PRODUCTION OF AUTOREACTIVE I REGION-RESTRICTED T CELL HYBRIDOMAS

By LAURIE H. GLIMCHER AND ETHAN M. SHEVACH

From the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205

The syngeneic or autologous mixed leukocyte reaction (MLR) is the proliferation that occurs when T lymphocytes are co-cultured with Ia-positive autologous or syngeneic non-T cells. An autologous MLR was first described in mice (1) and has since been studied in human (2-4), murine (5, 6), and guinea pig (7) systems. Although the characteristics of both the responder and stimulator cell populations have been clarified, some of the central questions regarding the syngeneic MLR remain unanswered. Is anti-self reactivity the property of all antigen-specific T cells or is there a unique T cell subpopulation that responds to self-Ia molecules in the absence of foreign antigen? If the latter is correct, what is the physiologic role of a population of T cells that presumably possesses receptors for self I region-encoded antigens?

In the present report, we describe the initial characterization of several autoreactive T cell hybridomas derived from the fusion of (BALB/c × A/J)F1 [CAF1] lymphocytes with the BW 5147 tumor line. These lines secrete interleukin 2 (IL-2) in serum-free medium only when challenged with I-A\(^d\)-positive stimulator cells. The availability of large numbers of cloned autoreactive T cells should greatly facilitate further analysis of the role of this unique cell population in the regulation of the immune response.

Materials and Methods

Animals. B10.A and A/J strains were purchased from The Jackson Laboratory, Bar Harbor, ME. The B10.A(4R) strain was purchased from Simonsen Laboratories, Gilroy, CA, and the D2.GD strain was the kind gift of Dr. David Sachs (National Cancer Institute, NIH). BALB/c AnN, (BALB/c × A/J)F1 [CAF1], and B10.D2SnN strains were obtained from the Division of Research Services, NIH.

Production of T Cell Hybridomas. The procedure for the production of T cell hybridomas was similar to that described by Heber-Katz et al. (8) and Kappler et al. (9). Antigen-specific T cell blasts were prepared by culturing 4 × 10\(^6\) nylon wool-passed lymph node (LN) cells from CAF1 mice, which had been immunized in the footpads 7 d previously with 10 \(\mu\)g of pork insulin (PINS) (Sigma Chemical Co., St. Louis, MO) with 10\(^8\) \(\alpha\)-irradiated CAF1 spleen cells in 2 ml Eagle's Hank's amino acids (NIH media production) plus 10% fetal calf serum (FCS) and 10 \(\mu\)g/ml PINS. After 3 d of culture, cells were harvested, enriched for blasts on a Ficoll density gradient, and mixed with the hypoxanthine, aminopterine, thymidine (HAT)-sensitive T cell line BW 5147, an AKR thymoma obtained from the Salk Institute Cell Distribution Center (La Jolla, CA), in a ratio of 1:2. Fusion was carried out using 50% (vol/vol) polyethylene glycol 1,000 (Baker Chemical Co., Phillipsburg, NJ) for 8 min. 3 × 10\(^5\) cells were plated out into 96-well Costar plates (Costar, Data Packaging, Cambridge, MA) containing 7 × 10\(^3\) \(\alpha\)-irradiated (2,000 rad) peritoneal washout cells in 0.2 ml of fusion medium. The fusion medium was 1:1 EHAA:RPMI 1640 (Biofluids, Rockville, MD) containing 20% FCS, HAT, l-glutamine,
penicillin-streptomycin, 2-mercaptoethanol, and Hepes buffer. After 7 d, hybridomas were picked and transferred to 24-well Costar plates, expanded, and tested for antigen-specific IL-2 production.

