW5147 and hybrids were selected by culture with hypoxanthine, aminopterin, and thymidine. Ag-specific, I region-restricted hybrids were identified on the basis of their ability to produce interleukin 2 (IL-2) upon antigenic stimulation as described by Kappler et al. (22) and as detailed below. The T cell hybridomas used in this report are shown in Table I. All hybrids were passaged in vitro in Dulbecco's modified Eagle's media (Gibco Laboratories, Grand Island, NY) with 4.5 g glucose per liter, 10% heat-inactivated fetal calf serum, and antibiotics.

[2] Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institutes of Laboratory Animal Resources, National Research Council (DHEW publication No. (NIH) 78-23, revised 1978).

*Monoclonal Antibodies.* Monoclonal antibody from the hybridomas MKD6 (22) (α-I-Ad, a gift from Dr. J. Kappler and Dr. P. Marrack, National Jewish Hospital, Denver, CO) and 10.2.16 (31) (α-I-$A^k$, made available by Dr. L. A. Herzenberg, Stanford University, Stanford, CA) was purified from culture supernatant by affinity chromatography on protein A-Sepharose. Culture supernatant from HO2.2ADH4 (32) (αLyt 2.2, made available by Dr. P. D. Gottlieb, University of Texas, Austin, TX) and purified HO13.4 (33) (α-Thy-1.2, made available by Dr. M. Gefter, Massachusetts Institute of Technology, Cambridge, MA) were prepared as previously described (34).

*Cell Culture.* T cell hybridomas were stimulated in microculture as described by Kappler et al. (22). An optimal number of T cell hybrids ($2.5-10 \times 10^4$) were cultured in duplicate or triplicate in the presence or absence of Ag and/or accessory cells, in 200 μl of RPMI 1640 (M. A. Bioproducts, Walkersville, MD) supplemented as previously described (34) in flat-bottomed microtiter wells (Falcon 3072; Falcon Labware, Becton, Dickinson & Co., Oxnard, CA) for 20–26 h at 37°C. Spleen cells were exposed to 1660 rad gamma irradiation and used as a source of accessory cells. In some experiments the accessory cells were depleted of T cells by exposure to 400 μg/ml of ammonium sulfate-purified monoclonal α-Thy-1.2 (HO 13.4) and 1 ml α-Lyt-2.2 culture supernatant (HO 2.2 ADH4) per $10^8$ cells for 30 min at 0°C followed by incubation with 1 ml of a 1:6 dilution of rabbit complement (Low Tox; Accurate Chemical & Scientific Corp., Westbury, NY) at 37°C for 30 min. Where indicated, the cloned, in vitro passaged, Ia-positive A-20 B lymphoblastoid line was used for a source of clonal accessory cells. This BALB/c cell line was originally isolated by Dr. Richard Asofsky (35) and has been shown to be a potent source of Ag-presenting cells (36, 37); it was kindly provided by Dr. J. Kappler and Dr. P. Marrack (National Jewish Hospital).

Antigen pulsing was accomplished by incubating $2 \times 10^7$ spleen cells/ml in $17 \times 100$-mm Falcon tubes or $10^6$ A-20 cells/ml in macrowells with the indicated concentration of Ag in complete RPMI media for the indicated time at 37°C. Subsequently, the Ag-pulsed cells were washed three times and the splenocytes were irradiated. Preparation of trinitrophenyl (TNP)-pulsed accessory cells was performed as previously described (38). The number of accessory cells added to culture is shown in the respective experimental protocols.

*IL-2 Assay.* T cell hybridoma activation was determined by measurement of the lymphokine IL-2, as modified from the methods of Kappler et al. (22) and Gillis et al. (39) as previously described (34). Briefly, 100 μl of culture supernatant was harvested, exposed to 8,000 rad gamma irradiation and in some cases diluted with culture media. IL-2 content was measured by adding $5 \times 10^3$ IL-2-dependent cells (L2.2 [34] or HT2 [22]) and incubating for 20–26 h with 1 μCi of tritiated thymidine added over the last 4–6 h. Cultures were harvested on glass fiber filter strips with the aid of a semi-automated cell harvester (PHD cell harvester; Cambridge Technology Inc., Cambridge, MA) and the incorporation of label into DNA determined by scintillation counting. Except where noted, the concentration of IL-2 measured in the culture supernatant was limiting.

## Results

*Specificity of MHC-restricted GAT-specific T Cell Hybridomas.* Our laboratory has recently derived a series of MHC-restricted T cell hybridomas with specificity for the Ir gene-controlled terpolymer GAT by fusing Ag-stimulated proliferating T cell blasts with the AKR thymoma, BW5147, as modified from Kappler et al. (22, 30). As we have previously shown and as is illustrated for a representative hybrid in Table II, experiment 1, the hybrids can be activated to produce the lymphokine IL-2 (T cell growth factor) after stimulation with GAT and syngeneic irradiated spleen cells as a source of accessory cells. No IL-2 is produced constitutively or by antigenic stimulation alone. As further illustrated in Table II, experiments 2 and 3, the T cell hybrid/accessory cell interaction is H-2-restricted and is inhibited by monoclonal α-I-A antibody, as we have previously described (30).

