ANALYSIS OF THYMOCYTE MHC SPECIFICITY WITH THYMOCYTE HYBRIDOMAS

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The peripheral T cell repertoire has been extensively characterized. One of its outstanding features is the phenomenon of major histocompatibility complex (MHC) restriction. Foreign antigens are co-recognized by inducer T cells in association with self-Ia molecules (1, 2). This specificity is dominated by the genetically polymorphic portions of the Class II MHC gene products and is determined by the genotype of the thymic environment in which the T cells have matured (3, 4). Much less is known about the specificity of intrathymic T cells.

Our laboratory and several others have detected a T cell proliferative response specific for self-MHC in cultures of thymocytes (5–9). These responses require the interaction of thymocytes with Ia-bearing accessory cells. In our hands, thymocyte proliferation occurs spontaneously in the absence of exogenous factors, although it may be dependent on factors generated within the culture (6). Interestingly, all the components required for this response reside in a normal thymus. We have speculated that this in vitro response may reflect the commitment of T cell specificity to MHC gene products that occurs in the thymus. Thus far, all analyses of thymocyte specificity have been limited to the responses of heterogeneous cell populations. It is of interest to define the clonal specificity of the proliferating thymocytes in these cultures, which may reflect at least a portion of the intrathymic T cell repertoire directed to self-Ia. To this end, we generated thymocyte hybridomas from these in vitro cultures with somatic cell hybridization techniques. The present report analyzed the specificity of these hybrids, for self and allo MHC molecules, as well as for private and public Ia determinants.

Materials and Methods

Mice. BALB/c mice, ages 6–10 wk, were purchased from Charles River Breeding Laboratories, Inc., Kingston, NY. ACA, DBA-1, RI11/S/J, C3H.NB, B10.PL, C3H/HeJ, C57BL/10, SJL, A/J, B10.A, B10.BR, B10.A(4R), and C3D2F1 mice, ages 4–6 wk, were purchased from The Jackson Laboratory, Bar Harbor, ME. B10.M and B10.AQR mice were kindly provided by Dr. Martin E. Dorf and Dr. Man-Sun Sy, Harvard Medical School, Boston, MA.

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Abbreviations used in this paper: Ag, antigen; APC, antigen-presenting cell; BSA, bovine serum albumin; DME, Dulbecco's modified eagle media; FCS, fetal calf serum; Hepes, N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid; IL-1 interleukin 1; IL-2, interleukin 2; LPS, lipopolysaccharide; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; NMS, normal mouse serum; PBS, phosphate-buffered saline; PNA, peanut agglutinin.

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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 No. (NIH) 78-23, revised 1978).

**Monoclonal Antibodies.** Hybridoma culture supernatants grown to the point of media exhaustion were prepared by centrifugation and filtration and used as sources of monoclonal antibodies. In some cases, monoclonal antibodies were further purified by affinity chromatography on protein A–Sepharose. The hybridomas, their specificities, and their sources are as follows: MKD6, α1-A\textsuperscript{d}q, was a gift from Drs. John Kappler and Philippa Marrack, National Jewish Hospital, Denver CO. (10); 14.4.4S, α1-E\textsuperscript{k}d, was made available by Dr. D. Sachs, National Institutes of Health (11); 10.2.16, α1-A\textsuperscript{k}f, and 11.4.1, αK\textsuperscript{b}, were both made available by Dr. L. A. Herzenberg, Stanford University (12); J11D, α cortical thymocytes, was made available by Dr. J. Sprent, University of Pennsylvania (13); 3JP, (Y-3P) α1-A\textsuperscript{b}p\textsuperscript{r}a\textsuperscript{r}n\textsuperscript{r}u\textsuperscript{r}k\textsuperscript{r} was a gift from Dr. C. Janeway, Yale University (14); H02.2ADH4, α Lyt-2.2 was made available by Dr. P. D. Gottlieb, University of Texas (15); H013.4, α Thy-1.2, was provided by Dr. M. Gefter, Massachusetts Institute of Technology (16); α Lyt-1.2, was the kind gift of Dr. Man-Sun Sy, Harvard Medical School. Supernatants were used in culture at a final concentration of 25% or less.

**Interleukin 1.** Supernatants containing IL-1 were generated by culturing 2 × 10\textsuperscript{6} of P388D1 subclone (a gift from Dr. R. Robb, E. I. Dupont) in RPMI 1640 with 1% fetal calf serum (FCS), antibiotics, 60 μg/ml lipopolysaccharide (Difco), and 5 × 10\textsuperscript{-5} M 2-mercaptoethanol for 144 h as previously described (5). Supernatants were extensively dialyzed and sterile filtered. Such preparations had 50% maximal activity at a dilution of 1:64 to 1:128 as tested in an IL-1 assay (17).

**Cell Preparation.** Peanut agglutinin lectin (PNA) (Sigma Chemical Co., St. Louis, MO) was used to separate thymocytes into two populations, PNA positive and PNA negative, according to the method of Kruisbeek (5, 18). Briefly, 2 × 10\textsuperscript{6} thymocytes were incubated with 500 μl of 500 μg/ml PNA in Dulbecco’s modified Eagle’s medium (DME) buffered with 25 mM Hepes without bicarbonate for 10 min at room temperature. 1 ml of DME with 5% FCS was added and this solution was layered over 8 ml of DME with 20% FCS. Cells were allowed to sediment for 30 min at room temperature and the top 2 ml, containing PNA-negative thymocytes, were collected. This fraction was centrifuged and resuspended in 2 ml 0.15 M D-galactose solution for 10 min at room temperature, and subsequently washed several times.