Assay of T Cell Hybridomas for Activity. \( 1 \times 10^6 \) T hybridoma cells were co-cultured with \( 2.5 \times 10^5 \) CAF1 spleen cells in the presence or absence of antigen in 0.2 ml of fusion medium without HAT. In some experiments, Iscove's medium (10) was substituted for fusion medium. After 2 d of culture, supernatants were collected and assayed for IL-2 content in a secondary culture by using HT-2 cells, an IL-2-dependent T cell line developed by Dr. James Watson, University of Auckland, Auckland, New Zealand, and provided by Dr. Phillipa Marrack, National Jewish Hospital, Denver, CO. 2-4 \( \times 10^3 \) HT-2 cells were cultured for 24 h in the presence of 35% primary culture supernatant, and the degree of stimulation was measured by the incorporation of \([^{3}H] \)thymidine (6.8 Ci/ml; New England Nuclear, Boston, MA) into DNA.

B Cell-B Lymphoma Antigen-presenting Hybridomas. TA3 is an I-A\(^{b}\)/I-E\(^{d}\}-bearing B cell-B lymphoma hybridoma with potent I region-restricted stimulatory capability, prepared by Dr. T. Hamano (Laboratory of Microbial Immunity, NIH) by fusing lipopolysaccharide (LPS)-activated CAF1 B cells with a drug-marked variant of a BALB/c B lymphoma (see ref. 11).

Monoclonal Antibodies. The monoclonal antibodies 34-5-4 (\( \alpha I-\text{A}^{d} \)), 14-4-4 (\( \alpha I-E^{d,k} \)), and 17-3-3 (directed against the combinatorial Ia molecule, \( \beta_{\text{B cells}} \)) were the kind gift of Dr. David Sachs, NCI, NIH. The 10-2-16 monoclonal antibody (\( \alpha I-\text{A}^{k} \)) was the gift of Dr. Richard Hodes, NCI, NIH. The 34-5-4, 14-4-4, and 10-2-16 antibodies were in ascites form and were used at a dilution of 1:1,000 (vol/vol) in culture. The 17-3-3 antibody was a culture supernatant and was used at a dilution of 1:20 (vol/vol).

Results and Discussion

Our initial attempts at the production of autoreactive T cell hybridomas involved the fusion of blasts obtained from primary or secondary syngeneic MLR cultures with the BW 5147 line, using the methods described by Kappler et al. (9) and Heber-Katz et al. (8). No hybridomas specific for syngeneic stimulator cells could be isolated from such fusions. However, in the course of the production of antigen-specific T cell hybridomas from CAF1 mice specific for PINS, we noticed that a large percentage of these hybridomas responded to H-2-matched spleen cells in the absence of the priming antigen. 44 positive wells were randomly picked, expanded for testing, and of these 26 had activity. The specificity of a number of these T cell hybridomas is shown in Table I. None of these hybridomas produced IL-2 in the absence of stimulator cells or in the presence of insulin alone. 8 hybridomas (group III) responded to H-2-matched stimulator cells only in the presence of the priming antigen (PINS), 14 demonstrated an antigen-independent response (group I), and 4 (group II) exhibited a mixed response, i.e., they had some response to CAF1 spleen cells alone but gave a superior response to CAF1 spleen cells in the presence of PINS. Two hybridomas (B3 and B4), whose proliferative response was restricted to the BALB/c parental strain (Table I), were selected for further study and cloned by limiting dilution.

Studies (12) with heterogenous T cell populations have shown that antigens encoded in the I subregion of the MHC are the target antigens in the syngeneic MLR. We therefore used stimulators from parental H-2-recombinant strains to map the genes that encode the antigen required for stimulation of the B3 autoreactive hybridoma (Table II). Spleen cells from the CAF1, B10.D2, and D2.GD, all of which bear K\(^{d}\)/I-A\(^{d}\), elicited a good response, whereas cells from B10.A and B10.A(4R), which bear K\(^{b}\)/I-A\(^{k}\), were nonstimulatory. These results indicate that antigens encoded in the K region and/or the I-A subregion of the H-2\(^{d}\) haplotype are involved in stimulating the autoreactive hybridoma B3.