We have recently shown that unmodified Ia determinants can make a substantial

TABLE II

*MHC and Nominal Antigen Specificity of a Representative BALB/c × BW5147 GAT-selected T Cell Hybrid*

| Experiment | Hybrid | Accessory Cell* | | Monoclonal antibody‡ | Antigen§ | CPM ± SEM‖ |
|---|---|---|---|---|---|---|
| | | Strain | H-2 | | | |
| 1 | RF9.140 | — | — | — | — | 148 ± 18 |
| | | — | — | — | GAT | 238 ± 14 |
| | | BALB/c | H-2$^d$ | — | — | 183 ± 16 |
| | | BALB/c | H-2$^d$ | — | GAT | 27,973 ± 415 |
| | | D2GD | H-2$^{g2}$ | — | — | 313 ± 43 |
| | | D2GD | H-2$^{g2}$ | — | GAT | 37,637 ± 610 |
| 2 | RF9.140 | BALB/c | H-2$^d$ | — | — | 262 ± 47 |
| | | BALB/c | H-2$^d$ | — | GAT | 22,728 ± 636 |
| | | C57BL/10 | H-2$^b$ | — | — | 247 ± 10 |
| | | C57BL/10 | H-2$^b$ | — | GAT | 395 ± 50 |
| 3 | RF9.140 | BALB/c | H-2$^d$ | — | — | 626 ± 49 |
| | | BALB/c | H-2$^d$ | — | GAT | 15,516 ± 376 |
| | | BALB/c | H-2$^d$ | α-I-A$^d$ | GAT | 898 ± 88 |
| | | BALB/c | H-2$^d$ | α-I-A$^k$ | GAT | 15,756 ± 18 |
| 4 | RF9.140 | BALB/c | H-2$^d$ | — | GAT | 19,919 |
| | | BALB/c | H-2$^d$ | — | GA | 928 |
| | | BALB/c | H-2$^d$ | — | GT | 1,155 |
| | | BALB/c | H-2$^d$ | — | GA + GAT | 21,872 |
| | | BALB/c | H-2$^d$ | — | GT + GAT | 1,962 |
| | — | — | — | — | — | 1,486 |

Microcultures were prepared with 5 × $10^4$ cell hybrids with or without the indicated additions in a final volume of 0.2 ml and cultured at 37°C for 20–26 h after which time supernatant was removed, x-irradiated, and tested for IL-2 content as described in Materials and Methods.

* $10^6$ x-irradiated splenocytes from the indicated strains were used as the source of accessory cells.

‡ 25 μg/ml final concentration of affinity-purified monoclonal antibody from the MKD6 (α-I-A$^d$) or 10.2.16 (α-I-A$^k$) cell lines.

§500 μg/ml final concentration for each antigen.

‖CPM from an IL-2 assay on culture supernatant expressed as the arithmetic mean ± SE for triplicate cultures. Experiment 4 was performed in duplicate so SE are not given.

contribution to the triggering of some Ag-specific T cell hybridomas (30). We were interested in further exploring the role of nominal antigenic determinants in the interaction with Ia molecules and the T cell receptor(s). Thus we tested the effect of the structurally related copolymers GT and GA on the activation of GAT-specific, I region-restricted T cell hybridomas. As is shown in Table II, experiment 4, neither of these structurally related copolymers can activate the RF9.140 GAT-specific hybridoma. However, we made the striking observation that inclusion of the nonresponder copolymer GT in cultures of RF9.140 with GAT and syngeneic accessory cells markedly inhibited the T cell hybridomas' activation.

*Specificity of the GT-mediated Inhibition of T Cell Hybrid Activation.* The GT-mediated inhibition of the GAT-specific T cell hybridomas' activation is reversible, as shown in Fig. 1 for two GAT-specific I-A$^d$-restricted T cell hybridomas. Thus under conditions where, relative to GT, GAT is equimolar or in only slight molar excess, inhibition is complete. However, as the GAT concentration is increased, the GT-mediated inhibition is completely abrogated. This finding strongly argues against GT inhibition arising from nonspecific effects (e.g., toxicity) and indicates that GT appears to

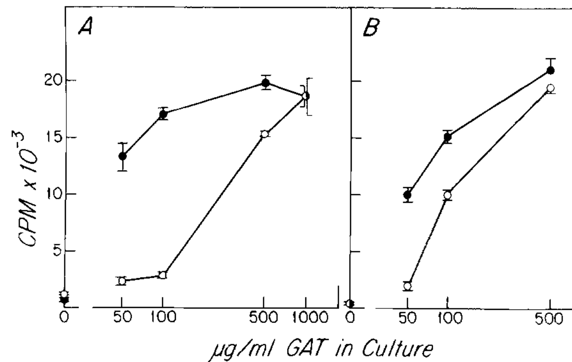


FIG. 1. Presence of GT in culture inhibits the activation of GAT-specific hybrids in a reversible manner. Microcultures were prepared with $5 \times 10^4$ T cell hybrids with $10^6$ x-irradiated splenocytes a source of APC, in the presence or absence of the indicated concentration of GAT, with (○) or without (●) 100 µg/ml of GT in a final volume of 0.2 ml. Supernatant was harvested after 24 h, x-irradiated and assayed for IL-2 content as described in Materials and Methods, and the results are presented as the arithmetic mean of triplicate cultures ± SEM. (A) Cultures with RF9.140, a BALB/c × BW5147 GAT hybrid and $BDF_1$ spleen cells. (B) Cultures with RF7.24.3, a BALB/c × BW5147 GAT hybrid with BALB/c spleen cells.

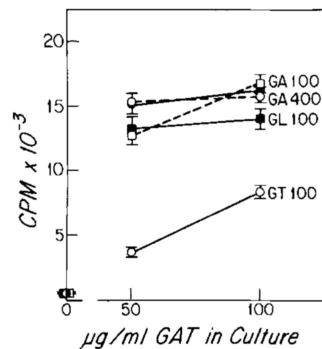


FIG. 2. Copolymer inhibition of GAT-specific hybrid activation is specific for GT. Microcultures were prepared as described in Fig. 1 with RF9.140 and BALB/c spleen, except that cultures were tested with the indicated concentration of GAT alone (●), or with or without GAT and the following copolymers: 100 µg/ml GT (—○—), 400 µg/ml GA (- -○- -), 100 µg/ml GA (□), or 100 µg/ml GL (■).

function as a competitive inhibitor. All GAT-specific I-$A^d$-restricted T cell hybrids tested thus far (four of four) have shown this same pattern (data not shown).

