T CELL REPLACING FACTOR SUBSTITUTES FOR AN I-J⁺ IDIOTYPE-SPECIFIC T HELPER CELL

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Brief Definitive Report

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Over the past several years, evidence has accumulated demonstrating the existence of at least two T helper (Th) subpopulations involved in the activation of B lymphocytes. The evidence has come from several experimental systems that can be grouped into two major categories. The first category involves anti-hapten responses in which only the total response is followed without regard to particular B cell clones in the responding population. The second category involves anti-hapten responses characterized by a dominant, well-defined idiotype(s). In the former category, several laboratories (1–6) have shown that at least two subpopulations of Th cells are required for an optimum anti-hapten response. One population, Th₁, requires the physical linkage of hapten to carrier, “cognate recognition” (2, 4–6) and appears to be an Ia-negative population (3–6). The second population, Th₂, does not require cognate recognition to function (2, 4–6), bears I region-encoded products (3–6), can be replaced by T cell replacing factors (TRF) (3, 5, 6), and in one system appears to recognize immunoglobulin (Ig) determinants in association with antigen (1).

In the second category, the expression of the dominant idiotype (Id) associated with the anti-hapten response depends upon two Th subpopulations. As with the first category, Th₁ are absolutely necessary for the activation of Id⁺ B cells, and the physical linkage of hapten to carrier is required in their collaborative function with B cells (7, 8). Th₂ cells have specificity for idiotypic determinants found on Ig (7–9) and appear to depend on the presence of circulating idiotypes in vivo for their normal development (8). In reconstitution experiments there is evidence that the Id-specific Th₂ population is carrier dependent but does not require a hapten-carrier conjugate for the activation of the dominant idiotype-bearing B cells (8).

The information to date leaves open the question of whether the Th₂ cells from the two categories are in fact similar or represent quite different Th populations. We present evidence, using the previously described phenyltrimethylamino (TMA) hapten system (10, 11), that Lyt-1⁺ Id-specific Th₂ cells, like the Th₂ cells of category one, bear I region gene products (I-J) and appear to activate Id⁺ B cells by elaborating a soluble factor(s).

Materials and Methods

Immunizations. Male A/J mice aged 6–8 wk (The Jackson Laboratories, Bar Harbor, ME) were immunized intraperitoneally either with 100 μg of keyhole limpet hemocyanin (KLH)
(Calbiochem-Behring Corp., San Diego, CA) or ovalbumin (OVA) (Sigma Chemical Co., St. Louis, MO) emulsified in Freund's complete adjuvant to obtain carrier-specific T cells. B cells were primed intraperitoneally with 1.0 μg of TMA coupled to Escherichia coli lipopolysaccharide (LPS) (Sigma Chemical Co.) (12). The hapten conjugates, TMA-KLH and TMA-OVA, were prepared as described (10).

Preparation and Treatment of T Cells. 1 wk after immunization, nylon wool-passed T cells or immunosorbent nonadherent cells were treated with monoclonal anti-Lyt-2 antibodies and rabbit-anti-arsanilate affinity-purified antibodies (B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA) followed by treatment with low toxic rabbit complement (C) (Cedarlane Laboratories Ltd.). In some experiments, T cells were first treated with anti-Lyt-2 plus C, followed by a two-step treatment (10⁷ cells/ml) with anti-I-J antisera (1:10) and C (3). Anti-I-J antisera were prepared by immunizing B10.A (3R) mice with concanavalin A (Con A)-activated T blasts from B10.A (5R) mice as previously described (3). Sera were exhaustively absorbed with cells from C57Bl/6J mice as described (3).

Removal of Idiotype-specific T Cells. The method of Woodland and Cantor (7) was used, except that culture dishes were prepared with 200 μg of Na₂SO₄-precipitated normal A/J Ig or affinity-purified A/J anti-TMA antibody (60% Id⁺) (10). Briefly, T cells (3 × 10⁶ to 5 × 10⁶ cells/ml) were incubated on plates for 40 min at 37°C, the nonadherent population collected, and the procedure repeated. Nonadherent cells (60-80% recovery) were then washed and treated with anti-Lyt-2 plus C.