To further define the stimulatory antigen, we included selected monoclonal anti-Ia
reagents in the cultures (Table III). As a source of homogeneous stimulator cells in the blocking experiments, we used an I-A<sup>d</sup>/I-E<sup>d</sup>-bearing B cell-B lymphoma hybridoma, TA3 (identical results were obtained using CAF<sub>1</sub> or BALB/c spleen cells as stimulator cells). Addition of a monoclonal anti-I-A<sup>d</sup> antibody (34-5-4) decreased the production of IL-2 by two B3 reclones, B3/C4 and B3/C5, by >99%. Three other monoclonal reagents (10-2-16, 14-4-4 and 17-3-3) reactive with other specificities present on the TA3 cells did not inhibit the production of IL-2 by the autoreactive hybridomas. These three reagents did, however, appropriately inhibit the production of IL-2 by two other antigen-specific hybridomas, one specific for hen egg lysozyme in the context of I-A<sup>d</sup> (10-2-16) and the other specific for cytochrome c in the context of the combinatorial Ia molecule (14-4-4 and 17-3-3) (data not shown).

To rule out the possibility that the "autoreactive" hybridomas were recognizing FCS components, such as albumin or bovine insulin in association with syngeneic Ia antigens, experiments were performed using a modification of protein-free Iscove's

---

**Table I**

| Hyridoma number | <sup>[3H]Tdr Incorporation\* | CAF<sub>1</sub> spleen | CAF<sub>1</sub> spleen + PINS | A/J spleen | BALB spleen |
|-----------------|-------------------------------|------------------------|-----------------------------|------------|------------|
| Group I         |                               |                        |                             |            |            |
| A2              | 17,170                        | 19,218                 |                             |            |            |
| A13             | 17,070                        | 16,966                 |                             |            |            |
| A17             | 9,090                         | 10,024                 |                             |            |            |
| B3              | 39,419                        | 27,945                 | 844                         | 19,612     |            |
| B4              | 18,742                        | 18,613                 | 302                         | 10,641     |            |
| B17             | 34,720                        | 28,444                 |                             |            |            |
| Group II        |                               |                        |                             |            |            |
| A5              | 9,070                         | 16,496                 |                             |            |            |
| A12             | 12,597                        | 27,415                 |                             |            |            |
| A23             | 8,270                         | 18,539                 |                             |            |            |
| B18             | 14,852                        | 30,800                 |                             |            |            |
| Group III       |                               |                        |                             |            |            |
| A16             | 690                           | 15,653                 |                             |            |            |
| B14             | 809                           | 23,913                 |                             |            |            |

*<sup>1</sup> <sup>1</sup> x 10<sup>6</sup> T hybridoma cells were co-cultured with 2.5 x 10<sup>3</sup> spleen cells in the presence or absence of antigen in 0.2 ml of fusion medium without HAT. After 2 d of culture, supernatants were collected and assayed for IL-2 content in a secondary culture on HT-2 cells. 2-4 x 10<sup>8</sup> HT-2 cells were cultured for 24 h in the presence of 35% primary culture supernatant, and the degree of stimulation was measured by the incorporation of [3H]thymidine into DNA.

**Table II**

| Source of stimulator cells | MHC alleles | Proliferative response (cpm) |
|----------------------------|-------------|------------------------------|
| (BALB/c x A/J)<sub>F<sub>1</sub></sub> | dddddddd | 54,187                       |
| B10.D2                     | dddddddd   | 35,327                       |
| B10.A                      | kkkkkkkk   | 1,820                        |
| D2.GD                      | dbbhbhbb   | 47,414                       |
| B10.A(4R)                  | kbhbhbhb   | 1,001                        |

10<sup>6</sup> T hybridoma cells were co-cultured with 2.5 x 10<sup>3</sup> of the designated spleen cells, and the assay was performed as detailed in the legend to Table I.
TABLE III