Accessory cells were density fractionated on discontinuous BSA gradients as described by Steinman and Cohn (19). Briefly, splenocytes were depleted of red cells with Tris- ammonium chloride treatment. After washing, these cells were suspended at 10\textsuperscript{8} cells/ml of bovine serum albumin (BSA; Armour Pharmaceutical Co., Chicago, IL), ρ = 1.082, transferred to centrifugation tubes and overlayed with BSA, ρ = 1.060. Gradients were spun at 10,000 g for 30 min at 4°C. The fraction banding at the interface was collected and washed. This low density fraction is enriched for Ia positive accessory cells. Accessory cells were exposed to 1,660 rad gamma irradiation with a \textsuperscript{157}Cs source (Gammacell, Atomic Energy Ltd., Canada).

**Culture Conditions.** 10\textsuperscript{7} PNA-negative thymocytes were cultured with IL-1-containing supernatant (1:8 dilution) in a final volume of 1.5 ml. Culture media consisted of RPMI 1640 supplemented with 20 mM Hapes, 2 mM glutamine, 1 mM nonessential amino acids, 10% heat-inactivated FCS (all from MA Bioproducts, MD), 100 μg/ml penicillin, 100 μg/ml Streptomycin, 0.25 μg/ml Fungizone (Gibco), and 5 × 10\textsuperscript{-5} M 2-mercaptoethanol with the addition of 2.5 × 10\textsuperscript{-5} M indomethacin (Sigma). Thymocyte blasts were isolated after 72 h of incubation. In another set of experiments, 5 × 10\textsuperscript{6} PNA-negative thymocytes were cultured with 5 × 10\textsuperscript{5} low density fraction cells for 144 h without exogenous IL-1. Blasts were then isolated on a Ficol-Hypaque gradient. Briefly, thymocyte blasts from either the IL-1 culture or low density fraction cell culture were isolated by overlaying media on a suspension of cells in 9.9% Ficoll 400/9.6% Hypaque and collecting
cells at the interface after centrifugation at 2,900 rpm at 20°C for 30 min. These cells were subsequently used in the generation of thymocyte hybridomas.

**Thymocyte Hybridomas.** Thymocyte hybridomas were derived as previously described (20). Thymocyte blasts were fused to the azaguanine-resistant AKR thymic, T lymphoma (BW5147) cells at a ratio of 4:1 using 40% polyethylene glycol. Hybridomas were selected with hypoxanthine, aminopterin, and thymidine media (HAT media), further expanded and screened for their ability to produce IL-2 upon stimulation with or without accessory cells. All hybridomas were passaged in vitro in DME (Gibco Laboratories, Grand Island, NY) with 4.5 g glucose per liter, 10% heat-inactivated FCS, and antibiotics.

**Hybridoma Stimulation and IL-2 Assay.** Thymocyte hybridomas were stimulated in microculture according to the method of Kappler et al. (10, 20). 10^5 hybridomas were cultured in 200 μl flat-bottom microtiter wells (Falcon 2072; Labware, Becton Dickinson and Co., Oxnard, CA) with or without 10^6 irradiated spleen cells as a source of accessory cells. Culture media was as described above, except for the omission of indomethacin. After 20–26 h of incubation at 37°C, 100 μl of culture supernatant was harvested and exposed to 8,000 rad gamma radiation with a ^137Cs source (Gammacell, Atomic Energy Ltd., Canada). IL-2 content was measured by adding 5 × 10^5 IL-2-dependent cells, HT2, and incubating for 20–26 h with 1 μCi of tritiated thymidine added over the last 4–6 h (10, 20). 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 was determined by scintillation counting. Data is expressed as the arithmetic mean of triplicate cultures ± the standard error of the mean (SEM). A summary of hybridomas illustrated in this report are listed in Table I.

**Immunofluorescent Staining and FACS Analysis.** 10^6 hybrids or control cells were incubated with 25 μl of directly fluoresceinated monoclonal antibody (mAb) (0.1 mg/ml) or 25 μl of mAb-containing supernatant on ice for 60 min followed by 25 μl of (0.1 mg/ml) rabbit anti-mouse immunoglobulin for an additional 60 min on ice. Stained cells were fixed with 0.5 ml of 2% paraformaldehyde for 15 min at room temperature followed by another 15 min incubation in 1 × phosphate-buffered saline (PBS). After fixation, the samples were analyzed with a FACS II (Becton Dickinson Electronics Laboratory, Mountain View, CA).