Preparation of Con A Supernatants. Normal A/J spleen cells were cultured with Con A (4 μg/10⁷ cells/ml) for 24 h at 37°C in complete media. The supernatant was collected, absorbed with Sephadex G-75, filter sterilized, and stored at -90°C until use (3).

Preparation of B Cells. TMA-LPS-primed spleen cells were treated with anti-Thy-1 monoclonal antibody secreted by the hybridoma T24/40.7 (kindly provided by J. Kappler and P. Marrack, National Jewish Hospital, Denver, CO.) and C (3). Routinely, ~60% viable spleen cells were recovered.

Culture Conditions and Assay. Cells were cultured in RPMI 1640 (GIBCO Laboratories, Grand Island Biological Co., Grand Island NY) supplemented with 5% fetal calf serum, 25 mM Hepes, 5 × 10⁻⁵ M 2-mercaptoethanol, penicillin, and streptomycin. Helper T cell activity was determined by titration into triplicate cultures containing 3 × 10⁶ hapten-primed B cells, and 1 μg/ml of appropriate antigen was added. 4 d after culture, cells from identical wells were pooled and assayed for total anti-TMA plaque-forming cells (PFC) using TMA-substituted sheep erythrocytes (10). Background PFC were detected using unsubstituted erythrocytes. CRI⁺ PFC were determined by incorporating 10 μl of anti-idiotypic antisera (anti-Id, 10 μg Id-binding capacity per ml) into the plaquing medium. The percent inhibition of PFC was calculated according to the formula: percent inhibition = ([1 - total PFC (anti-Id)]/total PFC) × 100 = percent CRI⁺ PFC.

Results and Discussion

In a preliminary communication (13) we reported that at least two Lyt-1⁺,2⁻ Th populations are required to trigger anti-TMA PFC response in vitro, of which 40-80% produce a cross-reactive idiotype(s) (CRI-TMA). As seen in Table I, Lyt-1⁺,2⁻ T cells from either OVA- or KLH-primed mice added to TMA-primed B cells support a secondary anti-TMA PFC response of which 50-60% secrete the CRI (lines 3 and 8). Furthermore, the data establish the participation of helper T cells in activating B cells via a homologous hapten-carrier bridge (compare lines 3, 4, 5, with 6, 7, and 8). If the T cell populations are first incubated in petri dishes coated with CRI⁺ anti-TMA antibodies and the nonadherent T cells added to the cultures, there is a marked reduction in total TMA PFC as well as the percent CRI⁺ PFC (lines 9 and 11). TMA-specific PFC responses are not altered if the primed T cells are incubated on petri dishes coated with normal mouse Ig, demonstrating the specificity of the absorption procedures (lines 10 and 12). The depleted T-OVA population can be reconstituted by the addition of unfractonated T-KLH, provided free KLH is also present in the
Table I

**CRI**^+ PFC Formation Depends on Lyt-1^+^,2^- Tn Cells Having Apparent Specificity for Both Idiotype(s) and Carrier Antigen**

| T cells in culture          | Line number | Antigen in vitro | Total TMA PFC/culture | Percent CRI^+ PFC |
|-----------------------------|-------------|------------------|------------------------|------------------|
| B cells only                | 1           | TMA-OVA          | 44                     | 22               |
| B cells only                | 2           | TMA-KLH          | 36                     | 12               |
| T-OVA                       | 3           | TMA-OVA          | 220                    | 59               |
| T-OVA                       | 4           | TMA-KLH          | 48                     | 8                |
| T-OVA                       | 5           | TMA-KLH + OVA    | 64                     | 10               |
| T-KLH                       | 6           | TMA-OVA          | 44                     | 11               |
| T-KLH                       | 7           | TMA-OVA + KLH    | 37                     | 14               |
| T-KLH                       | 8           | TMA-KLH          | 224                    | 50               |
| T-OVA (CRI-absorbed)        | 9           | TMA-OVA          | 72                     | 10               |
| T-OVA (NM Ig absorbed)      | 10          | TMA-OVA          | 210                    | 56               |
| T-KLH (CRI absorbed)        | 11          | TMA-KLH          | 40                     | 2                |
| T-KLH (NM Ig absorbed)      | 12          | TMA-KLH          | 210                    | 52               |
| T-OVA (CRI absorbed) + T-KLH| 13          | TMA-OVA          | 80                     | 14               |
| T-MOA + KLH                 | 14          | TMA-OVA + OVA    | 85                     | 10               |
| T-MOA + KLH                 | 15          | TMA-OVA + KLH    | 240                    | 65               |