It is possible that inclusion of any amino acid copolymer in the hybridoma cultures could interfere with the T cell hybrid activation. Thus, to further define the specificity of inhibition, we tested the effect of the addition in culture of either the copolymer GA or GL. As can be seen in Fig. 2, neither GA nor GL appreciably interferes with the activation of the hybrid RF9.140 nor cross-reacts with GAT. This lack of inhibition is observed even if a fourfold higher concentration (µg/ml) of GA (Fig. 2) or GL (data not shown) is used. Experiments using the RF7.24.3 GAT-specific hybrid have confirmed these findings (data not shown). Thus the inhibition of the GAT response appears unique to the GT copolymer, and is unlikely to arise solely from general effects, e.g., related to the charge of the inhibitor.

The experiments presented thus far do not distinguish whether GT is a general

inhibitor or is in fact specific for the GAT response. GT might indeed interfere with some aspect of the T cell/accessory cell/Ag interaction irrespective of the particular nominal antigenic specificity. To examine this point we performed several experiments. First we verified that GT inhibition does not occur with hybridomas of an unrelated nominal antigenic specificity. GT does not inhibit I-$A^d$-restricted T cell hybridomas with specificity for ovalbumin (OVA) (Fig. 3 A; the DO11.10 hybrid was a kind gift of Kappler and Marrack), keyhole limpet hemocyanin (KLH) (Fig. 3 B; AODK10.4 also provided by Kappler and Marrack), and TNP (Fig. 3 C). In fact the responses are often higher when GT is added. As can be seen in Fig. 3, the responses of these hybrids are of lesser magnitude relative to GAT-specific hybrids; therefore, lack of inhibition cannot reflect a stronger, and hence less suppressible, response. In all cases parallel cultures with a GAT-specific hybrid showed inhibition by GT (data not shown). We next tested the effect of GT upon the activation of the hybrid RF10.14, a GAT-specific T cell hybridoma we recently described (29) with specificity for both unmodified I-$A^d$ and I-$A^d$ plus GAT, resulting in both autoreactive and Ag-specific activation (30). If the GT-mediated inhibition were unrelated to the nominal antigenic specificity, then it would be predicted that the RF10.14 hybrid should be completely inhibited. As can be seen in Fig. 4, the RF10.14.22 hybrid produces no IL-2 constitutively (group A) but is triggered partially by syngeneic accessory cells (group B) and maximally by syngeneic accessory cells with GAT (group D). We have previously determined that both the Ag-specific and autoreactive activation use the same I-$A^d$ molecule (30). If GT is included in culture, the Ag-specific activation is completely abrogated (compare groups D and E), but the autoreactive activation is not (compare groups B and C). This experiment indicates that GT specifically interferes with the Ag-specific portion of the response to GAT and is not generally inhibitory. The data also have implications for the mechanism of the GT inhibition that will be discussed below. Thus, the GT-mediated inhibition is highly specific for

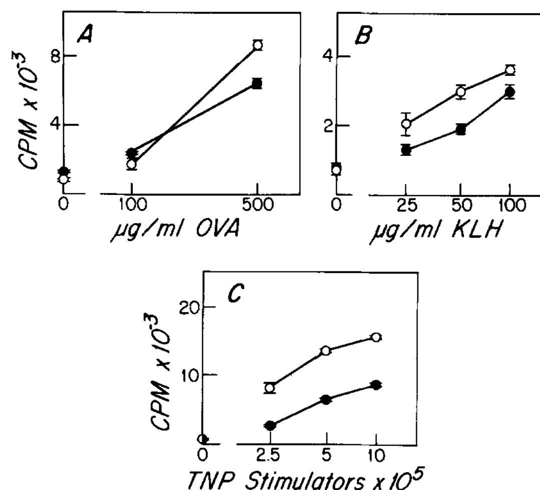


FIG. 3. GT does not inhibit T cell hybridomas of other nominal antigenic specificities. Microcultures were prepared with: (A) $10^5$ DO11.1, an I-$A^d$-restricted, OVA-specific hybrid; (B) $10^5$ AODK 10.4, an I-$A^d$-restricted, KLH-specific hybrid or (C) $10^5$ RF15.7, an I-$A^d$-restricted, TNP-specific hybrid, and the indicated soluble Ag- or TNP-coupled BALB/c splenocytes in the absence (●) or presence (○) of 100 µg/ml of GT, and handled as described in Fig. 1.

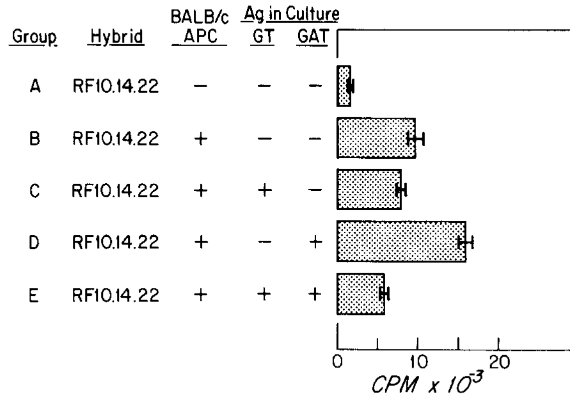


FIG. 4. GT inhibition is specific for the GAT-specific portion of the hybrid response. Microcultures were prepared as described in Fig. 1, except using $10^5$ hybrids, BALB/c spleen, with or without 500 μg/ml GAT and 100 μg/ml GT. RF10.14.22 is a clone of the RF10.14 BALB/c × BW5147 hybrid which is both autoreactive and Ag-specific (30).