**Results**

**Generation of Thymocyte Hybridomas.** We have used somatic cell hybridization techniques to construct a panel of thymocyte hybridomas that are autoreactive, MHC specific, and IL-2 producing. Medullary (PNA-) thymocytes were activated in vitro under two conditions: either in a 72-h culture with exogenous IL-1 alone or in a 144-h coculture with irradiated syngeneic or semi-syngeneic accessory cells in the absence of exogenous factors. This was of interest as the activation

| Hybrid | Origin | Autoreaction | Cross-reaction (H-2) |
|--------|--------|--------------|---------------------|
| YH 3.28 | C3H/Hej | I-A^b/I-E^a | d,b,s,q,l,p |
| YH 3.70 | I-E^a | None |
| YH 3.102 | C3H/Hej | I-E^a | None |
| YH 3.139 | I-E^a | b |
| YH 3.146 | I-E^a | None |
| YH 5.10 | C3D2F1 | I-A^a | None |
| YH 5.14 | I-A^a | b |
| YH 5.16 | I-E^a | b,s,p |

* C3D2F1 (H-2^k) thymocytes activated by C3H/Hej (H-2^k) accessory cells.
of thymocytes, under both conditions, requires an Ia-bearing accessory cell and is inhibited by α Ia mAbs (5). The characteristics of hybrid clones obtained from thymocytes activated under both conditions are comparable except for the frequency with which they are observed. Therefore, in the subsequent sections, they will be considered together. Medullary thymocytes were chosen because they respond vigorously under these conditions, thus allowing sufficient numbers of cells for hybridization (5, 6).

The results of five independent fusions are summarized in Table II. Our data indicate that thymocytes are amenable to hybridization and the frequency of viable hybrids is similar to our experience with peripheral T cell hybridizations (20). Moreover, many hybrids are functional and can be activated to produce IL-2 by irradiated, syngeneic accessory cells. The frequency of autoreactive hybrids is as high or higher than the frequency of antigen-specific hybrids arising in fusions with antigen-stimulated peripheral T cells or alloreactive hybrids in mixed lymphocyte reaction (MLR) fusions (20, 21). No additional autoreactive hybrids were identified with the inclusion of IL-1 in screening cultures (data not shown). It would therefore appear that these thymocyte hybridomas are either capable of inducing IL-1 or are IL-1 independent. This, however, has not been formally analyzed. Many of the hybrids have been highly stable, especially after cloning. Cells with weaker responses tend to be less stable, as we have observed with peripheral T cell hybrids.

Phenotypes of the Thymocyte Hybrids. The cell surface phenotype of several functional thymocyte hybrids was characterized by direct or indirect immunofluorescent staining with mAbs and FACS analysis. Results are summarized in Table III. All the hybrids bear Thy-1.2, which is the phenotype of the parental strains, C3H/Hej and C3D2F1. Thus, they are derived from the thymocytes activated in our in vitro culture system. All are Lyt-1+2−, which is characteristic of mature, inducer T cells. Furthermore, they are J11D−. That is not surprising since they are derived from PNA−, medullary thymocytes. In addition, they lacked surface Ig and Ia.

Specificity of the Thymocyte Hybridomas. All of the hybridomas described in the

| Fusion | Culture condition | Hybrids tested | Autoreactive hybrids | Frequency |
|--------|-------------------|----------------|----------------------|-----------|
| I      | C3H/Hej + IL-1    | 97             | 10                   | 10%       |
| II     | C3H/Hej + IL-1    | 99             | 13                   | 13%       |
| III    | C3H/Hej + IL-1    | 170            | 20                   | 12%       |
| IV     | C3H/Hej → C3H/Hej | 5              | 3                    | 60%       |
| V      | C3D2F1 → C3H/Hej  | 11             | 9                    | 82%       |

55% 14%

In fusion I–III, thymocytes were cultured with IL-1 and subsequently fused to the AKR lymphoma, BW5147. In fusion IV + V, thymocytes were cocultured with syngeneic or, semisyngeneic, X-irradiated accessory cells as indicated by thymocyte → accessory cell.
**Table III**

| Hybrid     | Thy-1.2 | Lyt-1.1 | Lyt-2.1 | J11D | α 1-A<sup>a</sup> | α 1-E<sup>a</sup> |
|------------|---------|---------|---------|------|-----------------|-----------------|
| YH 3.70   | +       | +       | -       | -    | -               | -               |
| YH 3.102  | +       | +       | -       | -    | -               | -               |
| YH 5.16.3 | +       | +       | -       | -    | -               | -               |

* Hybridomas were labeled either with directly fluoresceinated mAbs or with mAbs followed by fluoresceinated rabbit-anti-mouse Ig and analyzed with a FACS. Control experiments showed that all mAbs stain specifically.

**Table IV**

| Accessory cell | YH 3.70   | YH 5.10   |
|---------------|-----------|-----------|
| Strain        | cpm ± SEM | cpm ± SEM |
| --------------|-----------|-----------|
| C3H/Hej       | 44,770 ± 3,831 | 22,212 ± 782 |
| BALB/c        | 3,110 ± 591 | 2,201 ± 191 |
| C57BL/10      | 2,635 ± 825 | 3,203 ± 932 |
| SJL           | 1,305 ± 117 | 2,357 ± 59  |
| DBA-1         | 1,711 ± 67  | 2,341 ± 235 |
| B10.BR        | 52,118 ± 2,963 | 33,427 ± 1,643 |
| A/J           | 30,072 ± 2,217 | 20,706 ± 2,833 |
| B10.A         | 18,598 ± 2,365 | 25,837 ± 777 |
| B10.AQR       | 77,233 ± 2,078 | 36,324 ± 542 |
| B10.A(4R)     | 1,867 ± 217  | 47,889 ± 1,287 |

Microcultures were prepared with 10<sup>5</sup> hybrids with or without 10<sup>6</sup> X-irradiated spleen cells from indicated strains. Cultures were incubated for 24 h and 100 μl of supernatant removed, X-irradiated, and assayed for IL-2 content.

