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

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Over the past several years, evidence has accumulated demonstrating the existence of at least two T helper (T_h) 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 T_h cells are required for an optimum anti-hapten response. One population, T_h1, 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, T_h2, 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 T_h subpopulations. As with the first category, T_h1 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). T_h2 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 T_h2 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 T_h2 cells from the two categories are in fact similar or represent quite different T_h populations. We present evidence, using the previously described phenyltrimethylamino (TMA) hapten system (10, 11), that Lyt-1⁺ Id-specific T_h2 cells, like the T_h2 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 (10^7 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/6 J 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 Na_2SO_4-precipitated normal A/J Ig or affinity-purified A/J anti-TMA antibody (60% Id^+) (10). Briefly, T cells (3×10^6 to 5×10^6 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/10^7 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 mono-clonal 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 3×10^6 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^- T$_h$ 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 unfractionated T-KLH, provided free KLH is also present in the

TABLE I

CRI$^+$ PFC Formation Depends on Lyt-1$^+$,2$^-$ T$_h$ 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	57	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
	14	TMA-OVA + OVA	85	10
	15	TMA-OVA + KLH	240	65

* The appropriate Lyt-1$^+$,2$^-$ T cells were added at 4 × 10^6 cells/culture (previously determined to be optimum [13]) to triplicate cultures containing 3 × 10^6 TMA-primed B cells. In lines 13, 14, and 15, 2 × 10^6 T-KLH were added to 4 × 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.

culture (compare lines 3, 9, 13, 14, and 15). If, however, the same T-KLH population is first absorbed on CRI-coated dishes, thus removing potential Id-specific cells, they can no longer reconstitute the CRI$^+$ PFC response in the presence of free KLH (data not shown). These results suggest the requirement of Id-specific T$_h$2 and its activation by specific free carrier to obtain a PFC response dominated by CRI. Overall, these results suggest the participation of two classes of T$_h$ in activating TMA-specific B cells. This agrees well with other idiotype systems (7-9), which have similarly described the need for two T$_h$.

Tada et al. (4) have reported the existence of a T$_h$ subpopulation that is I-J$^+$ and does not require hapten linked to the carrier to function. To determine whether an I-J$^+$ T$_h$ cell(s) was involved in the Id$^+$ TMA response, the Lyt-1$^+$,2$^-$ T-KLH were first treated with anti-I-J serum plus complement (C) before addition into culture. Our anti-I-J serum was produced against Con A T cell blasts, a method we have previously shown to induce highly T cell reactive anti-Ia sera (3). As seen in Table II, this treatment does not result in the loss of total TMA PFC, but there is a significant reduction in the percent CRI$^+$ PFC compartment (line 2). To determine the completeness of the anti-I-J serum treatment, the treated T cells were added up to 1 × 10^6 in culture with no increase in CRI$^+$ PFC (data not shown). To test whether it is the T$_h$2 cells that bear I-J region gene products, the T-KLH were first treated with anti-I-J serum plus C before addition into cultures containing a T-OVA population depleted of T$_h$2 cells. As in the previous experiment, this treatment did not reduce the total TMA response but did prevent the T-KLH from restoring the CRI$^+$ PFC (compare lines 4, 6, and 7). The possibility remained, however, that the Id-specific and the I-J$^+$ T$_h$ were separate synergizing populations, and the removal of either one from our T-KLH population would prevent restoration of the CRI$^+$ PFC. To rule this out, we mixed a population of T-KLH depleted of Id-specific cells (by plate absorption) with T-KLH depleted by anti-I-J plus C treatment. If separate populations existed, they would complement each other in this mixture. However, in

TABLE II

Id-specific Lyt-1$^+$,2$^-$ T$_h$ 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	136	12
T-OVA (CRI absorbed) + T-KLH	C only	6	TMA-OVA + KLH	394	71
T-OVA (CRI absorbed) + T-KLH	Anti-I-J + C	7	TMA-OVA + KLH	300	7
T-OVA (CRI absorbed) + T-KLH	Anti-I-J + C (B10.A (5R) absorbed)	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 T$_h$2 on CRI-coated plates, the removal of T$_h$2 by anti-I-J treatment did not reduce the total TMA PFC. This might be explained by the removal of an I-J$^+$ 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 T$_h$2 Id-specific cells bear I-J-encoded determinants and also define a new function and property of this cell type, i.e., its requirement for optimal CRI$^+$ PFC formation and the apparent existence of surface receptors with specificity for Ig idiotypes. Whether this latter property is universal for all T$_h$2 cells involved in antihapten antibody responses remains unknown.

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

TABLE III

Con A Sn Replaces I-J$^+$, Lyt-1$^+$,2$^-$ T$_h$ 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	C only	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)	—	—	10	50	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$_h$1 cells in addition to T$_h$2 activity.

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