

MECHANISMS OF IDIOTYPE SUPPRESSION  
I. In Vitro Generation of Idiotypic-Specific Suppressor  
T Cells by Anti-Idiotypic Antibodies and Specific Antigen\*

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Essentially all anti-phosphorylcholine (PC)<sup>1</sup> antibodies produced by BALB/c mice bear an idiotype that is indistinguishable from that of TEPC-15 myeloma protein (T15) (1, 2). These mice, therefore, are virtually tolerant to stimulation by PC antigen after a neonatal injection of antibodies specific to the T15 idiotype (T15id). We have previously identified suppressor cells specific for the anti-PC response in idiotypically suppressed mice (3). The cells responsible for active suppression are represented in both the T and non-T-cell populations (3, 4). Similar suppressor cells have been also found in other systems of idiotype suppression (5, 6), suggesting that such idiotype-specific suppressor cells may play an important role in maintaining suppression, perhaps after the initial depletion of cell populations bearing specific Ig markers (7).

In this study, the mechanisms of idiotype suppression have been investigated by examining the generation of specific suppressor cells in vitro. We report here that anti-T15id antibodies, together with specific antigen, generate idiotype-specific suppressor T cells in vitro. This result suggests that suppressor T cells found in idiotypically suppressed mice may have been similarly generated.

Materials and Methods

*Mice.* 7- to 10-wk-old BALB/c mice were purchased from Cumberland View Farms, Clinton, Tenn.

*Antigens.* A rough strain of *Streptococcus pneumoniae*, R36a was obtained from the American Type Culture Collection, Rockville, Md. A vaccine of R36a was prepared by treatment with 0.5% formalin in 0.15 M NaCl (3) and the vaccine was used as a PC antigen. As a control antigen, 2,4-dinitrophenyl-lysyl-Ficoll (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) (DNP-Lys-Ficoll), prepared by the method of Sharon et al. (8), was used throughout the experiments in this study.

*Antisera.* The TEPC-15 myeloma protein (T15) was purified by using a PC-conjugated Sepharose 4B column (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) as described previously (4, 9). Anti-T15 antibodies were prepared in the ascitic fluid of A/He mice by multiple injections of T15 protein according to the method of Tung et al. (10). The ascites was clarified by centrifugation and the fluid was further absorbed using immunoabsorbent columns conjugated with PC-binding myeloma proteins possessing different idiotypes (i.e. MOPC-167

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<sup>1</sup> *Abbreviations used in this paper:* Anti-id, anti-idiotype; BSS, Hank's balanced salt solution; DNP-Lys-Ficoll, 2,4-dinitrophenyl-lysyl-Ficoll; KLH, keyhole limpet hemocyanin; PC, Phosphorylcholine; PFC, Plaque-forming cells; SRBC, sheep erythrocytes; T15, TEPC-15 myeloma protein; T15id, Idiotype of TEPC-15 myeloma protein.

and McPC-603). The resulting anti-idiotypic (anti-T15id) ascitic fluid was specific for only T15id when examined by radioimmunoassay using  $^{125}\text{I}$ -T15 Fab,  $^{125}\text{I}$ -M603 Fab, and  $^{125}\text{I}$ -M167 Fab: 20  $\mu\text{l}$  of a 1:100 dilution of the ascitic fluid precipitated 45% of T15, but only 3% of M603 and 1% of M167 (details will be published elsewhere). A/He mice were injected similarly with a mixture of Freund's complete adjuvant and saline without T15 protein. The resulting ascitic fluid was used as a control for anti-T15id ascites.

Rabbit anti-mouse Ig was purchased from Miles Laboratories Inc., Miles Research Products, Elkhart, Ind. The antiserum was further absorbed with thymocytes of BALB/c mice. Pretreatment of normal BALB/c spleen cells with the anti-Ig serum (1:80) plus complement resulted in 92% reduction of the PFC to PC after *in vitro* stimulation.

Anti-Thy 1.2 serum, prepared in AKR mice against C3H thymocytes as described by Reif and Allen (11) was supplied by Dr. Nicholas M. Ponzio in our department. This antiserum (1:50) did not reduce the anti-PC response of spleen cells, although 46% of the cells were killed by the treatment with this antiserum and agarose-absorbed rabbit complement (12). The detailed procedure of the treatment was described previously (3).

