

T LYMPHOCYTE-MEDIATED SUPPRESSION OF MYELOMA FUNCTION IN VITRO*

IV. Generation of Effector Suppressor Cells Specific for Myeloma Idiotypes

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An increasing body of experimental evidence (1) supports the concept that subsets of T lymphocytes that suppress humoral and cell-mediated immune responses participate in a defined pathway that is governed by complimentary receptor-antireceptor interactions. Cells at various stages of this pathway can be distinguished by surface phenotype, specificity, and functional effects. The earliest evidence supporting this notion came from the studies of Eardley et al. (2), who showed that Lyt-1^+2^- cells act on Lyt-1^+2^+ precursors and trigger them to mature into potent suppressor T cells (T_s). More recent experiments analyzing suppression of delayed hypersensitivity responses to the haptens, azobenzenearsonate (ABA), and nitrophenyl, indicate that antigen-reactive T_s (termed T_{s1}) induce second-order anti-idiotypic T_{s2} , which suppress the hapten-specific immune response, probably by inducing or functioning in concert with a third, antigen-specific cell termed T_{s3} (3, 4).

To define the mechanisms of action of T_s , we and others have attempted to develop clonal systems in which homogeneous neoplastic cells can be used as targets of immunoregulatory stimuli. In particular, we have shown that immunoglobulin (Ig) secretion by the BALB/c myeloma, MOPC 315 (IgA, $\lambda 2$, specific for 2,4-dinitrophenyl and 2,4,6-trinitrophenyl), can be inhibited by culturing these cells with T_s , specific for the M315 idiotype (5). This phenomenon is especially intriguing because the inhibition of Ig secretion is selective, in that membrane receptor expression is not blocked (5), and, in a somatic cell hybrid line that secretes two idiotypically unrelated Ig, T_s , specific for each idiotype, block secretion of only that Ig (6). In addition, T_s induced by immunizing BALB/c mice with the M315 IgA have been shown to suppress IgA secretion and synthesis by MOPC 315 cells in vivo and in vitro without affecting receptor expression or the synthesis of non-Ig protein (7, 8). Two features of our results that were observed consistently were that significant suppression of myeloma cell function required 3–4 d co-culture of MOPC 315 cells and T_s , and the maximum inhibition was 40–70% by hemolytic plaque assays (5, 6). Because of these findings, biochemical analysis of myeloma suppression was difficult. Since we induced T_s by intravenous immunization with M315 IgA-coupled syngeneic splenocytes, we

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reasoned that these cells corresponded to T_{S1} and needed to differentiate further in vitro to develop into effector Ts (3, 4). We report our attempts to generate effector Ts using a protocol that was first established in studies of the suppression of ABA-specific immunity. In principle, this protocol involves immunization of mice with a soluble extract of T_{S1} cells and subcutaneous priming with antigen- (or, in the present system, M315 IgA-) modified splenocytes (3). Our results indicate that it is possible to generate effector Ts reactive with myeloma idiotypes and that such Ts differ from T_{S1} in the kinetics of their effect and in surface phenotype.

Materials and Methods

Induction of Suppressor T Cells. BALB/c mice (12–16 wk of age, purchased from Cumberland Farms, Clinton, TN) were injected intravenously with $5\text{--}10 \times 10^7$ viable syngeneic splenocytes to which affinity-purified M315 IgA was coupled, using the covalent coupling reagent 1-ethyl 3-(3-diaminopropyl) carbodiimide, as described previously (5). Spleen cells were removed 6 or 7 d later and were depleted of erythrocytes by treatment with Tris-buffered 0.83% NH_4Cl . B lymphocytes were removed by binding to dishes coated with affinity-purified rabbit anti-mouse Ig (9); such preparations routinely contained <2% surface Ig-bearing cells by immunofluorescence and showed a >90% depletion of lipopolysaccharide responsiveness. These cells are referred to as T_{S1} . Soluble extracts of T_{S1} cells (T_{SF1}) were prepared by four cycles of freezing and thawing and centrifugation at 12,000 g for removing cellular debris. To induce effector Ts, BALB/c mice were immunized subcutaneously with 3×10^7 viable M315 IgA-coupled syngeneic splenocytes at two sites on the flank and were given daily intravenous injections of 2×10^7 cell equivalents of T_{SF1} for the next 5 d. Spleen cells were removed 1 d later, and T lymphocytes were prepared as for T_{S1} .

