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Autologous anti-idiotypic antibody response is regulated by the level of circulating complementary idiotype

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SUMMARY

BALB/c mice injected with lyophilized vaccine from *Streptococcus pneumoniae* R36a (Pn) predominantly responded with antibody molecules the vast majority of which expressed the public idiotype T15 and were directed to the immunodominant epitope phosphorylcholine (PC). However, after a single immunization with Pn vaccine young (3-month-old) BALB/c mice did not produce any specific anti-T15 antibody response. In contrast, young D1.LP mice were able to mount a specific anti-T15 response upon primary immunization with pneumococcal vaccine. The anti-PC response in the two mouse strains differed in that the proportion of antibody molecules that expressed the T15 idiotype for Pn-primed D1.LP mice showed a smaller proportion of PC-specific antibody expressing the T15 idiotype. Neonatal injection of anti-T15 monoclonal antibodies led to a long-term suppression of the PC-specific $T15^+$ B-cell clones but at young/adult age these mice maintained the ability to produce a normal amount of PC-specific antibody. Interestingly, the idiotypically-suppressed BALB/c mice mounted a significant anti-T15 response during the primary response to Pn. We interpreted these data as showing that the level of circulating idiotype may regulate the production of the complementary anti-idiotypic antibody. In addition, *in vitro* experiments demonstrated that the lack of the anti-T15 response during primary antibody response in BALB/c mice is probably because of a state of tolerance that is regulated by T cells.

INTRODUCTION

Antibody response to antigens is sometimes accompanied by the appearance of autoantibodies that bind to idiotypic determinants on the variable region of the antigen-specific antibody. Since Jerne proposed his theory of an idiotype (Id) network,[1] idiotypic determinants have been considered to play an important role in the regulation of immune response.

Anti-Id antibody has been implicated in the down-regulation of antigen-specific response and B-cell tolerance,[2,3] the decline of antigen-specific response in aging,[4,5] suppression of allograft rejection in pregnancy[6] and in the pathogenesis of various diseases related to immune disorders.[7] The antibody response to phosphorylcholine (PC), an immunodominant epitope of certain pneumococci, has been extensively studied both at cellular and molecular level[8,9] and it was found that the PC-specific response was dominated by antibody molecules encoded by V_H-1(S-107), D_HFL16.1, J_H1, V_κ22 and J_K5 and that expressed the public idiotype T15. The myeloma protein TEPC-15 (IgA/κ) represents the prototype of the anti-PC response.[8] Furthermore, using specific monoclonal antibodies, individual idiotopes of the T15 family were mapped on the variable region of TEPC-15.[10]

For the above-mentioned reasons the anti-PC response may be considered an excellent model to investigate various aspects of the anti-idiotypic response. It was demonstrated that the PC-response differed according to the mouse strain in regard to its magnitude and Id repertoire.[11] For example, BALB/c mice were able to mount a vigorous response to PC while D1.LP was one of the poorest responder strains to Pn administration and in this mouse strain the response was characterized by a much lower proportion of $T15^+$ antibody.[12] We decided to address the question as to whether a different level of circulating T15 idiotype may regulate the anti-T15 antibody response and we speculated that the high level of T15 in BALB/c mice may be able to render these mice tolerant to the T15 Id itself.

In contrast, D1.LP mice, due to the low level of T15 antibody, should not acquire tolerance to T15 Id. Therefore, one could expect that modification of the idiotypic repertoire in a certain mouse strain may up-regulate or down-regulate the antibody response to a specific idiotype. It is well known that neonatal injection of specific anti-T15 monoclonal antibodies led to a long-term suppression of B cells expressing the complementary Id.[13,14] However, at young/adult age the Id-suppressed mice were able to mount a robust, undiminished antibody response, but such a response was characterized by the lack of the dominant T15 Id. By this procedure we obtained

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a group of young BALB/c mice showing an idiotypic repertoire of the PC-specific response similar to that of D1.LP mice.

Our results indicated that the level of circulating idiotype may induce a state of tolerance, regulated by T cells, to self-Id.