report were detected by their activation with syngeneic accessory cells. We next investigated the specificity of this interaction by testing with accessory cells of different genotypes. The results are illustrated by representative experiments in Table IV and summarized in Table V. All of the autoreactive hybrids were found to be MHC specific. For example, as shown in Table IV, YH 3.70 reacts with C3H/Hej (H-2<sup>k</sup>) and B10.BR (H-2<sup>k</sup>), but not B10 (H-2<sup>b</sup>). Where examined, polymorphic genes outside of the H-2 complex do not affect reactivity. This is illustrated in Table IV where C3H/Hej (H-2<sup>k</sup>), B10.BR (H-2<sup>k</sup>) and A/J (kkkd) all activated YH 3.70.

The precise specificity of this interaction was determined by the use of H-2 recombinant inbred mice. As shown in Table IV, YH 3.70 reacts with B10.AQR (qkkd) but not B10.A4R (kkbb). This mapped the specificity of YH 3.70 to I-E<sup>k</sup>. YH 5.10 reacts with B10.A4R (kkbb) and B10.AQR (qkkd), but not DBA-1 (qqqq). Thus, the specificity of YH 5.10 is for I-A<sup>k</sup>. Table V summarized the results obtained with 20 autoreactive hybrids. As can be seen, all are MHC-specific and map to either I-A<sup>k</sup> or I-E<sup>k</sup> with the exception of YH 3.28, which recognizes both I-A<sup>k</sup> and I-E<sup>k</sup>. This latter hybrid will be further analyzed below. As shown in Table V, 40% of the hybrids are I-A<sup>k</sup> specific, 55% are I-E<sup>k</sup> specific,
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### TABLE V

| Specificity of 20 Autoreactive Hybridomas | No. | Percentage |
|------------------------------------------|-----|------------|
| MHC Specific*                            | 20/20 | 100 |
| I-A\(^a\) Specific\(^\dagger\)            | 9/20  | 45 |
| I-E\(^k\) Specific\(^\dagger\)            | 12/20 | 60 |
| Restricted to self I\(^\alpha\)           | 8/20  | 40 |
| Alloreactive\(^\ddagger\)                 | 12/20 | 60 |

| Reactive with: | No. | Percentage |
|---------------|-----|------------|
| 2 MHC         | 5/20 | 25 |
| 3 MHC         | 2/20 | 10 |
| 4 MHC         | 2/20 | 10 |
| >4 MHC        | 2/20 | 10 |

Hybridomas were tested with accessory cells of different genotypes as described in Table IV. 20 hybrids were stable enough to allow complete evaluation.

* MHC Specific: Reacts with both C3H/Hej (H-2\(^k\)) and B10.BR (H-2\(^k\)), but not with another B10 H-2 congenic.

\(^\dagger\) I-A\(^a\) Specific: Reacts with B10.A(4R) (kkbb) and B10.AQR (qkkd) but not DBA-1 (qppq).

\(^\ddagger\) I-E\(^k\) Specific: Reacts with B10.AQR (qkkd), but not B10.A(4R) (kkbb).

NOTE: YH 3.28 is reactive to both I-A\(^k\) and I-E\(^k\) and is included in both categories.

\(^\dagger\) Only reactive to H-2\(^k\). Number of haplotypes examined varied from 6 to 9.

\(^\ddagger\) Cross-react with other haplotypes.

### TABLE VI

| Accessory cell | Monoclonal* | YH 3.146 cpm ± SEM | YH 5.14 cpm ± SEM |
|----------------|-------------|---------------------|-------------------|
| C3D2F1 (H-2\(^{kde}\)) | —           | 657 ± 12            | 1,204 ± 51        |
| α I-A\(^k\)     | 40,534 ± 2,236 | 22,770 ± 1,434      | 21,347 ± 538      |
| α I-A\(^d\)     | 35,112 ± 387 | 1,119 ± 53          |                   |
| α I-E\(^k\)     | 36,005 ± 394 | 21,347 ± 538        |                   |
| α I-E\(^d\)     | 1,247 ± 71   | 15,052 ± 661        | 29,137 ± 1,949    |

* Microcultures were prepared as described in Table IV except for the addition of mAbs where indicated. α I-A\(^k\), α I-A\(^d\), and α I-E\(^k\), were culture supernatants from hybridomas 10.2.16, MKD6, and 14.4.4s, respectively. α K\(^k\) was obtained from the hybridoma 11.4.1 and was purified by affinity chromatography. α I-A\(^k\) and α I-A\(^d\) were used in 1:16 final dilution. α I-E\(^k\) was used in 1.8 dilution. α K\(^k\) was used at 25 μg/ml. α I-A\(^k\) was capable of blocking an I-A\(^k\)-specific hybridoma, RF 26.1.17 (data not shown).

and 5% are I-A\(^k\) and I-E\(^k\) specific. (Percentage was calculated from a total of 20 hybrids).

To confirm the result of the mapping experiments, we tested the effect of monoclonal α H-2 antibodies on selected hybrids. Representative experiments are shown in Table VI; YH 3.146 is blocked by α I-E\(^k\) but not α I-A\(^k\) or α I-A\(^d\), whereas YH 5.14 is blocked by α I-A\(^k\) but not α I-E\(^k\) or α I-A\(^d\). This blocking is highly specific since each of these α Ia MAbs bind the F\(_1\) accessory cell used to
stimulate the hybrids. The results of these specific, reciprocal inhibitions agree completely with the mapping studies described above.