Myeloma Cell Cultures and Assays. These techniques have been described previously (5). Briefly, tissue culture-adapted MOPC 315 cells were centrifuged over Ficoll-Isopaque, and 10^5 viable myeloma cells were cultured with varying numbers of T lymphocytes in flat-bottomed 96-well Linbro wells (Flow Laboratories, McLean, VA) in triplicate. Cultures were done in 0.2 ml of RPMI 1640 supplemented with 2 mM L-glutamine, penicillin, streptomycin, nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol, and 10% heat-inactivated fetal calf serum (all from Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) in a humidified atmosphere of 5% CO_2 . At the end of culture, the cells were harvested, washed once, and aliquots were used to (a) measure viable myeloma cell recovery, viability being assessed by trypan blue dye exclusion, and (b) determine the numbers of IgA-secreting cells by a reverse hemolytic plaque assay using Staphylococcus protein A-coated sheep erythrocytes as indicator cells (5). Results are expressed as IgA plaque-forming cells (PFC) per 10^3 viable myeloma cells recovered, and statistical analyses were done by Student's t tests.

Other Techniques. For depletion of various T cell subsets, aliquots of cells were treated with monoclonal anti-Thy-1.2, anti-Lyt-1.2, or anti-Lyt-2.2 antibody (New England Nuclear, Boston, MA) in serum-free L-15 for 40 min at room temperature. After one wash, the cells were treated with 1:8–1:10 dilution of low-tox rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) in L-15 for 45 min at 37°C , and viable cell recovery was determined after three washes. Dish binding experiments were done as previously described (5, 6), using plastic dishes coated with various myeloma Ig to deplete T cells of appropriate specificities.

Results and Discussion

Previous experiments had established that M315-specific T_{S1} , induced by intravenous immunization with M315-coupled spleen cells, suppressed IgA secretion by the MOPC 315 myeloma after 3–4 d of co-culture (5). In the first series of experiments, we compared the kinetics of suppression induced by culturing MOPC 315 cells with T_{S1} and T cells generated by subcutaneous immunization with M315-coupled splenocytes, together with administration of T_{SF1} . The pooled data illustrated in Fig. 1 show that T_{S1} produce significant inhibition of IgA PFC after >2 d culture with

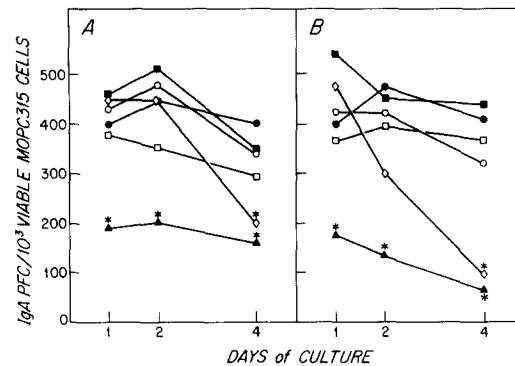


FIG. 1. 10^5 MOPC 315 cells were cultured alone (●), or with 10^6 (A) or 2×10^6 (B) splenic T lymphocytes from normal BALB/c mice (■), or with mice immunized as follows: ○, 3×10^7 M315-coupled splenocytes s.c.; □, TsF₁ for 5 d i.v.; ◇, 5×10^7 to 10×10^7 M315-coupled splenocytes i.v. (Ts₁); and ▲, 3×10^7 M315-coupled splenocytes s.c. plus TsF₁ for 5 d i.v. (effector Ts). IgA PFC were measured by reverse hemolytic plaque assays 1, 2, and 4 d after culture initiation. Groups showing statistically significant suppression ($P < 0.01$ compared to MOPC 315 cells alone by Student's *t* tests) are marked with asterisks. Data are pooled from four experiments.

MOPC 315 cells, whereas T cells induced by TsF₁ and subcutaneous immunization suppress >50% of the PFC within 1 d and 80–90% by 4 d. T lymphocytes from normal BALB/c mice, or mice immunized only with M315-coupled cells subcutaneously, or TsF were not significantly suppressive. We have operationally termed the cells mediating rapid suppression (i.e., within 1 d) “effector Ts.” By analogy with other systems (3), it can be predicted that M315-specific TsF₁ induces a second-order Ts₂ whose specificity is anti-(anti-M315), and subcutaneous immunization with M315-coupled cells leads to the generation of the effector Ts (Ts₃). However, we have not yet been able to demonstrate the existence of Ts₂ in the MOPC 315 system because of the lack of a definitive marker for specificity. Moreover, it has not been possible to generate effector Ts by in vitro culture of Ts₁, as has been done for nitrophenyl-reactive Ts (4).