MATERIALS AND METHODS

Animals and antigens

BALB/c mice of either sex and female D1.LP mice were purchased from Charles River Laboratories (Milan, Italy) and then caged in a clean restricted-access room; BALB/c mice were bred in our facility. Lyophilized vaccine from *Streptococcus pneumoniae* strain R36a (Pn) for *in vivo* immunization was prepared as follows: bacteria from frozen stock were grown on trypticase soy agar plate with 5% sheep blood and the growing colonies were then inoculated in Todd–Hewitt broth supplemented with 1% yeast extract (both from DIFCO Lab., Detroit, MI). One subsequent passage was made in broth for 5 to 6 hr and the early log phase pneumococci were harvested by centrifugation and washed in cold phosphate-buffered saline (PBS). The bacteria were then lyophilized overnight. The *in vivo* primary response was evaluated in mice injected with 15 μg of Pn vaccine (i.p.) resuspended in 250 μl of PBS. Furthermore, formaldehyde-treated Pn antigen for *in vitro* stimulation of lymphocytes was prepared according to previously described procedure.[15]

Neonatal injection of anti-T15 monoclonal antibodies

Anti-T15 hybridomas AB1-2 and B24-44 were grown in the appropriate medium. The origin, maintenance and specificity of the monoclonal antibodies (mAb) are described elsewhere.[10,16] The mAb were then purified by ammonium sulphate precipitation[17] and then stored at $-80°$ until used. BALB/c mice were bred in our facility. On days 1 and 3 after birth the newborn mice were injected (i.p.) with an equimolar mixture of anti-T15 mAb resuspended in saline solution (25 μg of antibody in 100 μl/mouse).

Lymphocytes preparation and cultures

Spleen cells from Pn-primed BALB/c mice (either normal or idiotypically suppressed) were removed in sterile conditions, the cell suspensions were prepared and then pooled. Two different procedures were used to isolate T or B cells. B-cell-rich fractions were prepared by cytotoxic depletion of T cells from splenocyte suspensions using two treatments with mAb HO-13-4 (anti-Thy 1.2) plus complement; T-cell-rich fractions were prepared by panning of lymphocytes on Petri dishes coated with goat anti-mouse immunoglobulin (Sigma Chemicals, St Louis, MO). CD4 and CD8 cells were then isolated by cytotoxic treatment with mAb 3.155 (anti-CD8) and GK1.5 (anti-CD4) respectively, as described for B-cell preparation. Flow-cytometric assay determined that both B and T cell-enriched fractions were 95–98% pure. The *in vitro* antibody response to PC was initiated in flat-bottomed wells of 96-well plates. Each well contained 10^6 B cells either alone or with T cells (1×110^5–3×10^5). Each day 10 μl of feeding medium, prepared as described by others,[18] were added to the cultures. Cultures containing antigen were set up in 12 replicas; control cultures in triplicate. The cells were maintained in culture for 6 days and then the anti-PC and anti-T15 antibody responses were evaluated by enzyme-linked immunospot assay.

Enzyme-linked immunospot assay (ELISPOT)

The *in vivo* and *in vitro* antibody responses were evaluated, at the level of single antibody-forming cell (AFC) by ELISPOT assay. Nitrocellulose filter discs (0·45 μm pore size and 47 mm diameter; Schleicher and Schuell, Keene, NH) were incubated with either PC–bovine serum albumin (BSA) (50 μg/ml) or purified myeloma proteins TEPC-15 or MOPC-315 overnight at 4° in a humid chamber. Membranes were rinsed with ice cold PBS and then blocked with PBS containing 2% fetal calf serum (FCS) and 2% casein (Sigma) for 3 hr at 37°. Spleen cells were added at various number in 0·5 ml of ELISPOT medium (RPMI-1640, 5% FCS, and Cytodex 1) for 4–6 hr at 37° 5% CO_2. The membranes were then treated with PBS–ethylene diamine tetraacetic acid (EDTA) (10 mM) for 10 min; rinsed and incubated with horseradish peroxidase (HRP)-labelled antibody against mouse κ-chain (anti-PC response) or IgG immunoglobulins (anti-Id response) overnight at 4°. The membranes were then washed and finally incubated with HRP-color development reagents (BioRad, Richmond, CA) for 5–10 min. The reaction was stopped with deionized water and the visible spots were scored. A variation of the same technique was used to enumerate PC-specific AFC producing $T15^+$ antibody. Splenocytes were incubated on PC–BSA coated filters as described above. The filters were then developed with a mAb, AB1-2 that reacts with specific T15 idiotope. The anti-Id was biotin-labelled with biotin-*N*-hydroxysuccinamide (Vector Lab., CA) using a protocol supplied by the manufacturer. The discs were then rinsed and incubated with streptoavidin labelled with alkaline phosphatase (Boeringher Mannheim, Germany) for 2 hr at room temperature in a humid chamber. After developing bound enzyme activity with nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (Boeringher Mannheim) the visible spots were enumerated.