*Enrichment of B and T Cells.* Essentially, the method of Julius et al. (13) was applied to enrich splenic B- and T-cell populations. Briefly,  $1-2 \times 10^8$  spleen cells were loaded aseptically on a 10-ml nylon wool column equilibrated with RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 2% fetal calf serum. After an incubation at 37°C for 45 min, a nonadherent cell population was separated from the column by adding warm (37°C) medium, dropwise. An adherent cell population was collected by forcing cold medium through the column using a plunger. Approximately 80% of the applied cells were recovered from the column and 50-60% of the recovered cells were in the T-cell-enriched fraction.

*Spleen Cell Cultures.* The Mishell-Dutton technique of immunization of mouse spleen cells (14) was used, with the exception that the culture medium was RPMI-1640 with 25 mM HEPES buffer (Grand Island Biological Co.). Individual cultures contained  $1.2 \times 10^7$  spleen cells and were immunized with either  $2.5 \times 10^8$  pneumococci or 1 ng of DNP-Lys-Ficoll in 25  $\mu\text{l}$ . For induction of idiotypic suppression, 100  $\mu\text{l}$  of a 1:200 dilution of anti-T15id ascites in the culture medium was added to individual cultures. The final volume of individual cultures was adjusted to 1 ml. Unless otherwise indicated, the cultures were incubated for 4 d to elicit immune responses *in vitro*.

*Generation of Suppressor Cells In Vitro.* Normal BALB/c spleen cells ( $1.2 \times 10^7$ ) were cultured for 3 d in a multiwell tissue culture plate (Falcon 3008, Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) with a 1:2,000 final dilution of anti-T15id ascites and  $2.5 \times 10^6$  R36a cells as described above. The precultured cells were washed three times with Hank's balanced salt solution (BSS) and then cocultured with  $1.2 \times 10^7$  freshly prepared normal BALB/c spleen cells in the presence of the same quantity of R36a. After 4 d of additional incubation, immune responses of individual cultures to PC were determined by a hemolytic plaque assay.

*Hemolytic Plaque Assay.* The number of plaque-forming cells was determined by a slide modification of the Jerne-Nordin hemolytic plaque technique (15). To detect plaque-forming cells (PFC) to PC, sheep erythrocytes (SRBC) were coated with R36a C-polysaccharide employing chromic chloride (16) and used as the specific target cells (1). For target cells of DNP-specific PFC, SRBC were conjugated with trinitrobenzene sulfonic acid to yield TNP-SRBC (17).

*Enumeration of PFC Producing Anti-PC Antibodies with T15id.* The number of PFC secreting antibodies bearing T15id was determined by the method of Cosenza and Köhler (18). Spleen cells immunized with R36a for 4 d *in vitro* were incubated for 1 h with a 1:400 final dilution of anti-T15id ascites in BSS. The number of plaques was counted after an additional 1-h incubation with complement, and compared with the number of plaques developed in the absence of anti-T15id antibody.

## Results

*In Vitro Generation of Idiotypic-Specific Suppressor Cells.* The possibility that anti-idiotypic (anti-id) antibodies were involved in the generation of idiotypic-specific

TABLE I  
*Generation of Idiotype-Specific Suppressor Cells In Vitro by Treatment with Anti-Idiotype Antibodies*

Spleen cells/culture*		PFC/culture		
Precultured	Fresh normal	PC total‡	T15(+ )§	DNP total
			%	
0	$2.4 \times 10^7$	$3,520 \pm 130$	97	$2,016 \pm 207$
0	$1.2 \times 10^7$	$2,510 \pm 270$	98	$2,620 \pm 331$
$1.2 \times 10^7$ + control	$1.2 \times 10^7$	$1,130 \pm 130$	93	$1,423 \pm 338$
$1.2 \times 10^7$ + anti-T15id	$1.2 \times 10^7$	$65 \pm 35$	1	$1,945 \pm 261$

\* Normal BALB/c spleen cells were incubated for 3 d with either control or anti-T15id ascitic fluid (1:2,000) in the presence of an immunizing dose ( $2.5 \times 10^6$  cells) of R36a. The treated cells were washed three times with BSS and then added to fresh normal BALB/c spleen cells. Individual cultures were adjusted to a 1-ml final volume. The cell mixture was incubated for 4 d with either  $2.5 \times 10^6$  R36a or 1 ng of DNP-Lys-Ficoll.

‡ Individual cultures were assayed for their specific PFC using either PC-SRBC or TNP-SRBC, respectively. The number of PFC per culture represents the geometric mean and standard error of determinations for individual triplicate cultures.