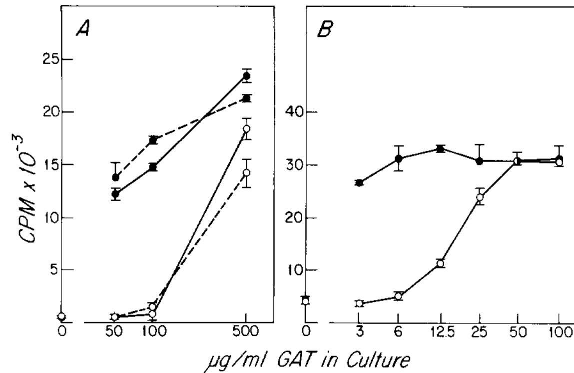


FIG. 5. T suppressor cells do not account for GT-mediated inhibition. Cultures were prepared with $5 \times 10^4$ RF7.24.3 cells as described in Fig. 1, except that in (A) x-irradiated BALB/c spleen cells were either untreated (——) or treated with α-θ, α-Lyt-2 + complement (- - -); cultures were constructed with these two splenocyte populations with (○) or without (●) 100 μg/ml GT; and in (B) $2 \times 10^4$ A20 tumor cells were substituted for splenic accessory cells; cultures were prepared with (○) or without (●) 100 μg/ml of GT.

the GAT response.

*GT Inhibition of GAT Responses Is Not Due to T Suppressor Cells.* The copolymer GT is a nonresponder Ag in all inbred strains of mice. The basis for nonresponsiveness in some strains (e.g., H-$2^d$) is due, at least in part, to the induction of suppressor T cells (40, 41). The possibility must be envisaged that GT induces suppressor T cells in the spleen cells used as a source of accessory cells, which then suppress the hybrid activation. This possibility is considered unlikely as the culture period is short and the splenocytes are irradiated. However, to formally exclude this mechanism, we depleted the accessory cell population of T cells by treatment with both monoclonal α-Thy-1.2 and α-Lyt-2.2 antibody and complement. As can be seen in Fig. 5A, GT causes the same degree of inhibition whether or not the accessory cell population contains T cells. Additionally, a cloned source of accessory cells can be used in this system with

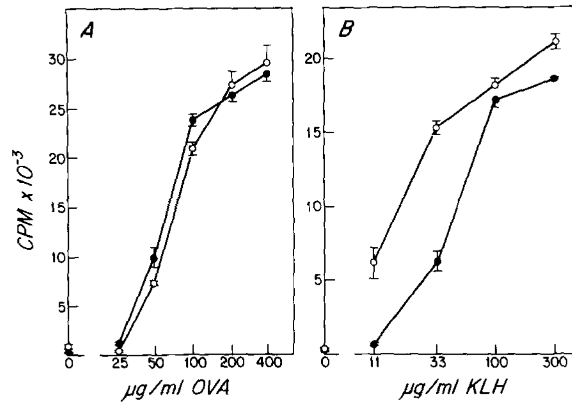


FIG. 6. GT does not inhibit hybrid responses to OVA or KLH with A20 APC. $10^5$ DO.11.10 hybrids (A) or $10^5$ AODK 10.4 hybrids (B) were cultured with $2 \times 10^4$ A20 cells and the indicated Ag concentrations in the absence (●) or presence (○) of 100 μg/ml of GT.

identical results. As recently described, Ia-positive B lymphoblastoid cell lines can function to present Ag to MHC-restricted T cells (36, 37). As shown in Fig. 5 B, the H-$2^d$ B lymphoblastoid cell line, A-20, is a potent APC in our system. Further inclusion of GT in the cultures of RF9.140 with A-20 cells and GAT completely inhibits this response, and the inhibition is reversed by high concentrations of GAT. As illustrated in Fig. 6, the GT inhibition of GAT cultures with A-20 cells is specific, as neither an OVA- (Fig. 6 A) or KLH- (Fig. 6 B) specific response is inhibited by the presence of GT in culture. Additionally, mixing experiments with an OVA-specific hybrid and a GAT-specific hybrid in the same culture with both relevent Ag shows no GT suppression of the OVA response (data not shown). These experiments rule out the possible role of a suppressor T cell or factor in this phenomenon.

*Localization of the Inhibitory Effect of GT.* The experiments presented thus far indicate that GT, but not other related copolymers, specifically inhibits the activation of GAT-specific T cell hybridomas, and that T suppressor cells are not involved. However, the locus of the inhibition is not defined. GT may compete with GAT either at the level of the APC or at the responding T cell. To distinguish between these alternatives, experiments were performed where either the accessory cells or T cell hybridomas were preincubated with GT and then tested for functional ability. As shown in Fig. 7 A, if BALB/c accessory cells are pulsed with GAT for 18 h and subsequently washed to remove free Ag, they will function to activate RF9.140. However, if GT is present during the preincubation period, there is marked interference with effective Ag pulsing, which is partially reversed at high GAT concentration. In our work, Ag pulsing of splenocytes appears to be less efficient for subsequent presentation relative to the use of fresh splenocytes with soluble Ag. Thus the level of response under these conditions is smaller (compare Fig. 7 A with Figs. 1 and 2). We therefore repeated this experiment using the A-20 Ag-presenting B lymphoblastoid line. As shown in Fig. 7 B, preexposure of A-20 cells to GAT results in excellent Ag presentation. If however the A-20 cells are pulsed with both GAT and GT, the ability to present Ag is markedly inhibited, although high concentrations of GAT reverse this inhibition. This pattern is identical to that described above in experiments where GT was added directly to culture.