Statistical analysis

The *in vivo* results were analysed by the Wilcoxon Rank test; the variability of the *in vitro* cultures was analysed by the Mann–Whitney *U* test. In both cases the *P* values were considered significant at $P \leq 0{\cdot}05$.

RESULTS

Anti-T15 response in BALB/c and D1.LP mice

Previous experiments indicated that the ELISPOT assay may detect specific anti-Id AFC with very high efficiency.[19] Three-month-old BALB/c mice were immunized with a single (i.p.) injection of Pn vaccine. A kinetic study of anti-PC and anti-T15 responses was performed. As expected, a sharp increase of the PC-specific AFC (Table 1) was detectable on day 3, the response reached a peak on day 6 and then slowly declined starting on day 9. At the same time we failed to detect any specific anti-T15 response. The anti-Id response in young D1.LP showed a different pattern (Table 2) for on day 6 we observed a significant increase of specific anti-T15 AFC. The anti-Id response further increased on day 9, reaching the peak on day 15.

The auto anti-Id response appeared to be heavily influenced by the genetic make-up of the host.

Table 1. Anti-T15 response in BALB/c mice—primary immunization (ELISPOT plaques/spleen)

No.	Day	PC*	Anti-TEPC-15†	Anti-MOPC315†	P‡
4	0	50 ± 10	35 ± 11	35 ± 10	—
4	3	5500 ± 900	35 ± 15	31 ± 9	—
4	6	45500 ± 8500	20 ± 7	20 ± 10	—
4	9	43000 ± 9000	40 ± 9	30 ± 15	—
3	15	29000 ± 6500	30 ± 12	40 ± 15	—

*Anti-PC response in 3-month-old BALB/c mice upon Pn primary immunization. The response was evaluated, by ELISPOT assay, at different times after the antigenic stimulation (day 0 = not immunized). The values represent the number of κ-chain PC-specific AFC. Means were from number (No.) of mice/group (±SE).

†Anti-idiotypic (T15) response on filters coated with either TEPC-15 myeloma protein ($T15id^+$, IgA/κ) or MOPC-315 myeloma protein ($T15id^-$, IgA/κ). The values represent the number of anti-Id AFC of IgG isotype.

‡Probability (*P*) values were calculated by Wilcoxon Rank test. The *P* values were omitted when not significant. The *P* values refer to anti-T15 and anti-MOPC-315 responses only.

Pn-primary response in BALB/c, D1.LP and Id-suppressed BALB/c strain

At the age of 3 months Id-suppressed mice were immunized with a single dose of Pn vaccine. In Table 3 we compared the magnitude and idiotypic profile of PC-specific response of normal BALB/c, normal D1.LP and Id-suppressed BALB/c mice during the primary response. BALB/c mice (Group I) showed a vigorous response to PC and the vast majority of specific antibody were $T15^+$. The presence of the T15 idiotype was assessed by the presence of the idiotope AB1-2 which is considered a canonical marker of the T15 idiotype.[20] Idiotype-suppressed mice (Group II) displayed a slightly reduced PC-response and the proportion of $T15^+$ AFC dropped to 12%. As expected the D1.LP (Group III) showed a lower response to immunization with Pn vaccine as compared to BALB/c mice. In the D1.LP mouse strain the percentage of $T15^+$ AFC was 25%. Thus, we had the possibility to test the hypothesis that manipulation of the idiotypic repertoire may regulate the production of the complementary anti-Id antibody.

Anti-idiotypic response in Id-suppressed mice

In Table 4 we reported the results of the study of PC and T15-specific responses in Id-suppressed BALB/c mice upon Pn primary immunization. The profile of anti-Id response is comparable to that of D1.LP mice; also in this case we detected a significant level of anti-T15 AFC on day 6 after the immunization, followed by a marked increase on days 9 and 15. It appeared that a high level of $T15^+$ antibody may down-regulate the anti-T15 response suggesting the possibility of a state of tolerance to self-Id.