Blocking Studies with Monoclonal Anti-Ia Antibodies Confirm That the Autoreactive Hybridomas Recognize Determinants in the I-A\(d\) Subregion

| Monoclonal antibody | Experiment 1 | Experiment 2 | Experiment 3 |
|---------------------|--------------|--------------|--------------|
|                     | B3/C5        | B3/C4        | B3/C5        |
| None                | 36,084       | 31,906       | 32,449       | 31,985       | 17,375       |
| 34:5-4(aI-A\(t\))   | 767          | 2,340        | 307          | 682          | 441          |
| 10-2-16(aI-A\(k\))  | 23,797       | 34,041       | —            | —            | —            |
| 14-4-4(aI-E\(\alpha\)) | 37,978       | 38,569       | 36,979       | 33,178       | 18,074       |
| 17-3-3(aI-Ad\(\beta\)) | 35,142       | 34,273       | 34,986       | 38,521       | 19,215       |

* B3/C5 and B3/C4 are reclones of the original autoreactive hybridoma B3. These hybrids are Ia negative, as determined by cytotoxicity with the above reagents. 10\(^5\) T hybridoma cells were co-cultured with 10\(^4\) irradiated (10,000 rad from a \(\beta\)cesium source) TA3 cells (see ref. 11) in the presence of the designated antibodies in Iscove’s medium prepared without exogenous bovine serum albumin or insulin. The remainder of the assay was performed as described in Table I.

TABLE IV

Two Autoreactive Hybridomas Respond to Self H-2\(^d\) Determinants in Serum-free, Insulin-free Medium

| Stimulator cell | \(^{[3}H\)-TdR incorporation |
|-----------------|-----------------------------|
|                 | B3                          | B4                          |
| Experiment I    |                             |                             |
| A/J             | 422                         | 761                         |
| CAF\(_I\)        | 61,407                      | 31,625                      |
| TA3             | 70,173                      | 30,658                      |
| Experiment II   |                             |                             |
| CAF\(_I\)        | 19,493                      | 31,149                      |
| TA3             | 27,835                      | 17,375                      |
| Experiment III  |                             |                             |
| TA3 (grown in Iscove’s medium) | 26,017 | 26,017 |

\(10^6\) hybridoma cells were co-cultured with the designated stimulator cells in modified Iscove’s medium (10) prepared without exogenous bovine serum albumin, insulin, or transferrin, and the supernatants were assayed for IL-2 activity as detailed in the legend to Table I. medium (10) prepared without bovine serum albumin, insulin, or transferrin. The results of two such experiments (Table IV) demonstrated that the induction of IL-2 production by the appropriate parental strain stimulator cells in two of the autoreactive hybridomas (B3 and B4) was unimpaired in the presence of such serum-free, transferrin-free, and insulin-free medium. To definitively rule out the possibility that foreign serum components that adsorbed to either the hybridoma or stimulator cell surface were responsible for activation of the hybridomas, both B3 and TA3 were grown in Iscove’s media for 48 h before use in the experiments, with no difference in the results obtained (Table IV, experiment 3). The addition of PINS or FCS to these cultures had no effect on the response obtained (not shown). Although it is possible that significant amounts of serum components remain cell associated after 48 h of culture in serum-free medium, we believe this is unlikely because a T cell hybridoma specific for FCS components cultured under these same conditions was inactive in the absence of added FCS.

In spite of the fact that the addition of PINS had no augmenting effect on IL-2 production by these autoreactive hybridomas, it is surprising that such hybridomas
could only be identified during the course of studies designed to produce PINS-specific, I region-restricted hybridomas. The possibility must, therefore, still be considered that some relationship exists between the autoreactive and antigen-specific T cell populations. First, it is intriguing to speculate that these cells bear antigen-specific receptors as well as high-affinity anti-self receptors. Indeed, recent work by Dos Reis and Shevach (13), which demonstrated direct activation of antigen-specific T cell clones by unmodified self-Ia antigens, suggests that some antigen-specific T cells can be directly activated in an antigen-independent manner by Ia-positive cells through their receptors for self-Ia molecules.

Alternatively, it is possible that the autoreactive T cell is directly activated by the antigen-specific T cell via an idiotypic-anti-idiotypic receptor network, although this possibility seems unlikely because the autoreactive cells can be activated by unmodified Ia antigens. Further studies to evaluate the relationship between autoreactive and antigen-specific T cells by using assays of T cell specificity, such as help or suppression of insulin-specific antibody production by B lymphocytes, are now in progress.