The specificity of the effector Ts was assessed by their ability to bind to different myeloma proteins. As shown in Table I, suppressive activity can be depleted by absorbing effector Ts on dishes coated with M315 IgA but not on dishes coated with MPC11 (IgG_{2b,k} of unknown specificity) or M460 proteins. MOPC 460 secretes an IgA, k antibody that has the same specificity for dinitrophenyl and trinitrophenyl as MOPC 315 but has largely dissimilar idiotypic determinants (10). Furthermore, M315-specific effector Ts do not inhibit MPC 11 cells and cause >70% selective suppression of IgA secretion by the MOPC 315-MPC11 somatic cell hybrid. This selective effect is observed with hemolytic plaque assays as well as gel electrophoretic analysis of immunoprecipitated Ig produced by the somatic cell hybrid (G. Moser, M. Takoaki, I. Fox, M. I. Greene, and A. K. Abbas, manuscript in preparation).

Finally, antibody and complement treatment of suppressor cells showed that the effector Ts are Thy-1.2⁺, Lyt-1⁺2⁺ cells (Table II). In contrast, the suppressive effect of Ts₁ can be abrogated by treating the cells with either anti-Lyt-1 plus complement or anti-Lyt-2 plus complement, indicating that these cells are either Lyt-1⁺2⁺ or that T lymphocytes bearing both Lyt-1⁺ and Lyt-2⁺ markers are necessary for Ts₁ to induce or mature into effector Ts in vitro (2).

TABLE I
Specificity of M315-reactive Effector Ts

Experi- ment	T lymphocytes	Days of culture	IgA PFC/ 10 ⁵ MOPC 315 cells	P*
			<i>mean ± SE</i>	
1	None	1	209 ± 4	—
	Normal BALB/c, 2 × 10 ⁶	1	177 ± 29	NS‡
	Effector Ts, 2 × 10 ⁶	1	133 ± 21	<0.005
	Effector Ts, depleted on M315-coated dish, 2 × 10 ⁶	1	195 ± 13	NS
	Effector Ts, depleted on M460-coated dish, 2 × 10 ⁶	1	87 ± 19	<0.001
	None	2	209 ± 10	—
	Effector Ts, 10 ⁶	2	96 ± 20	<0.005
	Effector Ts, 2 × 10 ⁶	2	61 ± 10	<0.001
	Effector Ts, depleted on M315-coated dish, 10 ⁶	2	288 ± 31	NS
	Effector Ts, depleted on M315-coated dish, 2 × 10 ⁶	2	216 ± 36	NS
	Effector Ts, depleted on M460-coated dish, 10 ⁶	2	85 ± 23	<0.001
	Effector Ts, depleted on M460-coated dish, 2 × 10 ⁶	2	81 ± 11	<0.001
	None	2	288 ± 6	—
	Effector Ts, depleted on M315-coated dish, 2 × 10 ⁶	2	256 ± 30	NS
2	Effector Ts, depleted on MPC11 IgG-coated dish, 2 × 10 ⁶	2	63 ± 3	<0.001

10⁵ MOPC 315 cells were cultured in triplicate with splenic T lymphocytes from normal BALB/c mice or mice immunized to generate effector Ts. The Ts were untreated or depleted on myeloma protein-coated dishes, as described in Materials and Methods. IgA PFC were measured by reverse hemolytic plaque assays. Data from two representative experiments out of four are shown.

* Calculated by Student's *t* tests, using groups containing MOPC 315 cells alone as controls. Groups showing statistically significant suppression are italicized.

‡ Not significant.

These experiments demonstrate that two different populations of Ts can be experimentally induced that are specific for the idiotypic determinants of a myeloma protein and whose effect is to inhibit antibody secretion by the myeloma cells in vitro. The first, termed Ts₁, is generated by intravenous immunization with myeloma protein-coupled syngeneic splenocytes, requires 3–4 d culture with the myeloma to induce significant functional suppression, and is phenotypically either Lyt-1⁺2⁺ or requires Lyt-1⁺ and Lyt-2⁺ cells for its terminal maturation. The second type of Ts, the effector Ts, can be generated in vivo by subcutaneous immunization with myeloma protein-coupled splenocytes, followed by the administration of a soluble extract of Ts₁ cells. Effector Ts inhibit myeloma function within 1 d, induce a greater degree of suppression than Ts₁, and are phenotypically Lyt-1⁺2⁺ T cells. It is likely that these effector Ts are similar to the Lyt-1⁺2⁺ idiotype-specific Ts induced by multiple immunizations with myeloma protein in adjuvant (8). The advantage of the protocol used in the present experiments is that within the limits of detection it induces only Ts and not other idiotype-reactive T cell subsets or anti-idiotypic antibodies. The two types of M315-specific Ts described herein may be related in one of several ways. First, it is possible that the Ts₁ population contains cells that expand or mature during coculture with the myeloma targets and develop into effector Ts. The differences in the Ly phenotypes of the Ts₁ and effector Ts could be explained by a requirement for additional T cell subsets during the process of expansion or differentiation in vitro.