§ The number of PFC producing anti-PC antibodies with T15id was determined by specific inhibition of plaque formation by treatment with anti-T15id antibodies.

suppressors was examined by direct treatment of normal spleen cells in vitro with anti-id antibodies. To determine the suppressor cell activity, normal spleen cells that were incubated for 3 d with anti-T15id antibodies and R36a were subsequently washed and cultured with fresh normal BALB/c spleen cells in the presence of R36a or a control antigen, DNP-Lys-Ficoll. The spleen cells which had been preincubated with anti-T15id antibodies were capable of suppressing the anti-PC response of fresh normal spleen cells, whereas spleen cells treated with the control ascites were not inhibitory (Table I). The suppression was specific to PC because those precultured cells did not inhibit the immune response of normal spleen cells to DNP-Lys-Ficoll. These results indicated that preincubation of normal spleen cells with anti-T15id antibodies resulted in generation of a suppressor cell population that was specific with the anti-PC response.

Idiotype analysis was performed using the plaque-inhibition technique (18) to examine the specificity of idiotype suppression mediated by the in vitro generated suppressor cells (Table I). Either spleen cell cultures of normal BALB/c mice or those receiving spleen cells pretreated with R36a and control ascites, induced a normal level of PFC producing anti-PC antibodies with T15id: the number of T15id-producing PFC was >93% of the total PFC. In contrast, BALB/c spleen cells cocultured with suppressor cells were able to mount neither a normal level of anti-PC response (<6% of control response) nor a significant proportion of T15id-bearing PFC (only 1%) in the anti-PC response. This result indicates that anti-T15id-induced suppressor cells can interfere specifically with the induction of PFCs that produce anti-PC antibodies bearing T15id.

*Requirement of a Specific Antigen for Generating Idiotype-Specific Suppressor Cells.* We extended our study to determine whether the presence of specific antigen was necessary for the generation of idiotype-specific suppressor cells in vitro. Table II clearly demonstrates that the presence of the PC-antigen, R36a, was necessary in addition to anti-T15id antibodies for inducing these suppressor cells. The possibility that the antigen alone might induce antigen-specific suppressor T cells was also explored. Cells

TABLE II  
*Requirement of Specific Antigen for Generation of Idiotypic-Specific Suppressor Cells In Vitro*

Cells added to fresh culture*			Anti-PC response	
Cell No.	Antibodies	Antigens	PFC/culture	T15(+) %
0			3,413 ± 266	NT‡
1.2 × 10 <sup>7</sup>	BSS	R36a	2,560 ± 206	97
1.2 × 10 <sup>7</sup>	Control	R36a	3,038 ± 337	93
1.2 × 10 <sup>7</sup>	Anti-T15id	R36a	543 ± 69	6
1.2 × 10 <sup>7</sup>	Control	DNP-Lys-Ficoll	1,835 ± 175	92
1.2 × 10 <sup>7</sup>	Anti-T15id	DNP-Lys-Ficoll	1,805 ± 81	89

\* Normal spleen cells, were cultured for 3 d with BSS, control or anti-T15id ascitic fluid. As a stimulating antigen, either 2.5 × 10<sup>6</sup> R36a or 1 ng of DNP-Lys-Ficoll was added to the groups of cultures. The treated cells were washed and then added to 1.2 × 10<sup>7</sup> fresh normal BALB/c spleen cells for an additional 4-d incubation in the presence of R36a.

‡ Not tested.

preincubated with either R36a alone or control ascites plus R36a, did not exhibit suppressor cell activity under these experimental conditions. In contrast, only the cells cultured with anti-T15id antibodies plus R36a resulted in suppression of both T15id production and anti-PC response when added to normal spleen cells.

In addition, the possibility that anti-T15id antibodies may generate idiotypic-specific suppressor cells in the presence of an antigenic stimulation with non-PC-containing antigen was also examined. Cells treated with anti-T15id antibodies plus DNP-Lys-Ficoll did not cause a specific decrease in the response to PC when compared with the effect of control ascites plus DNP-Lys-Ficoll, although a slight reduction of the anti-PC response was noticed in both cultures. No significant alteration of the idiotypic profile was observed in the cultures receiving pretreated spleen cells with either anti-T15id plus DNP-Lys-Ficoll or control ascites plus R36a or DNP-Lys-Ficoll (Table II). These results indicate that generation of idiotypic-specific suppressor cells in vitro requires specific antigen in addition to anti-id antibodies.