Taken together, these data demonstrate that in our cultures of in vitro activated thymocytes, there is a high frequency of cells that can be activated by syngeneic accessory cells. On a clonal level, these cells have specificity for self-Class II MHC molecules, either I-A\(^k\) or I-E\(^k\) or both.

**Autoreactive Thymocyte Hybrids Do Not Appear to Require Corecognition of a Nominal Antigen.** To test whether thymocyte hybrids were truly autoreactive, we attempted to eliminate foreign antigens from culture. We chose to use a clonal APC, TA3, for these studies to eliminate the possibility of foreign antigen pulsing of accessory cells in vivo. TA3 is an H-2\(^{dk}\) bearing B cell–B lymphoma hybridoma derived by Glimcher (22). Representative thymocyte hybrids and TA3 were extensively washed and grown in syngeneic normal mouse serum for several generations to rule out carrying over of culture antigens. Subsequently, both hybrids and TA3 were tested under serum-free conditions. As shown in Table VII, hybrid activation is still detectable under these conditions. Similar results were obtained when both cells were grown in FCS and tested under serum-free conditions in parallel. We therefore fail to detect a requirement for an exogenous nominal antigen. Taken together, our findings suggest that recognition of self-Class II MHC molecules is sufficient for the activation of these hybridomas.

**Recognition of Public and Nonpolymorphic MHC Determinants.** To further characterize the fine specificity of self-MHC specific hybrids, we tested a large number of our clones for reactivity toward a number of different MHC haplotypes. The results for several representative hybrids are shown in Table VIII and our

| Condition* | Hybrid | Accessory cell* | cpm ± SEM |
|------------|--------|----------------|-----------|
| 1          | YH 5.70 | -              | 751 ± 110 |
|            |        | +              | 17,563 ± 277 |
| 2          | YH 5.70 | -              | 693 ± 68  |
|            |        | +              | 26,179 ± 647 |
| 1          | YH 5.16 | -              | 641 ± 75  |
|            |        | +              | 2,081 ± 327 |
| 2          | YH 5.16 | -              | 524 ± 14  |
|            |        | +              | 3,071 ± 353 |

* Condition 1: Both hybrids and TA3 had been washed ×3, grown in 1% syngeneic NMS for 3 d and washed ×3 before use. Condition 2: Both hybrids and TA3 were grown in 10% FCS and washed ×3 before usage. Cultures were prepared with media lacking FCS. The results in this table were obtained from the same experiment. In addition, YH3.70 and TA3 have been passaged in NMS for 1 wk before testing (~10 generations) with identical results (data not shown).

* The B-B lymphoma hybrid, TA3, was used as a clonal source of accessory cells (19).
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Table VIII
Representative Patterns of Alloreactivity

| Accessory cell | Strain   | H-2 | YH 3.139 | YH 5.15 | YH 5.16 | YH 3.28 |
|---------------|----------|-----|----------|----------|----------|----------|
| C3H/Hej       | k        | ++  | ++       | ++       | ++       |
| BALB/c        | d        | -   | -        | -        | -        | ++       |
| C57BL/10      | b        | ++  | ++       | ++       | ++       |
| SJL           | s        | -   | -        | ++       | +        |
| DBA-1         | q        | -   | -        | -        | +        |
| B10.M         | f        | -   | -        | -        | NT       |
| RIll S/J      | r        | -   | -        | -        | NT       |
| C3H.NB        | p        | -   | -        | +        | +        |
| B10.PL        | u        | -   | -        | -        | NT       |

The responses were scored on the basis of the stimulation index: stimulated cpm/background cpm.
+ indicates an index of 2–6.
++ indicates an index of 6 or more.
NT, not tested.

general experience is summarized in Table V. ~60% of our hybrids recognize more than one MHC specificity. Since only a limited number of haplotypes were tested, this is probably an underestimate. In most cases, the alloreactivity is weaker than the autoreactive response. Interestingly, several of the clones, ~35%, react against several independent haplotypes and therefore appear to recognize broadly shared public Ia determinants. It should be noted that all of the hybridomas in this study initially arose at clonal frequency. Thus, it is probable that the multiple reactivities reflect the specificity of a single clone. Since the existence of T cells with specificity for public MHC determinants is of interest, we sought to verify this point. Two very cross-reactive hybrids were cloned by limiting dilutions at 0.33 hybrids per well. The results for representative subclones are illustrated in Tables IX–XII. As can be seen, subclones of YH3.28 retain pan-reactivity with all MHC haplotypes tested, even upon multiple clonings. Unfortunately, all of the pan-reactive hybrids thus far identified have been relatively unstable, even after cloning. Also, YH 5.16, which cross-reacts with H-2k,b,s,p, also retains these cross-reactivities when subcloned (Tables IX and X) and has been more stable. In addition, we demonstrated that such alloreactivities can be blocked by specific mAbs (Tables X and XII). These two hybrids show that thymocytes can recognize public Ia determinants. Further, YH 3.2.8.3 may be recognizing a nonpolymorphic determinant on Ia molecules as suggested by the above data, however, extensive testing has been limited by the instability of this clone. In addition, it is clear that an I-E reactive hybrid can cross-react with an I-A molecule as demonstrated by YH 3.2.8.3 (Table XII) and YH5.16 (Table X). Taken together, these results indicate that self-specific thymocytes can also display alloreactivity. Furthermore, within the thymocyte repertoire for self-MHC molecules are cells with receptors for public and possibly nonpolymorphic MHC determinants. This finding stands in marked contrast to cells in the peripheral T cell pool, as will be discussed below.

