THE ROLE OF Ia MOLECULES IN THE ACTIVATION OF T LYMPHOCYTES

III. Antigen-specific, Ia-restricted, Interleukin 2-producing T Cell Hybridomas with Detectable Affinity for the Restricting I-A Molecule*

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It is well established that most T cells recognize nominal antigens in association with self-major histocompatibility complex (MHC) molecules (1, 2). Despite intense investigation, several aspects of this phenomenon are not understood. The nature of the T cell's receptor(s), its corresponding ligand(s), and their interactions is imprecisely understood. Although MHC molecules, e.g., Ia antigens, are known to be critical for T cell activation, it is still unproven whether unmodified Ia determinants are directly recognized by the T cell receptor(s). Some theories (3), in the extreme, postulate no direct Ia recognition. Alternatively, some theories (4) postulate that both MHC and nominal antigen determinants are jointly recognized as a neoantigenic determinants distinct from either native molecule. It is also unclear how the T cell repertoire with specificity for self-Ia plus antigen is generated and selected. It has been postulated that developing T cells with high affinity for self are mutated and/or deleted with selection for cells with low affinity for self which then require restricted recognition of antigen in the context of self-MHC antigens for activation (5). The range of affinity for unmodified self that is not eliminated and thus represented in the repertoire is unknown. In this report we describe the properties of L-glutamic acid\textsuperscript{6},L-alanine\textsuperscript{9},L-tyrosine\textsuperscript{10} (GAT) plus Ia-restricted T cell hybridomas that show variable reactivities with the appropriate Ia in the absence of antigen. The implications of these findings for several of the questions posed above are discussed.

Materials and Methods

Mice. BALB/c mice ages 6-10 wk were purchased from Charles River Laboratories, Inc., Wilmington, MA, C57BL/10, C57BL/6, and A/J mice ages 6-10 wk were purchased from The Jackson Laboratory, Bar Harbor, ME. D2GD and C3HLG mice were kindly provided by Dr. Martin Dorf, Harvard Medical School.

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 Institute of Laboratory Animal Resources, National Research Council (DHEW publication [NIH] 78-23, revised 1978).

T Cell Hybridomas. T cell hybridomas were derived as previously described (6). Briefly, nylon wool-nonadherent lymph node cells from GAT/complete Freunds adjuvant footpad-

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primed mice were stimulated with 100 μg/ml GAT in 24-well plates. T cell blasts, usually 7 d post-stimulation, were isolated by centrifugation on Ficoll-hypaque gradients and fused to BW5147 azaguanine-resistant tumor cells at a ratio of 4:1 using 40% polyethylene glycol. Hybrids were selected with hypoxanthine, aminopterin, thymidine media and in all cases arose at clonal frequency.

**Hybrid Stimulation and Interleukin 2 (IL-2) Assay.** Hybrids were stimulated under the conditions described by Kappler et al. (7). Briefly, the indicated number of hybrids was cultured with or without 500 μg/ml GAT, in the presence or absence of 10^6 irradiated spleen cells, as a source of antigen-presenting cells, for 20–26 h at 37°C in 96-well flat-bottomed microtiter trays. Culture medium was RPMI 1640 supplemented as previously described (6). The presence of IL-2 in the culture supernatant was assayed with the L2.2 cell line as previously described (6) or 5 × 10^5 HT-2 cells (7, derived by Dr. J. Watson (University of Auckland, Auckland, New Zealand), kindly provided by Dr. J. Kappler and Dr. P. Marrack (both of the National Jewish Hospital, Denver, CO)). Data is expressed as the mean counts per minute of duplicate or triplicate cultures.

**Monoclonal αIA Antibodies.** Culture supernatants from the hybridomas MKD6 (7, αIAd provided by Dr. J. Kappler and Dr. P. Marrack) and 3JP (αIAb, a gift of Dr. C. Janeway (Yale University, New Haven CT)) were prepared as previously described (6) and added to cultures at a final concentration of 25%.