*T-Cell Nature of the Suppressor Population.* The cell type of the suppressor population was determined using anti-Thy 1.2 and anti-Ig sera in the presence of complement (Table III). The suppressor cells which were generated in vitro by incubation with anti-T15id antibodies and antigen were treated with BSS, complement alone, or antibodies to Thy 1.2 or mouse Ig and complement. The treated cells were washed and resuspended into 0.5 ml of medium. These cells were mixed with 0.5 ml of syngeneic spleen cell suspension containing 1.2 × 10<sup>7</sup> cells. The cell mixture was cultured for an additional 4-d period and, then assayed for their anti-PC production. When the suppressor cells were treated with either BSS, complement alone, or anti-Ig plus complement, they were still capable of suppressing the anti-PC response of normal spleen cells. However, treatment of suppressor cells with anti-Thy 1.2 plus complement abrogated virtually all the suppressive activity (Table III), indicating that the suppressor population is T cell in nature.

*Generation of the Suppressor Population from Isolated T Cells.* To confirm the cell type of suppressor, T- and B-cell populations of normal spleen were enriched by using a nylon wool column (13). These T and B cells were pretreated separately for 3 d with anti-T15id antibodies and R36a. The pretreated cell populations were washed and

TABLE III  
Susceptibility of Anti-Idiotype-Induced Suppressor Cells to the Treatment with Anti-Thy 1.2 and Complement

Precultured spleen cells*		PFC/culture
Pretreatments	Treatments	
0		1,697 ± 250
$1.2 \times 10^7$ control	—	1,372 ± 162
$1.2 \times 10^7$ anti-T15id	—	205 ± 35
$1.2 \times 10^7$ anti-T15id	C'	378 ± 28
$1.2 \times 10^7$ anti-T15id	anti-Ig + C'	250 ± 30
$1.2 \times 10^7$ anti-T15id	anti-Thy 1.2 + C'	1,283 ± 33

\* Normal spleen cells were incubated with either control or anti-T15id ascites for 3 d in the presence of R36a. The cells were then washed three times with BSS and treated with either BSS, C' alone, or antibodies to Thy 1.2 or Ig plus C'. The treated cells were washed and resuspended in 0.5 ml of fresh medium. These cells were then mixed with 0.5 ml of normal BALB/c spleen cell suspension containing  $1.2 \times 10^7$  cells. The cell mixture was cultured for 4 d before their PFC was assayed against PC-SRBC.

TABLE IV  
Generation of Idiotype-Specific Suppressor Cells from Nylon Wool Nonadherent Lymphocytes by Treatment with Anti-Idiotype Antibodies and Specific Antigen

Cells added to fresh culture*		PFC/culture	Control response
Cell types‡	Treatment		
			%
B cells	Control	5,070 ± 491	100
B cells	Anti-T15id	5,017 ± 423	99
T cells	Control	5,078 ± 358	100
T cells	Anti-T15id	1,100 ± 160	21
None		6,617 ± 101	

\* Nylon wool-separated spleen cells ( $1 \times 10^7$ ) were incubated for 3 d with either control or anti-T15id ascites in the presence of R36a. The pretreated cells were then added to  $1.2 \times 10^7$  freshly prepared normal spleen cells for an additional 4-d culture with R36a.

‡ Nylon wool adherent and nonadherent spleen cell populations were used as B and T cells, respectively.

then mixed with fresh normal spleen cells. After 4 d of culture with R36a, individual cultures were assayed for their PFC response to PC. T-cell populations, treated with anti-T15id antibodies, were capable of suppressing anti-PC response of normal spleen cells, whereas an aliquot of the same T-cell population treated with control ascites were not. In contrast, the B-cell population, treated with either control or anti-T15id ascites, did not inhibit the anti-PC response (Table IV). These results, therefore, indicate that the idiotype-specific suppressor cells induced in vitro are only derived from the T-cell population and the presence of B cells is not necessary for the suppressor induction.

### Discussion

We have demonstrated here that the presence of PC-specific antigen along with the anti-T15id antibody is necessary for generating T15id-specific suppressor T cells in vitro. A similar mechanism requiring specific antigens may also be operating in

induction and stimulation of suppressor cells *in vivo*. A previous report by Bangasser et al. (6), suggesting that the presence of antigen can enhance the generation of idio-type-specific suppressor cells *in vivo*, supports this possibility. In contrast, without deliberate antigenic stimulation, the presence of idio-type-specific suppressor cells has been demonstrated in mice rendered unresponsive to PC by neonatal injection with anti-T15id antibodies (3). This discrepancy may be explained by the fact that PC-antigen is widely distributed in a variety of microorganisms that normally reside within the gastrointestinal and respiratory tracts (19). Therefore, deliberate antigenic stimulation may not be necessary to generate suppressor cells specific for anti-PC *in vivo*.