MHC Specificity of Clones from an F1→P Fusion. The results presented above
Table IX

Analysis of a Cross-reactive Thymocyte Hybridoma

| Expt. | Hybrid | Accessory cell | cpm ± SEM |
|-------|--------|----------------|-----------|
| 1     | YH 5.16. C3D2F₁ → C3H/Hej | C3H/Hej k k k k | 3,358 ± 253 |
|       |       | BALB/c d d d d | 1,877 ± 139 |
|       |       | C57BL/10 b b b b | 10,312 ± 2,494 |
|       |       | SJL s s s s | 49,261 ± 6,416 |
|       |       | DBA-1 q q q q | 2,166 ± 190 |
|       |       | A.C.A f f f f | 1,887 ± 68 |
|       |       | RIII S/J r r r r | 1,625 ± 130 |
|       |       | C3H.NB p p p p | 6,421 ± 12 |
|       |       | B10.PL u u u u | 1,515 ± 89 |
|       |       | C3H/Hej k k k k | 12,001 ± 191 |
|       |       | B10.BR k k k k | 22,822 ± 2,397 |
|       |       | A/J k k k d | 8,372 ± 1,441 |
|       |       | B10.A k k k d | 9,946 ± 1,000 |
|       |       | B10.AQR q k k d | 47,436 ± 1,539 |
|       |       | B10.A(4R) k k b b | 1,327 ± 80 |

* Microcultures were prepared as described in Table IV.

Table X

Monoclonal αIa Antibody Blocking of a Cloned Cross-reactive Thymocyte Hybridoma

| Hybrid | Accessory cell | Monoclonal* Ab | cpm ± SEM |
|--------|----------------|----------------|-----------|
| Clone  | Strain H-2     |                |           |
| YH 5.16.3 C3D2F₁ → C3H/Hej | C3H/Hej k | αI-A₁ | 3,103 ± 106 |
|        | C3H/Hej k | αI-E₁,d | 20,984 ± 634 |
|        | C3H/Hej k | αI-E₂,d | 2,099 ± 363 |
|        | C57BL/10 b | αI-E₁,p | 13,119 ± 913 |
|        | C57BL/10 b | αI-E₂,p | 38,016 ± 1,145 |
|        | SJL s | αI-E₁,p | 20,581 ± 477 |
|        | SJL s | αI-E₂,p | 38,016 ± 1,145 |
|        | C3H.NB p | αI-E₁,p | 3,780 ± 132 |
|        | C3H.NB p | αI-E₂,p | 8,282 ± 160 |

Microcultures were prepared as described in Table VI.

* All monoclonals were used in 1:4 dilution. αI-A₁, αI-E₁,d, and αI-E₂,d were supernatants derived from 10.2.16, 14-4.4s, and 3JP respectively. 3JP and 14-4.4s both block specifically in control experiments (data not shown).

Demonstrated that thymocytes activated in vitro by IL-1 or by coculturing with syngeneic accessory cells contain a high frequency of autoreactive, Ia-specific clones. We have previously shown that the Ia molecules on accessory cells are essential to this activation (5, 6). Most of the hybrids in this study (Fusion I–IV) were derived from thymocytes activated in the presence of syngeneic accessory cells. The exception to this design was the fifth fusion. In this experiment, C3D2F₁, H-2<sup>k</sup>', thymocytes were cocultured with parental, C3H/Hej (H-2<sup>k</sup>), accessory cells which present a more limited array of Ia molecules. As shown in
ANALYSIS OF THYMOCYTE MHC SPECIFICITY

TABLE XI
Multiple Cross-reactivities of a Cloned Hybridoma*

| Hybrid | Origin | Accessory cell | cpm ± SEM |
|--------|--------|----------------|-----------|
| Expt.  | Clone  | Strain K A E D |           |
| 1      | YH 3.28.1 | C3H/Hej | 1,307 ± 323 |
|        |        | C3H/Hej k k k k | 4,350 ± 347 |
|        |        | BALB/c d d d d | 7,820 ± 354 |
|        |        | C57BL/10 b b b b | 9,725 ± 3,241 |
|        |        | SJL* s s s s | 2,588 ± 81 |
|        |        | DBA-1 q q q q | 12,667 ± 1,385 |
|        |        | C3H/Hej k k k k | 3,924 ± 220 |
|        |        | C3H.NB p p p p | 6,195 ± 679 |

* Microcultures were prepared as described in Table IV.
† The pan reactivity illustrated is representative of multiple experiments. The weak reaction seen with SJL has been consistently observed (stimulation index = 2.2, n = 4) and is therefore considered a positive reaction.