**Results and Discussion**

T cell hybridomas were derived from the fusion of T cell blasts, selected for proliferation to the terpolymer GAT, to BW5147, and screened for the ability to produce IL-2 in response to GAT in the presence of the appropriate accessory cells. Initial fusions were derived from BALB/c (H-2d) responding cells. As shown in Table I, two antigen (Ag)-specific phenotypes were identified. The first phenotype, which is unique, is illustrated by the hybrid RF10.14, which displays specificity for syngeneic accessory cells alone, i.e., is triggered partially in the absence of GAT, although it is still GAT-specific, as maximal triggering occurs with both GAT and syngeneic accessory cells. As can be seen, IL-2 is not produced constitutively or in response to antigen without accessory cells. The genetics of the hybrid accessory cell interaction were analyzed using a series of inbred and recombinant inbred mouse strains as donors for accessory cells. The results in Table I show that factor production stimulated either with or without Ag is H-2 restricted (BALB/c vs. B10) and maps to the I-A-B subregion (BALB/c and D2GD vs. C3HLG and AJ). There is no evidence of alloreactivity against the inappropriate presenting cells (IAb^k^k^f^). The other phenotype that was identified is illustrated by the hybrid RF7.24.3 (Table I) and is conventional, i.e., demonstrating reactivity only to Ag with accessory cells but to neither stimulus alone. As also can be seen in Table I, this hybrid displays the same genetics as is expected for this immune response gene-controlled polymer.

To further document the specificity of the hybrid for self IA and self I-A plus nominal Ag, blocking studies were performed with monoclonal αIA antibodies. As shown in Table II, both the autoreactive (RF10.14 + accessory cells) and GAT-specific responses (RF10.14 + accessory cells + GAT) are inhibited by an αIAd monoclonal antibody (Mab) but not αIAb. The nonautoreactive hybrid, RF7.24.3, also showed αIAd-specific blocking. Reciprocal controls are shown with two representative hybrids from a C57BL/10 α GAT by BW5147 fusion that also illustrate both patterns of reactivity. Thus, the Ag-specific yet autoreactive hybrid (RF13.27) and the Ag-specific nonautoreactive hybrid (RF13.64) are both blocked by the αIAb but not αIAd. Control experiments with these Mab, F1-presenting cells, and the GAT
**Representative Phenotypes of Ag-specific I Region-restricted T Cell Hybridomas**

| Hybrid   | Accessory cell | K | A | B | J | E | C | S | G | D | GAT | cpm |
|----------|----------------|---|---|---|---|---|---|---|---|---|-----|-----|
| RF10.14  |                |   |   |   |   |   |   |   |   |   |     |     |
| BALB/c   | d              | d | d | d | d | d | d | d | d | d | 2,766|
| A/J      | k              | k | k | k | k | k | d | d | d | d | 5,766|
| C57BL/6  | b              | b | b | b | b | b | b | b | b | b | 287  |
| C57BL/6  | b              | b | b | b | b | b | b | b | b | b | 195  |
| BALB/c   | d              | d | d | d | d | d | d | d | d | d | 121  |
| BALB/c   | d              | d | d | d | d | d | d | d | d | d | 2,766 |
| D2GD     | d              | d | d | d | d | b | b | b | b | b | 20,424 |
| D2GD     | d              | d | d | d | b | b | b | b | b | b | 7,245 |
| C3H-LG   | d              | f |   |   |   |   |   |   |   |   | 164  |
| C3H-LG   | d              | f |   |   |   |   |   |   |   |   | 392  |
| RF7.24.3 |                |   |   |   |   |   |   |   |   |   |     |     |
| BALB/c   | d              | d | d | d | d | d | d | d | d | d | 13,930|
| BALB/c   | d              | d | d | d | d | d | d | d | d | d | 24,024|
| D2GD     | d              | d | d | b | b | b | b | b | b | b | 458  |
| D2GD     | d              | d | d | b | b | b | b | b | b | b | 7,245 |
| C3H-LG   | d              | f |   |   |   |   |   |   |   |   | 40,449|
| C3H-LG   | d              | f |   |   |   |   |   |   |   |   | 298  |