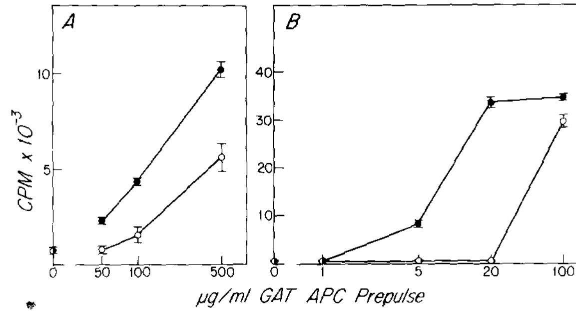


FIG. 7. GT inhibits Ag pulsing of APC. Microcultures were prepared with $5 \times 10^4$ RF9.140 cells as described in Fig. 1 except that all the Ag present in culture was introduced on Ag-prepulsed accessory cells. Accessory cell populations were exposed to the indicated concentration of GAT for ~18 h with (○) or without (●) 100 µg/ml of GT, washed extensively, and added to culture. (A) $10^6$ x-irradiated BALB/c Ag-pulsed splenocytes; (B) $2 \times 10^4$ Ag-pulsed A20 B lymphoblastoid cells. With the maximal response, the amount of IL-2 measured in this assay was not limiting (data not shown).

TABLE III

*Effect of Related Copolymers on Antigen Presentation by A20 Cells*

| Group | Hybrid | A-20 APC | APC antigen-pulsing conditions | | | | µg/ml Antigen in culture | CPM ± SEM |
|---|---|---|---|---|---|---|---|---|
| | | | 25 µg/ml GAT | | 100 µg/ml GT | 100 µg/ml GL | | |
| A | RF9.140 | + | − | | − | − | — | 568 ± 55 |
| B | RF9.140 | + | + | | − | − | — | 28,637 ± 1,201 |
| C | RF9.140 | + | + | | + | − | — | 637 ± 12 |
| D | RF9.140 | + | − | | + | − | — | 615 ± 19 |
| E | RF9.140 | + | + / − | Mixed | − / + | − | — | 31,287 ± 1,200 |
| F | RF9.140 | + | + | | − | + | — | 27,795 ± 234 |
| G | RF9.140 | + | − | | − | + | — | 416 ± 198 |
| H | RF9.140 | + | − | | − | − | 100 GAT | 42,451 ± 582 |
| I | RF9.140 | + | − | | + | − | 100 GAT | 37,468 ± 1,532 |
| J | RF9.140 | + | − | | − | − | 3 GAT | 13,371 ± 56 |
| K | RF9.140 | + | − | | + | − | 3 GAT | 2,803 ± 613 |
| L | RF15.7 | + | − | | − | − | — | 647 ± 69 |
| M | RF15.7 | + | − | | − | − | TNP 10 mM | 4,245 ± 486 |
| N | RF15.7 | + | − | | + | − | — | 550 ± 4 |
| O | RF15.7 | + | − | | + | − | TNP 10 mM | 4,013 ± 424 |

Microcultures were prepared with $5 \times 10^4$ (RF9.140) or $10^5$ (RF15.7) T cell hybrids and $2 \times 10^4$ Ag-pulsed or unpulsed A20 B lymphoblastoid cells as a clonal source of APC, with or without soluble GAT in 0.2 ml and handled as described in Table II. Group E contained $2 \times 10^4$ each of GAT-pulsed and GT-pulsed A20 cells.

The inhibition that is seen by preexposure of the accessory cell to GT is specific. As shown in Table III, pulsing A-20 cells with GT but not GL causes an inhibition of subsequent Ag presentation. Preexposure of A-20 cells solely to GT does not inactivate these cells since they are capable of presenting soluble GAT if high concentrations are used (Table III); such prepulsed cells efficiently present TNP to a TNP-specific hybrid (Table III). Also, GT does not inhibit KLH pulsing of A-20 APC (data not shown). If GT-prepulsed A-20 cells are tested at more limiting GAT concentrations, they are partially blocked in their ability to present GAT (Table III) indicating that preexposure to GT alone is sufficient to cause the inhibitory effect although it is more easily reversed.

The reciprocal experiment where the T cell hybridoma is preincubated with GT

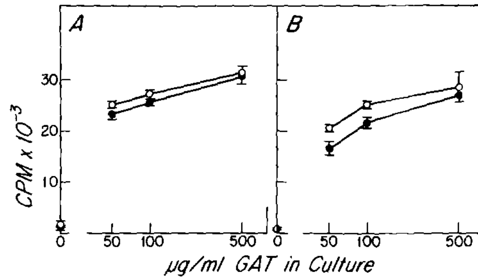


FIG. 8. T cell hybridomas are not sensitive to preexposure to GT. Microcultures were prepared with $10^6$ x-irradiated BALB/c splenocytes and (A) $5 \times 10^4$ RF9.140 or (B) $5 \times 10^4$ RF7.24.3 cells as described in Fig. 1, except hybrids were prepulsed with media (●) or 100 μg/ml GT (○) for 18 h at 37°C, washed, and tested in cultures.