TABLE XII
Inhibition of a Cross-reactive Thymocyte Hybridoma's Activation by a Ia mAbs

| Hybrid | Accessory cell | Monoclonal Abs | cpm ± SEM |
|--------|----------------|----------------|-----------|
| Clone  | Strain H-2 |               |           |
| YH 3.28.3 | C3H/Hej | —              | 703 ± 134 |
| C3H/Hej | k             | 9,596 ± 239 |
| C3H/Hej | α i-A4        | 1,868 ± 104 |
| C3H/Hej | α i-Ekd       | 5,267 ± 824 |
| C3H/Hej | α i-A4 + α i-Ekd | 751 ± 45 |
| BALB/c | d              | 3,908 ± 46 |
| BALB/c | α i-A4        | 1,360 ± 74 |
| BALB/c | α i-Ekd       | 1,459 ± 353 |
| BALB/c | α i-A4 + α i-Ekd | 885 ± 105 |

* Microcultures were prepared as described in Table VI. All monoclonals were used in 1:4 dilution. Specific monoclonals and their derivation are: α i-A4, 10.2.16; α i-Ekd, 14.4.4s; α i-A4, MKD6.

Table II, 9 out of 11 hybrids tested are autoreactive. Remarkably, all of the autoreactive hybrids are specific for the Ia alleles of the accessory cell in culture. Several cross-reactive hybrids were derived from this fusion but none recognized the opposite parental haplotype (Table I and data not shown). Although our analysis is limited to this single fusion, the results are quite clear. Other non-hybridoma studies to be reported separately have demonstrated that F1 thymocytes initially contain cells capable of recognizing both parental MHC haplotypes, and that H-2d accessory cells are capable of similar selection (23). Although not the main focus of the present report, these results suggest that in vitro, Ia-bearing accessory cells can select thymocytes bearing complementary receptors.

Discussion

The thymus plays a critical role in the selection of the T cell repertoire (3, 4). Thus, examination of the specificities of developing thymocytes may enhance
our understanding on the nature of MHC restriction. In the present study, we
used the techniques of somatic cell hybridization to generate a large panel of
thymocyte hybridomas. Our major findings are as follows: (a) Thymocytes are
amenable to fusion and the resulting hybridomas are functional, i.e., they secrete
IL-2 when specifically activated; (b) Thymocytes activated in vitro by IL-1 or
accessory cells contain a high frequency of autoreactive clones; (c) These auto-
reactive clones have specificity for Class II MHC molecules; (d) Individual clones
can be identified that react with self-I-A or I-E determinants; (e) There is no
apparent requirement for nominal antigens; (f) Autoreactive clones are fre-
quently alloreactive; (g) Thymocytes exist that recognize public Ia determinants.

Thymocyte hybridomas were derived from medullary thymocytes activated in
vitro with either IL-1 or accessory cells. Our previous studies showed that both
conditions result in thymocyte proliferation that is dependent on Ia molecules
on accessory cells (5, 6). Therefore, we could potentially derive MHC-reactive
clones for analysis. The result of several fusions, as shown in Table II, demon-
strated the ease of obtaining functional thymocyte hybridomas. Furthermore,
autoreactive hybrids were identified with high frequency. Thymocytes activated
by IL-1 alone contained 10–13% autoreactive clones, whereas thymocyte acti-
vation by accessory cells result in 60–82% autoreactive hybrids. The higher
frequency of the later may reflect either increased clonal expansion due to
extended culture (144 h vs. 72 h) or a greater selection pressure (no exogenous
IL-1). However, our data does not distinguish between these possibilities. The
overall frequency of autoreactive clones is as high or higher than the frequency
of Ag-specific hybrids resulting from fusion of antigen-stimulated peripheral T
cells or alloreactive hybrids from fusion of MLR-activated T cells (20, 21).

The phenotype of all of the autoreactive hybridomas is Thy-1+, Lyt-1+ and
negative for Lyt-2, Ia, Ig, and the marker defined by the J11D mAb. This is
consistent with the developmental stage of the thymocytes from which the
hybridomas were derived. Our previous data has suggested that the response of
autoreactive thymocytes is not dependent on nominal antigens (5). To further
examine this point with the hybrid clones, we assessed their activation in the
absence of foreign antigen. As an accessory cell source we chose a lymphoblastoid
tumor line, TA3, H-2kxd to eliminate the possibility of in vivo foreign antigen
pulsing. When both TA3 and hybrids were grown in syngeneic NMS for several
generations to rule out the possibility of carrying over of FCS antigens, we could
still detect significant IL-2 production. These results suggest that neither in vivo
nor in vitro acquired antigens are required for the response of these autoreactive
hybrids.

The specificity of these autoreactive thymocyte hybrids was analyzed by
utilizing accessory cells from intra-H-2 recombinants and by mAb blocking. Our
results demonstrated that all the autoreactive hybrids have specificity for self-
Class II MHC determinants. The non-MHC genetic background of the accessory
cells does not appear to affect the response within the strains tested in this report.
Thus, our data indicate that recognition of unmodified Ia is sufficient for
activation of the autoreactive thymic clones. Nonetheless, our data does not
formally exclude a role of an autologous, nonpolymorphic cellular antigen being
corecognized.
A substantial proportion of the self-MHC specific thymocyte hybrids were also found to react with allo-MHC. This cross-reactivity was maintained after cloning and therefore is the property of a single cell. To our knowledge, this is the first demonstration that an autoreactive T cell can also be alloreactive. This is consistent with the observations of alloreactivity of antigen-specific, MHC-restricted T cells (24, 25).