Microcultures were prepared with the indicated combinations of $10^6$ x-irradiated splenocytes, 500 μg/ml GAT and $10^6$ (RF10.14) or $2.5 \times 10^5$ (RF7.24.3) hybrids. Supernatants were harvested after 24 h and assayed for IL-2 content. In the absence of hybrids, no measurable IL-2 is induced by splenocytes with or without antigen (data not shown).

**Blocking of Antigen-specific and Autoreactive T Cell Hybrids with Monoclonal αIA Antibodies**

| Hybrid   | Accessory Cell | H-2 | Mab   | GAT | CPM |
|----------|----------------|-----|-------|-----|-----|
| RF10.14  |                |     |       |     |     |
| BALB/c   | H-2^d          | —   | —     | —   | 607 |
| BALB/c   | H-2^d          | —   | +     | +   | 27,447|
| BALB/c   | H-2^a          | —   | +     | —   | 406 |
| BALB/c   | H-2^a          | —   | —     | 658 |
| BALB/c   | H-2^a          | —   | —     | 910 |
| BALB/c   | H-2^a          | —   | —     | 28,670|
| RF13.27  |                |     |       |     |     |
| B10      | H-2^a          | —   | —     | —   | 11,677|
| B10      | H-2^a          | —   | +     | —   | 28,670|
| B10      | H-2^a          | —   | —     | 379 |
| B10      | H-2^a          | —   | —     | 829 |
| B10      | H-2^a          | —   | —     | 5,398|
| B10      | H-2^a          | —   | +     | 24,790|
| RF7.24.3 |                |     |       |     |     |
| BALB/c   | H-2^d          | —   | —     | —   | 694 |
| BALB/c   | H-2^d          | —   | +     | —   | 39,422|
| BALB/c   | H-2^a          | —   | —     | 458 |
| BALB/c   | H-2^a          | —   | —     | 9,599|
| BALB/c   | H-2^a          | —   | —     | 472 |
| BALB/c   | H-2^a          | —   | +     | 32,762|
| RF13.64  |                |     |       |     |     |
| B10      | H-2^a          | —   | —     | —   | 631 |
| B10      | H-2^a          | —   | +     | —   | 21,422|
| B10      | H-2^a          | —   | —     | 439 |
| B10      | H-2^a          | —   | —     | 754 |
| B10      | H-2^a          | —   | +     | 506 |

Conditions were identical to Table I using $10^6$ hybrids/culture and 25% MAb in the form of culture supernatant where indicated. The RF13 series hybrids were derived from a fusion with GAT-specific C57BL/10 T cell blasts.
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Table III

| Hybrid     | Accessory cell | Serum | GAT | cpm |
|------------|----------------|-------|-----|-----|
| RF10.14.36 | -              | -     | -   | 313 |
| BALB/c     | +              | -     | -   | 1,636 |
| BALB/c     | +              | +     |     | 4,892 |
| RF10.14.36 | -              | -     | -   | 567 |
| BALB/c     | -              | -     | -   | 2,718 |
| BALB/c     | -              |        | +   | 4,430 |

Culture conditions were as described in Table I except for the omission of fetal calf serum from the culture media where indicated. RF10.14.36 is a subclone of RF10.14 derived by limiting dilution.

hybridomas also showed specific blocking (data not shown). These findings confirm the results of the genetic mapping studies above and show that the same Ia molecule is critical for both the Ag-specific and autoreactive activation of these clones. Identification of both IA^d^ and IA^b^ autoreactive cells indicates that the phenomenon described above is not an isolated finding. The frequency of such cells in the repertoire is difficult to assess from our data as our screening was often visual (7) and initially directed towards “conventional” antigen-specific cells and further, the preselection for Ag-specific blasts might bias the frequency of clonal representation. However, the frequency of the Ag-specific autoreactive phenotype has been as high as 25% of the Ag-specific hybridomas in some fusions.