TABLE IV

*APC Antigen-pulsing Conditions*

| Experiment | Hybrid | A20 APC | Antigen | GT in culture | CPM ± SEM |
|---|---|---|---|---|---|
| 1 | RF9.140 | + | — | − | 876 ± 96 |
| | | + | GAT | − | 13,160 ± 546 |
| | | + | GAT | + | 2,636 ± 152 |
| | AODK10.4 | + | — | − | 643 ± 112 |
| | | + | KLH | − | 2,305 ± 83 |
| | | + | KLH | + | 4,574 ± 93 |
| 2 | RF9.140 | + | — | − | 869 ± 58 |
| | | + | GAT | − | 19,318 ± 103 |
| | | + | GAT | + | 4,902 ± 733 |
| | DO11.10 | + | — | − | 789 ± 213 |
| | | + | OVA | − | 9,824 ± 729 |
| | | + | OVA | + | 8,794 ± 97 |

Microcultures were prepared with $5 \times 10^4$ (RF9.140) or $10^5$ (AODK10.4 and DO11.10) hybrids with $5 \times 10^4$ Ag-pulsed or unpulsed A20 cells and 100 μg/ml GT where indicated, and were otherwise handled as described in Table II. A20 cells were pulsed for 18 h with either 10 μg/ml GAT or 250 μg/ml KLH or OVA; all of which are limiting Ag concentrations for pulsing (data not shown).

for 18 h, washed, and subsequently tested in culture is shown in Fig. 8. Preincubation of the T cell hybrids does not cause inhibition. This indicates that the mechanism of inhibition is not caused by the interaction of free Ag with the T cell hybrids. Further, the inhibition that is seen by prepulsing the APC cannot be attributed to the paralysis of the T cell hybridoma from carry-over of antigen. These experiments indicate that GT preexposure of the accessory cell but not the T cell hybridoma is both necessary and sufficient to produce the GT-inhibitory effect.

We next tested the effect of GT upon GAT presentation by GAT-prepulsed APC. Thus, A20 cells were Ag-pulsed for 18 h, a sufficient time for Ag uptake and processing (unpublished data) and were then tested for their ability to present GAT in the absence or presence of GT in culture. As shown in Table IV, GT inhibits the activation of the T cell hybrid to GAT-prepulsed APC. This effect is specific, as KLH- or OVA-prepulsed APC are not inhibited by the addition of GT to culture (Table IV). The most efficient inhibition however occurs if GT is present at the time of GAT

pulsing (Fig. 7).

It is possible that inhibition of presentation at the level of the accessory cell could arise from either GT competition with GAT for association in the accessory cell or from GT independently associating with the accessory cell and subsequently competing for I-A plus GAT recognition by the T cell. According to the latter scheme, inhibition would result from a decrease in the number of appropriate receptor/ligand interactions or by imparting a negative signal to the T cell. As shown in Table III, if GT-pulsed accessory cells are mixed with GAT-pulsed accessory cells, no inhibition is seen. Since the cultures should contain the same number of "inappropriate ligands", if independent, it is unlikely that GT mediates its inhibition as a direct consequence of independent associative recognition. The experiment with the GAT-specific autoreactive T cell hybridoma described above (Fig. 4) also strongly argues against independent recognition of GT resulting in a negative signal to the T cell hybridoma. Thus the mechanism of GT-mediated inhibition of the GAT response appears to most likely reflect a competition of GT for GAT association at the APC.

*Relation of GT Inhibition to MHC-restriction Specificity: Effect of GT Upon Presentation by I-A$^{bxd}$ $F_1$ APC.* We have previously described T cell hybridomas with specificity for GAT in association with I-A$^b$ (30). Preliminary experiments revealed that several of these hybrids were not inhibited by GT using C57BL10 accessory cells (data not shown). We therefore tested the effect of GT upon both RF9.140 (restricted to I-A$^d$) and RF13.64 (restricted to I-A$^b$) using H-2$^d$ × H-2$^b$ $F_1$ accessory cells. As shown in Fig. 9, RF13.64 is not inhibited by the inclusion of GT in culture (B and D) while under identical conditions, the RF9.140 hybrid is inhibited (A and C). This experi-

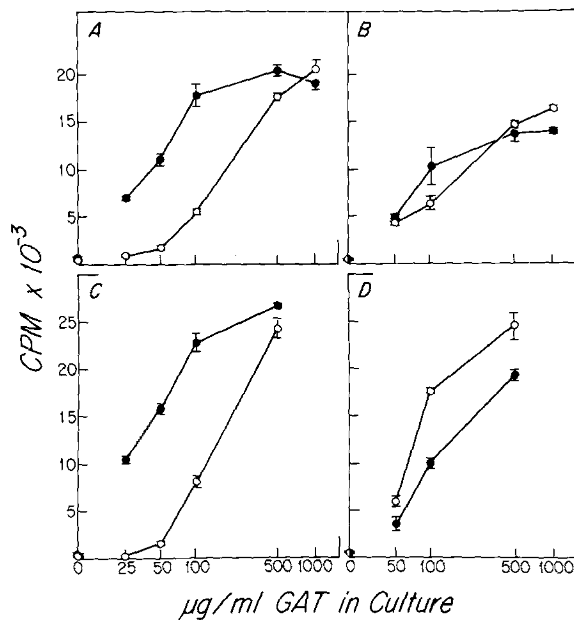


FIG. 9. GT inhibits an I-A$^d$-restricted but not I-A$^b$-restricted GAT hybrid. Microcultures were prepared as described in Fig. 1 with 5 × 10$^4$ RF9.140 (A and C) and 10$^5$ RF13.64 (B and D). Two representative experiments are shown: experiment 1 (A and B); Experiment 2 (C and D). Cultures were constructed with 10$^6$ x-irradiated CBF$_1$ splenocytes, with (○) or without (●) 100 μg/ml of GT. RF9.140 is a BALB/c × BW5147 hybrid and RF13.64 is a C57BL/10 × BW5147 hybrid.

ment strongly suggests that the GT-mediated inhibition cannot be accounted for by nonspecific inhibition of the accessory cells. Furthermore, GT is unlikely to exert its inhibition through a general interference with accessory cell Ag uptake or processing because if this occurred, the $F_1$ accessory cell should not be able to present GAT to RF13.64.