We have isolated clones that recognize several MHC haplotypes. Analysis with MHC recombinant inbred strains and mAbs indicate that they recognize public Ia determinants shared by different Ia subregions (I-A and I-E). One rare hybrid, YH3.28 appears to recognize nonpolymorphic determinants, being reactive to all MHC haplotypes tested. Nevertheless, it appears to be MHC-specific as assessed by mAb blocking studies. Our data indicates that cross-reactive cells are not infrequent in the thymocyte population. Studies by other laboratories have shown that various alleles of Class II MHC genes share substantial sequence homology (26). Moreover, many mAbs have the ability to bind to public Ia determinants (14, 27). Yet, it is quite clear that the specificity of the peripheral T cell pool is dominated by the polymorphic regions of Ia molecules. Accordingly, the vast majority of antigen-specific T cell clones are exquisitely self-MHC restricted. Rare clones exist that can corecognize more than one restriction element; but even here, the permissive range is highly restricted (28, 29). In addition, alloreactive cells, which appear to derive from cross-reactions of antigen-specific T cells, also focus on private MHC determinants. Examples of cross-reactive cells have been described (30). However, even when intentionally selected for cross-reactivity, the cross-reactions have been limited to a few haplotypes (31). Thus, the finding of highly cross-reactive clones from thymocyte fusions is of considerable interest. It indicates that the repertoire of thymocytes appears to differ from that of peripheral T cells in several respects. It comprises (a) frequent self-Ia reactive clones and (b) cells with specificity for public Ia determinants, including rare clones specific for common MHC or framework determinants. The reason why the peripheral T cell pool is nonreactive to such epitopes is unknown. Our data clearly shows that these epitopes do exist in a configuration that can be recognized by T cells.

The difference between the central and peripheral repertoire is most likely a consequence of selection. There are at least two obvious ways in which this could occur. First, constraints of associative recognition of antigen could restrict the peripheral repertoire. As we previously suggested, the genetically polymorphic sites on the Ia molecules may be critical for antigen-presentation (20). Thus, T cells with specificity for public determinants may be incapable of corecognizing most antigens. Second, imposition of self tolerance could limit the potential specificities. For example, the reactivity to public determinants shared with self may be deleted. Alternatively, cross-reactivity may be the manifestation of high affinity clones that are not allowed to exist in the periphery (30). These possibilities are not mutually exclusive. Further studies will examine these points.

The present study also extends our previous observations on the role of Ia molecules in thymocyte activation. We have previously shown that the thymocyte IL-1 response in the absence of mitogens is dependent on an Ia bearing accessory cell and is blocked by α Ia mAbs (5). The results were interpreted to indicate
that self-specific thymocytes are triggered by Ia molecules and are driven to expand in the presence of the second signal, IL-1. The use of somatic cell hybridization has allowed the direct demonstration of a high frequency of autoreactive clones in such cultures. The finding of individual clones with specificity for I-A or I-E correlates with our observation of partial blocking of the IL-1 response by α I-A or α I-E mAbs alone and an additive effect when mixed. We had also shown that thymocytes were capable of inducing IL-1 via an Ia-dependent mechanism and subsequently proliferating (6). We proposed that this response was essentially identical to the response to exogenous IL-1 and further would result in the selective expansion of self-MHC recognizing T cells. However, since α Ia mAbs block IL-1 production and the subsequent thymocyte proliferation, we could not be certain that the clonal expansion in culture was a response by Ia-specific thymocytes. The results of our fusions with thymocytes activated under these conditions confirm our interpretations. A very high frequency of self-Ia reacting thymocyte hybrids were derived from these proliferating cells under both conditions. Further, when MHC heterologous F1 thymocytes were cocultured with parental accessory cells, the clonal specificity that emerged was specific for the MHC haplotype of the accessory cells in culture. The interpretation of this result is dependent on whether both potential sets of MHC-specific thymocytes can expand under our culture condition. Experiments using nonhybridoma techniques have demonstrated this potential as expected (23). Thus, the F1 → P fusion reported here further suggests that a selection of thymocyte MHC specificity occurs under this in vitro condition.

Summary

Medullary, peanut agglutinin-negative (PNA−), thymocytes were activated in vitro with either exogenous interleukin 1 (IL-1) or accessory cells. T cell blasts from these cultures were subsequently fused to BW5147 to generate thymocyte hybridomas. Fusion frequencies similar to those obtained with peripheral T lymphocytes were observed. A high frequency of these hybrids are triggered to produce IL-2 in the presence of syngeneic accessory cells. Exogenous, nominal antigens do not appear to be required for this activation. Using accessory cells from a series of recombinant inbred mice, the specificity of this hybrid-accessory cell interaction could be mapped to either I-Ak or I-Ek or both. This was confirmed by blocking with α Ia monoclonal antibodies (mAbs). A high frequency of these self-reactive cells are also alloreactive. Interestingly, several clones were identified that appear to recognize public Ia determinants broadly shared by different alleles and genetic subregions. Such specificities appear to contrast with those of peripheral T lymphocytes whose specificity is dominated by the genetically polymorphic portion of the Ia molecule. These results document the clonal specificity occurring in the cultures of in vitro activated thymocytes and allow an analysis of at least a portion of the intrathymic repertoire for major histocompatibility complex (MHC) determinants. The implications of these findings are discussed.

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