TABLE II
Ly Phenotype of Ts₁ and Effector Ts

T lymphocytes		Days of culture	IgA PFC/ 10 ⁶ MOPC 315 cells	P*
<i>mean ± SE</i>				
None		2	303 ± 20	—
Normal BALB/c	10 ⁶	2	330 ± 38	NS‡
Normal BALB/c	2 × 10 ⁶	2	411 ± 66	NS
Effector Ts	10 ⁶	2	153 ± 23	<0.005
Effector Ts	2 × 10 ⁶	2	60 ± 12	<0.001
Effector Ts, anti-Lyt-1 + C	10 ⁶	2	176 ± 10	<0.005
Effector Ts, anti-Lyt-1 + C	2 × 10 ⁶	2	37 ± 8	<0.001
Effector Ts, anti-Lyt-2 + C	10 ⁶	2	433 ± 66	NS
Effector Ts, anti-Lyt-2 + C	2 × 10 ⁶	2	302 ± 52	NS
Effector Ts, anti-Thy-1 + C	2 × 10 ⁶	2	356 ± 41	NS
Effector Ts, C	2 × 10 ⁶	2	61 ± 17	<0.001
None		4	263 ± 30	—
Normal BALB/c	10 ⁶	4	359 ± 87	NS
Ts ₁	10 ⁶	4	91 ± 28	<0.01
Ts ₁ , anti-Lyt-1 + C	10 ⁶	4	314 ± 49	NS
Ts ₁ , anti-Lyt-1 + C	2 × 10 ⁶	4	300 ± 50	NS
Ts ₁ , anti-Lyt-2 + C	10 ⁶	4	341 ± 59	NS
Ts ₁ , anti-Lyt-2 + C	2 × 10 ⁶	4	289 ± 44	NS
Ts ₁ , anti-Thy-1 + C	2 × 10 ⁶	4	220 ± 67	NS
Ts ₁ , C	10 ⁶	4	136 ± 33	<0.02
Ts ₁ , C	2 × 10 ⁶	4	133 ± 29	<0.02

10⁶ MOPC 315 cells were cultured in triplicate with splenic T lymphocytes from normal BALB/c mice or mice immunized to generate Ts₁ or effector Ts, as described in Materials and Methods. T cells were treated as shown, and 10⁶ or 2 × 10⁶ viable cells remaining after treatment were cultured with MOPC-315 cells. Because anti-Thy-1.2 plus C lysed >90% of the T cells, cultures were done after adjusting treated cells to the initial lymphocyte concentration for this group. IgA PFC were measured by reverse hemolytic plaque assays. Data from one representative experiment out of four are shown.

* Calculated by Student's *t* tests, using groups containing MOPC 315 cells alone as controls. Groups showing statistically significant suppression are italicized plus complement (C).

‡ Not significant.

Alternatively, Ts₁ may initiate the sequence of complimentary receptor-antireceptor interactions that culminates in the generation of effector Ts (1). Studies of the suppression of physiologic immune responses have led to the suggestion that Ts₁ inhibit the inductive or afferent phase of the response because they only mediate suppression when administered at or before the time of immunization (11). However, our observations that Ts₁ manifest a delayed effect, even on myeloma targets that are obviously independent of inductive signals, suggests that the apparent "afferent suppression" of normal immune responses mediated by Ts₁ might be the result of expansion or maturation of these cells or induction of effector Ts, which occurs concomitant with the development of the immune response. Such a concept, which implies that Ts₁ generated with antigen-modified cells in vivo mediate suppression not by themselves but by developing into or inducing effector Ts, is receiving increasing support from several lines of experimental data (reviewed in ref. 1). The ability to induce two cell types at different stages of a suppressor pathway, and to measure their effects on a monoclonal, autonomous target, should facilitate analysis of the differentiation and mechanisms of action of suppressor T lymphocytes.

Summary

BALB/c mice immunized intravenously with syngeneic splenocytes, to which affinity-purified IgA produced by the MOPC 315 myeloma is covalently coupled,

develop suppressor T cells (T_{S1}) that inhibit IgA secretion by MOPC 315 cells after 3–4 d of co-culture. Immunization with M315-coupled splenocytes subcutaneously, followed by administration of a soluble extract of T_{S1} cells, leads to the generation of effector Ts that are also idiotypic specific and inhibit myeloma function within 1 d. Moreover, effector Ts are $Lyt-1^{-}2^{+}$, whereas T_{S1} are either $Lyt-1^{+}2^{+}$ or require $Lyt-1^{+}$ and $Lyt-2^{+}$ cells to mature into effector Ts in vitro. Such a protocol should be useful for analyzing the interactions that result in the maturation of Ts and in defining the mechanisms of action of Ts, whose effect can be measured on a homogeneous target population and that are specific for a well-characterized myeloma idiotypic.

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