## Discussion

These experiments were undertaken to analyze the interaction of nominal Ag with accessory cells and/or the T cell receptor(s). We chose the Ag GAT and its related copolymers because they are simple Ag, composed of only two to three amino acids, and are under control of MHC-linked Ir genes. The model system selected was an Ag-specific, MHC-restricted T cell hybridoma/accessory cell interaction in which clonal populations of all interacting partners could be used. The major findings of this report are: (*a*) Multiple H-2-restricted GAT-specific T cell hybridomas fail to respond to the related copolymers GA and GT. (*b*) The nonresponder copolymer GT markedly inhibits the response of BALB/c, I-$A^d$-restricted, but not B10, I-$A^b$-restricted, GAT-specific T cell hybridomas to GAT plus accessory cells. (*c*) The GT-mediated inhibition of hybridoma activation is specific, only occurring with the GAT response. (*d*) Other structurally related, similarly charged copolymers fail to block the hybridoma response. (*e*) The GT-mediated inhibition occurs at the level of the APC. (*f*) The inhibition is reversible. (*g*) GT can inhibit presentation by GAT-prepulsed APC. (*h*) This specific competition suggests a specific interaction of Ag with APC. Initial experiments tested whether MHC-restricted T cell hybridomas with specificity for the terpolymer GAT would cross-react with either of the two GAT-related copolymers, GA or GT. A series of BALB/c-derived, GAT-specific, I-$A^d$-restricted hybrids were thus tested and no cross-reactions were detected. The data does not distinguish whether GT/GA nonresponsiveness occurs because the hybrids are specific for a GAT determinant or because they are specific for GA or GT but fail to respond because the simpler GAT-related copolymers are not presented appropriately. Whatever the case, this lack of cross-reaction allows the related copolymers to be tested for their potential to act as competitive inhibitors. Thus if either of the related copolymers competes for GAT association at the T cell's receptor(s) or for presentation by the accessory cell, then under limiting conditions it should inhibit the GAT-specific T cell hybrid's activation.

The potential for cross-inhibition in the hybrid system was tested by the addition of related copolymers to GAT cultures. Inclusion of GT in cultures of BALB/c GAT-specific T cell hybridomas markedly inhibited the response to GAT. This inhibition is not caused by an excess of amino acid copolymers in culture, as GAT is not inhibitory even at very high concentration (Fig. 1) and the inhibition can be reversed by increasing the concentration of GAT (Fig. 1). The inhibitory effect appears specific for the GT copolymer insofar as both GA and GL, even in excess, are without effect.

Several experiments indicate that the GT-mediated inhibitory effect is selective. Inhibition is seen with several BALB/c GAT-specific T cell hybridomas. These hybrids arose from three independent fusions and are therefore unlikely to represent an unusual or fortuitous phenotype. In contrast, hybrids with specificity for other protein Ag in association with I-$A^d$, namely OVA and KLH, are not inhibited by GT. In addition, two independently derived BALB/c TNP-specific hybrids (Fig. 3C

and data not shown) are not inhibited by inclusion of GT in culture. Furthermore, we tested a GAT-specific hybridoma that autoreacts to unmodified I-$A^d$ alone, albeit to a lesser degree, and found that only the nominal Ag-specific portion of the response was inhibited. Taken together these results show that GT inhibition of the BALB/c GAT hybridomas is highly specific for nominal antigenic activation of hybrids in association with I-$A^d$ on APC. The finding of specific competitive inhibition suggests that there is an inhibitable locus of specific Ag association.

To localize the site of inhibition, APC or hybrids were preincubated with Ag and tested for subsequent function. As expected, exposure of APC to GAT results in successful 'pulsing' for subsequent presentation. Successful pulsing is however, inhibited by GT. Higher concentrations of GAT reverse the inhibition, paralleling the findings above and arguing against toxic effects. Also GT does not interfere with KLH pulsing (data not shown) and GT-pulsed accessory cells are fully competent to present unrelated Ag, e.g., TNP (Table III) and OVA (data not shown). Additionally, GT-pulsed accessory cells are not inhibitory to separately GAT-pulsed cells (Table III). On the other hand, if the T cell hybridomas are prepulsed with GT, from 1 to 18 h before culture, no inhibition ensues. Taken together these findings localize the GT inhibitory effect to the APC.

The localization of inhibition to the level of the accessory cell suggests but does not establish that GT specifically competes for GAT association with the APC. The alternative, that GT independently associates with the accessory cell and interferes by creating an impotent or tolerizing independent determinant or even by the generation of suppressor cells is highly unlikely based on several experiments. First, co-culture of separately pulsed GT and GAT-APC should cause inhibition if inappropriate determinants interfere with the appropriate receptor/ligand interaction, which is not observed. Next if GT formed an independent toleragenic determinant, then the GAT-specific autoreactive hybrid, RF10.14, should be totally inhibited, which also was not observed. Finally, T suppressors were ruled out by negative selection and use of clonal APC, and nonspecific suppressive effects were not detected in mixing experiments.