*The appropriate Lyt-1^+^,2^- T cells were added at 4 X 10^5 cells/culture (previously determined to be optimum [13]) to triplicate cultures containing 3 X 10^6 TMA-primed B cells. In lines 13, 14, and 15, 2 X 10^6 T-KLH were added to 4 X 10^6 CRI absorbed T-OVA. The cultures were stimulated with the appropriate hapten-carrier conjugates and when necessary with free carriers (lines 14 and 15). 4 d later the cultures were harvested and the total, as well as percent CRI^+ direct PFC/culture determined. This table is representative of seven separate experiments.*

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Id-specific Lyt-1+2- Th Cells Bear the I-J Subregion-encoded Gene Products*

| T cells in culture | T cell treatment | Line number | Antigen in vitro | Total TMA PFC/culture | Percent CRI+ PFC |
|--------------------|-----------------|-------------|------------------|-----------------------|-----------------|
| T-KLH              | C only          | 1           | TMA-KLH          | 274                   | 45              |
| T-KLH              | Anti-I-J + C    | 2           | TMA-KLH          | 362                   | 19              |
| T-KLH              | Anti-I-J + C (B10.A (5R) absorbed) | 3 | TMA-KLH          | 280                   | 44              |
| Unfractionated T-OVA |                | 4           | TMA-OVA          | 262                   | 45              |
| T-OVA (CRI absorbed) |                | 5           | TMA-OVA          | 216                   | 12              |
| + T-KLH            | C only          | 6           | TMA-OVA + KLH    | 394                   | 71              |
| T-OVA (CRI absorbed) |                | 7           | TMA-OVA + KLH    | 300                   | 7               |
| + T-KLH            | Anti-I-J + C    | 8           | TMA-OVA + KLH    | 372                   | 61              |

* Nylon wool-passed T cells were first treated with anti-Lyt-2 plus C followed by treatment with anti-I-J sera plus C as described in Materials and Methods. Cultures were performed as in Table I. This table is representative of six similar experiments with the exception of the absorption experiment using B10.A (5R) spleen cells which was performed once. Experiments not shown, this mixture failed to reconstitute an Id-depleted T-OVA population in the presence of TMA-OVA and KLH (similar to line 7). Lines 3 and 8 demonstrate the specificity of the antiserum because absorption with B10.A (5R) lymphocytes removes anti-I-J specific activity. Unlike removal of Tth2 on CRI-coated plates, the removal of Tth2 by anti-I-J treatment did not reduce the total TMA PFC. This might be explained by the removal of an I-J+ suppressor inducer T cell for TMA. Such a T cell (I-J+, Lyt-1+) has been described by others (14) but requires a Lyt-2+ cell for its expression. Such residual Lyt-2+ cells may contaminate either our T or B cell preparations. Overall, these results demonstrate that Tth2 Id-specific cells bear I-J-encoded determinants and also define a new function and property of this cell type, i.e., its requirement for optimal CRI+ PFC formation and the apparent existence of surface receptors with specificity for Ig idiotypes. Whether this latter property is universal for all Tth2 cells involved in antihapten antibody responses remains unknown.