Summary

We produced autoreactive T cell hybridomas that can be induced to secrete interleukin 2 by co-culture with either syngeneic splenic stimulator cells or syngeneic Ia-positive B cell-B lymphoma hybridomas. These autoreactive hybridomas arose from the fusion of pork insulin-primed lymph node T cells with the AKR thymoma BW 5147 and occurred at a higher frequency than the expected insulin-specific hybridomas. Mapping studies using recombinant strains and blocking studies using monoclonal anti-Ia antibodies localized the stimulatory determinant to the I-A\(^{\text{d}}\) subregion of the major histocompatibility complex. These T cell hybridomas did not appear to be directed at any foreign antigen present in the culture system because activation occurred in serum-free, insulin-free medium (Iscove's medium). Such hybridomas should prove to be a potent tool in studying the biologic significance and function of the autoreactive response.

We thank Dr. Teruaki Hamano for the preparation of the TA3 hybridoma, Dr. Ira Green for reviewing the manuscript, Mrs. Christina Chan and Mr. Charles Hoes for excellent technical assistance, and Ms. Shirley Starnes for the preparation of this manuscript.

Received for publication 24 April 1982 and in revised form 1 June 1982.

References

1. Howe, M. L. 1973. Isogenic lymphocyte interaction responsiveness of murine thymocytes to self antigens. J. Immunol. 100:1090.
2. Opelz, G., M. Kiuchi, M. Takanuki, and P. I. Terasaki. 1975. Autologous stimulators of human lymphocyte subpopulations. J. Exp. Med. 142:1327.
3. Kurtz, M. M., J. B. Innes, and M. E. Weksler. 1976. Lymphocyte transformation induced by autologous cells. IV. Human T lymphocyte proliferation induced by autologous or allogeneic non-T lymphocytes. J. Exp. Med. 143:1042.
4. Haussmann, P. B., and J. D. Stobo. 1979. Specificity and function of a human autologous reactive cell. J. Exp. Med. 149:1337.
5. Smith, J. B., and R. D. Pasternak. 1978. Syngeneic mixed leukocyte reaction in mice: strain distribution, kinetics, participating cells, and absence in NZB mice. J. Immunol. 121:1809.
6. Nussenzweig, M. C., and R. M. Steinman. 1980. Contribution of dendritic cells to stimulation of the murine syngeneic mixed leukocyte reaction. J. Exp. Med. 151:1196.
7. Yamashita, U., and E. M. Shevach. 1980. The syngeneic mixed leukocyte reaction: the genetic requirements for the recognition of self resemble the requirements for the recognition of antigen in association with self. J. Immunol. 124:1773.
8. Heber-Katz, E., R. H. Schwartz, L. A. Matis, C. Hannum, T. Fairwell, E. Apella, and D. Hansburg. 1982. Contribution of antigen-presenting cell major histocompatibility complex gene products to the specificity of antigen-induced T cell activation. J. Exp. Med. 155:1086.
9. Kappler, J. W., B. Skidmore, J. White, and P. Marrack. 1981. Antigen-inducible, H-2-restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. J. Exp. Med. 153:1198.
10. Mosier, D. E. 1981. Primary in vitro antibody responses by purified murine B lymphocytes in serum-free defined medium. J. Immunol. 127:1490.
11. Glimcher, L., T. Hamano, R. Asofsky, E. Heber-Katz, S. Hedrick, R. Schwartz, and W. E. Paul. 1982. I Region-restricted antigen presentation by B cell-B lymphoma hybridomas. Nature (London). In press.
12. Glimcher, L. H., D. L. Longo, I. Green, and R. H. Schwartz. 1981. Murine syngeneic mixed lymphocyte response. 1. Target antigens are self Ia molecules. J. Exp. Med. 154:1652.
13. Dos Reis, G. A, and E. M. Shevach. 1981. The syngeneic mixed leukocyte reaction represents polyclonal activation of antigen-specific T lymphocytes with receptors for self Ia antigens. J. Immunol. 127:2456.