All BALB/c-derived, GAT-specific T cell hybridomas thus far examined (four of four, from three independent fusions) demonstrate the phenomenon of GT-mediated inhibition (Figs. 1, 4, and data not shown). It is possible however, that some noninhibitable clones might be found as larger numbers of hybrids are examined. In contrast, preliminary studies of several B10-derived GAT-specific hybrids suggest that most of these (four out of five) are not inhibited by GT (work in progress). One of these B10 hybrids was tested with I-$A^{bxd}$ $F_1$ APC and showed no GT inhibition whereas parallel cultures with a BALB/c hybrid were inhibited (Fig. 9). The mechanism underlying this differential effect of GT on these two hybrids is not defined by the present data. One possibility is that the selective inhibition relates to the restriction element recognized by the T cell hybridoma. Thus, it is possible that the locus of inhibition is a site associated with the APC's I-A molecule and that the I-$A^b$-associated site interacts with GAT via a different determinant or with different affinity than the I-$A^d$-related site. Models have previously been proposed that postulate such specific Ag association with Ia molecules (23, 29). According to this scheme, the finding of an I-$A^b$-restricted hybrid that is inhibited by GT would suggest that a particular I-A molecule could have more than one distinct Ag-association site. An alternative possibility is that the differential inhibitory effect reflects differences in the respective

T cell hybridomas. Thus, noninhibited hybrids of B10 origin might differ in their specificity, e.g., they might be of higher affinity. It should be noted however, that the Ag dose-response curve is similar between the two sets of hybrids (Fig. 9). The data does not distinguish among these possibilities and subsequent experiments will focus on these issues.

The precise site of specific association and competition at the APC has not been identified. For successful presentation, an APC must acquire Ag and further may need to 'process' it (42–44). Additionally, there may be an interaction between nominal Ag and MHC molecules as has been suggested from analysis of the MHC and Ir gene effects upon the fine specificity of T cell responses (23–26). GT could potentially compete with GAT at any or all of these steps. Whether MHC molecules participate in APC Ag uptake, processing or Ag orientation, or whether any of these steps are linked, is unknown. The experiment using I-$A^{bxd}$ $F_1$ APC however, suggests that GT is not simply competing for a general uptake/processing mechanism independent of MHC gene products; otherwise both I-$A^b$ and I-$A^d$ associative recognition should be inhibited. Furthermore, GT causes inhibition of presentation by GAT-prepulsed APC (Table IV). Since under these conditions, APC have taken up and processed Ag (as subsequent metabolic inhibition of such APC doesn't interfere with presentation; data not shown), the locus of GT inhibition is most likely not solely at either of these points. Taken together, the data are most consistent with a direct inhibition of GAT association at the level of presentation. We have previously proposed that Ag specifically associate with Ia molecules on APC (29). The data in this report support this hypothesis but do not directly demonstrate such an association. The ability to inhibit presentation by GAT-prepulsed APC would suggest that the association is noncovalent.

This capacity of accessory cells to specifically associate with an Ag should occur through an Ag-recognition site that is not clonally distributed, in contrast to the lymphocyte (45), because a clonal source of APC can present multiple Ag. We speculate that other structurally distinct Ag use other recognition sites, a notion that is supported by recent studies of antigenic competition in the guinea pig (46). The methods described in this paper may serve as a general approach to define the nature of such other sites. As a practical consideration, the ability of related Ag to compete for association should depend on their relative affinity. In our system GT appears to be a very efficient inhibitor giving complete inhibition at less than equimolar ratios and furthermore is effective even if prepulsed. It is possible that GT associates more avidly, although this is speculative. Since GT is nevertheless a nonresponder Ag, nonresponsiveness may occur in spite of molecular interactions between Ia and Ag. Such nonresponsiveness may then result from the inability of the T cell receptor to properly interact with the complex or its components.

## Summary

The interaction of nominal Ag with major histocompatibility complex (MHC)-restricted T cells and accessory cells was studied by analyzing the effect of structurally related antigens on the response of antigen-specific MHC-restricted T cell hybridomas. The copolymer L-glutamic $acid^{50}$-L-$tyrosine^{50}$ (GT) completely inhibits the response of L-glutamic $acid^{60}$-L-$alanine^{40}$-L-$tyrosine^{10}$ (GAT)-specific, I-$A^d$-restricted T cell hybridomas to GAT plus accessory cells. This inhibition is specific, as hybridomas of

other specificities are not inhibited under identical conditions, and is unique to the GT antigen, as other similar copolymers are not inhibitory. The inhibitory effect is reversible by adding increasing amounts of GAT. Antigen-pulsing experiments localized the inhibition to the level of antigen-presenting cell (APC). GT-prepulsed APC are not inhibitory in cell-mixing experiments and can present other antigens. GT only inhibits the nominal antigen-directed component of a GAT-specific, autoreactive hybrid's response. Together these findings suggest that GT causes inhibition by competing for GAT association at the accessory cell. GT interferes with GAT presentation by an I-$A^{dxb}$ $F_1$ APC to a BALB/c, I-$A^d$-restricted, but not B10, I-$A^b$-restricted, T cell hybridoma, and GT inhibits presentation by GAT-prepulsed APC. The implications of these findings for MHC-restricted presentation of antigen are discussed.

The authors are especially grateful to Dr. John Kappler and Dr. Phillipa Marrack for their generous gifts of the DO11.10, AODK 10.4, and MKD6 hybridomas. We thank Dr. Ethan Shevach for helpful discussion. We wish to thank Drs. L. A. Herzenberg, P. D. Gottlieb, M. Gefter, and R. Asofsky for making available the 10.2.16, HO2.2.ADH4, HO13.4, and A20 cell lines, respectively. We thank Dr. Martin E. Dorf for mice and the gift of GL antigen, and Dr. Shyr-Te Ju for GA antigen. We acknowledge the help of Dr. Man-Sun Sy in the creation of the RF15.7 hybrid. The superb technical assistance of Mary Carol Mellen is gratefully acknowledged. We thank Mrs. Nancy Axelrod and Mrs. Mary Jane Tawa for preparation of this manuscript.

*Received for publication 11 January 1983.*

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