We (3) and others (5) have described a Tth2 population that bears surface Ia antigens and can be replaced by cell-free supernatants from in vitro 24-h Con A-stimulated splenic lymphocytes. To test whether such supernatants could replace the Id-specific, I-J+ Tth2 cells, A/J spleen cells were stimulated with Con A for 24 h and the cell-free supernatant media (Con A Sn) harvested. Con A Sn was added to cultures depleted of Tth2 activity by either anti-I-J + C treatment or absorption on CRI+ coated plates. As seen in Table III, Con A Sn added to intact cultures has no significant effect (lines 4 and 9) but clearly restores the CRI+ PFC response in cultures that have been depleted of Tth2 activity (lines 6 and 11). These results suggest that Con A Sn can replace the Tth2 cell. However, it could be argued that certain T cell growth factors in the Con A Sn amplify the few remaining Tth2 cells that may have not been removed after the antiserum or plate-absorption procedures. This is unlikely, as the supernatant from a T cell hybridoma, FS6-14.13 (kindly provided by Dr. J. Kappler and Dr. P. Marrack), which produces T cell growth factor but has no T cell-replacing activity for antibody responses (15), does not substitute for the idiotype-specific Tth2 in our hands (data not shown). Moreover, Con A Sn originating from certain non-H-2a strains does not reconstitute the response (data not shown), a
**Table III**

*Con A Sn Replaces I-J*, Lyt-1*,2* T<sub>h</sub> Specific for Idiotype*

| T cells in culture | T cell treatment | Supplementation | Line number | Total TMA PFC/culture | Percent CRI* PFC |
|-------------------|-----------------|-----------------|-------------|-----------------------|------------------|
| B cells only      | —               | —               | 1           | 12                    | 5                |
| B cells only      | Con A Sn        | —               | 2           | 20                    | 8                |
| T-OVA            | C only          | —               | 3           | 165                   | 55               |
| T-OVA            | Con A Sn        | Con A Sn        | 4           | 210                   | 43               |
| T-OVA Anti-I-J + C| —               | —               | 5           | 180                   | 17               |
| T-OVA Anti-I-J + C| Con A Sn        | —               | 6           | 240                   | 50               |
| B cells only      | —               | —               | 7           | 16                    | 9                |
| T-OVA            | —               | —               | 8           | 120                   | 81               |
| T-OVA            | Con A Sn        | —               | 9           | 105                   | 62               |
| T-OVA (CRI absorbed) | —            | Con A Sn        | 10          | 59                    | 6                |
| T-OVA (CRI absorbed) | —            | Con A Sn        | 11          | 138                   | 67               |

* Cultures were performed as in Tables I and II. Con A Sn was added into the cultures at a final 30% concentration on day 1 of culture. This table is representative of six separate experiments.

Restriction we have so far observed only for idiotype-specific responses. It should be noted in Table III (line 2) that B cells alone in the presence of antigen plus Con A Sn are not activated to CRI* TMA PFC above background, indicating the necessity for T<sub>h</sub>1 cells in addition to T<sub>h</sub>2 activity.

In conclusion, the results demonstrate that the T<sub>h</sub>2 cell necessary for the expression of a dominant Id(s) also shares characteristics of T<sub>h</sub>2 cells described in systems where idiotypes are not characterized. Namely, the Id-specific T<sub>h</sub>2 cell bears surface I-J-encoded Ia antigens and can be replaced with cell-free supernatant media from 24-h Con A-stimulated spleen cells. The latter findings suggest the effect of T<sub>h</sub>2 on CRI* antibody-producing cells can be mediated by secreted T cell factors.

**Summary**

An in vitro system for the study of idiotype (Id) expression on antitrimethylamino hapten antibody-producing cells and its regulation by two classes of helper T cells is described. These cells are distinguished in four ways: one requires a hapten-carrier bridge and gives a good response that is low in Id; it does not bind to Id-coated dishes and is not affected by anti-I-J plus complement. The other requires antigen but not a hapten-carrier bridge, is bound by Id-coated dishes and is killed by anti-I-J and complement. The Id-specific cell appears to be antigen specific and acts via a soluble factor(s).

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