*In vitro* generation of suppressor T cells by treatment with antigens alone has been demonstrated by a number of laboratories (20–22). Under the conditions used in this study, however, the specific antigen alone did not induce a significant level of suppressor cells. The lack of generation of antigen-specific suppressor T cells in our system by antigen alone appeared to be a result of the nature and concentration of antigen used; i.e. optimal immunizing dose of particulate antigen. In contrast, either higher concentrations of antigens (21) or a different form of antigen (tolerogen) containing the same antigenic determinants (22) have been used to induce antigen-specific suppressor T cells *in vitro*.

Recently, Bottomly et al. (23), using an adoptive transfer system, demonstrated that treatment of keyhole limpet hemocyanin (KLH)-primed T cells with anti-T15id antibodies resulted in generation of a suppressor T-cell population which specifically inhibits helper T-cell function for the secondary immune response of PC-primed B cells to PC-KLH. Therefore, anti-id antibodies, together with specific antigens, appear to be capable of inducing at least two separate suppressor T-cell populations which inhibit specifically either B cells (3) or helper T cells (23), possibly depending on the nature of the stimulating antigens, i.e. T-independent or T-dependent antigens, respectively.

Suppressor cells generated *in vitro* by treatment with anti-T15id antibodies and antigen were apparently derived from T-cell precursors, because only the nylon wool-enriched T-cell fraction can induce such specific suppressor cells (Table IV). Treatment of the nylon wool-adherent B cells with anti-T15id antibodies and antigen did not result in induction of suppressor cells. The presence of idio-type-specific non-T suppressor cells has however been demonstrated previously in athymic nude mice rendered tolerant to PC-antigen by neonatal treatment with anti-T15id antibodies (4). Moreover, B cells from either idiotypically suppressed, antigen-stimulated animals (24) or antigen-stimulated congenic animals that lack the particular (anti-arsenate) idio-type (25) were also able to suppress the production of antibodies bearing the suppressed idio-type. Therefore, the mechanism of the generation of an idio-type-specific suppressor B-cell population is most likely different from that of a suppressor T-cell population.

It is not yet clear how anti-id antibodies and specific antigen participate in the generation of idio-type-specific suppressor T cells. Although idio-type-specific suppressor T cells appear to directly recognize B cells bearing the corresponding idio-type (26), the presence of nylon wool-adherent B cells and macrophages may not be necessary for induction of the suppressor cells (Table IV). A suppressor T-precursor cell may require both triggering signals of anti-id antibodies and specific antigen to

be a functionally mature suppressor T cell. Alternatively, precursors of the suppressor T cell may differentiate to mature T cells after an interaction with anti-id antibodies and an antigen-specific helper T-cell population (27, 28) which had been induced by specific antigen. Recent reports demonstrating that generation of either suppressor or cytotoxic T cells requires a population of antigen-specific helper T cells (29, 30) suggest that PC-specific helper T cells are most likely participating in the generation of idiotype-specific suppressor T cells in this system.

### Summary

Normal BALB/c spleen cells are unresponsive in vitro to the phosphorylcholine (PC) determinant in the presence of anti-idiotypic antibodies specific for the TEPC-15 myeloma protein (T15) which carries an idiotypic determinant indistinguishable from that of most anti-PC antibodies in BALB/c mice. The possibility that idiotype-specific suppressor cells may be generated during the culture period was examined by coculturing the cells with untreated syngeneic spleen cells. Cells that had been preincubated with anti-T15 idiotype (anti-T15id) antibodies and a PC-containing antigen, R36a for 3 d, were capable of specifically suppressing the anti-PC response of fresh normal spleen cells, indicating that idiotype-specific suppressor cells were generated during the culture period. The presence of specific antigen also appeared to be necessary because anti-T15id antibodies and a control antigen, DNP-Lys-Ficoll, were not capable of generating such suppressor cells. Suppressor cells were induced only in the population of spleen cells nonadherent to nylon wool and the suppressive activity was abrogated by treatment with anti-Thy 1.2 serum and complement. These results indicate that anti-idiotypic antibodies and specific antigen can generate idiotype-specific suppressor T cells in vitro. These in vitro results may reflect in vivo mechanisms of idiotype suppression.

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