We have considered several other points to clarify whether the phenotype of Ag-specific yet autoreactive activation represents a cell with specificity, for both “modified” and unmodified Ia molecules. First, to ascertain whether the reactivity against syngeneic Ia with and without GAT was mediated by the same cell, RF10.14 was subcloned. RF10.14 was plated at 0.3 cells/well with 65% of the plated cells subsequently growing. All active clones demonstrated Ag-specific and autoreactive activation (Table III, and data not shown). Thus a single Ag-specific cell is triggered in the presence of syngeneic Ia without GAT being present. It is possible that the apparent autoreactivity of such hybrids is due to a cross-reactive recognition of proteins present in the culture media. As shown in Table III, the ability to stimulate IL-2 production by RF10.14 without Ag and in serum-free culture argues against such a possibility. Although we feel it is unlikely, we cannot formally rule out the possibility that an endogenous nonpolymorphic Ag, e.g., viral Ag, might serve this role.

These experiments strongly suggest that a single Ia-restricted Ag-specific cell can have detectable affinity for the restricting and unmodified Ia molecule. The implications of these findings depend whether the same portion of the Ia molecule is seen with or without Ag. Recognition of Ia with or without Ag is clearly blocked by the same Mab, but this is not necessarily discriminatory. Assuming that the sites are the same, then the data would strongly argue against theories where Ia molecules are not directly recognized by Ag-specific T lymphocytes. Although MHC-nominal Ag complex (neoantigenic) determinants may exist (8), our data argue that native Ia determinants may be recognized by some Ag-specific T cell clones. The fact that native Ia determinants are recognized, however, is compatible with either a distinct self receptor (dual receptors) or a single receptor model where Ia makes a substantial contribution to the receptor ligand interaction. It should be noted that to explain the
RF10.14 phenotype with a dual receptor model, one would have to postulate that partial triggering can occur by occupation of only one of the T cells receptors.

The final issue to be considered is how these findings relate to autoreactivity. If normal cells like the RF10.14 hybrid were detected without knowledge of their nominal antigen specificity, they would appear solely autoreactive. Such cells could account for the syngeneic mixed lymphocyte reaction where T cells are triggered to proliferate or produce IL-2 in response to self-Ia alone (9, 10). Our findings would then be analogous to the demonstration that the same clone can be specific for both allogeneic MHC antigens alone and self-MHC antigens plus nominal Ag (11). Thus, as is the case for alloreactivity, autoreactivity may not be a fundamentally distinct phenomenon but arises from those cells specific for nominal Ag plus MHC molecules. This is supported by studies (12, 13) at the population level, where it has been suggested from both positive and negative selection techniques that autoreactive and Ag-specific T cells are at least overlapping populations. A recent report characterizes solely autoreactive, non-Ag-specific T cell hybridomas produced from a fusion with insulin specific T cell blasts (14). An insulin-augmented, autoreactive phenotype was noted but not characterized. This latter cell type and possibly both autoreactive phenotypes (the former with an unknown nominal antigenic specificity) may represent the same phenomenon as described in this current report. The availability of cloned autoreactive T cell hybridomas should provide a tool to assess the functional significance of such cells and might permit comparisons of the affinities of T cell receptor(s) for self-Ia and self-Ia plus Ag.

Summary

Antigen-specific, I region-restricted, interleukin 2-producing T cell hybridomas were produced by fusing GAT-specific T cell blasts with BW5147. Two antigen-specific phenotypes were identified, one autoreactive and one nonautoreactive. All of the antigen-specific and autoreactive clones were H-2 restricted, mapping to the IA subregion by genetic analysis and monoclonal antibody inhibition. Both the antigen-specific and autoreactive stimulation are the property of a single cell and required no known exogenous antigens.

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