In vitro anti-Id response

We then tested the hypothesis that the state of tolerance to T15 may be related to T cells. B cells from Pn-primed mice were cultured in presence of T cells isolated either from normal or Id-suppressed BALB/c mice. As shown in Table 5 addition of T cells (at any number added) from both groups of donors were able to augment the anti-PC response. However, at the same time, only T cells from Id-suppressed mice were able to induce

Table 2. Anti-T15 response in D1.LP mice—primary immunization (ELISPOT plaques/spleen)

No.	Day	PC*	Anti-TEPC-15†	Anti-MOPC315†	P‡
4	0	40 ± 10	20 ± 15	25 ± 10	—
4	3	2500 ± 200	29 ± 11	18 ± 9	—
4	6	22000 ± 5500	225 ± 45	25 ± 10	0·03
4	9	25000 ± 3000	490 ± 120	20 ± 10	0·035
3	15	19000 ± 3500	700 ± 50	40 ± 15	0·025

*Anti-PC and anti-T15 responses in 3-month-old D1.LP mice upon Pn primary immunization. The responses were evaluated, by ELISPOT assay, at different times after the antigenic stimulation (day 0 = not immunized). The values represent the number of κ-chain PC-specific AFC. Means were from number (No.) of mice/group (±SE).

†Anti-idiotypic (T15) response on filters coated with either TEPC-15 myeloma protein ($T15id^+$, IgA/κ) or MOPC-315 myeloma protein ($T15id^-$, IgA/κ). The values represent the number of anti-Id AFC of IgG isotype.

‡Probability (*P*) values were calculated by Wilcoxon Rank test. The *P* values were omitted when not significant. The *P* values refer to anti-T15 and anti-MOPC-315 responses only.

Table 3. Antibody forming cells (AFC)/spleen in D1.LP, BALB/c and Id-suppressed BALB/c mice

Groups*	No.	AFC/spleen†	AB1-2$^+$AFC‡	%
I	6	45 500 ± 6500	38 600 ± 4000	84
II	5	38 500 ± 6000	4 600 ± 900	12
III	6	22 000 ± 5500	5 500 ± 2100	25

* Different groups of mice were immunized with a single injection of Pn vaccine and 6 days later the antibody responses were evaluated. Group I = normal BALB/c; Group II = Id-suppressed BALB/c; Group III = normal D1.LP. The values represent the arithmetic means from *n*/animals group ± SE.

† The κ-chain specific response was enumerated by ELISPOT assay.

‡ The amount of T15Id$^+$ PC-specific AFC was calculated enumerating the AB1-2 Id$^+$ AFC.

the production of specific anti-Id antibody. T cells from Id-suppressed mice when added did rescue the anti-Id response of Pn-primed B cells while T cells from normal Pn-primed mice did not do so. Addition of 1×10^5 T cells from Id-suppressed mice determined an increase, even though not statistically significant, of the number of anti-Id AFC as compared to the same amount of T cells from normal BALB/c (16 ± 4 and 8 ± 4 respectively). Addition of higher number of T cells from Id-suppressed mice led to a further increase of anti-Id response. In these cases the increases were marginally significant.

Purification of T-cell subsets from Id-suppressed mice (CD4 and CD8 cells) enabled us to determine that the ability to increase the specific anti-Id response was due to CD4 cells. In contrast, CD8 cells did not have any regulatory activity, either on anti-PC or on anti-T15 response. When CD4 cells from Id-suppressed mice were added to the cultures (Table 6) we observed a marginally significant increase of the anti-Id response. These experiments pointed out that a single immunization with Pn vaccine in mice neonatally injected with anti-T15 mAb induced the proliferation of T helper cells specifically involved in the generation of the anti-idiotypic antibody response.

DISCUSSION

The main finding of this study is that a high level of circulating idiotype may induce a state of tolerance to self-Id. A single injection of Pn vaccine in BALB/c mice induced a vigorous anti-PC response mediated by antibody a large fraction of which (> 85%) expressed the public idiotype T15. Despite the remarkable level of T15$^+$ antibody molecules, a kinetic study failed to detect any specific anti-T15 antibody response.

Study of anti-T15 response upon Pn primary immunization in the past, obtained contrasting results, probably depending on technical difficulties to detect anti-Id antibody. For example, other investigators have employed the haemolytic plaque assay for direct detection of anti-Id-producing cells, in which the purified immunoglobulin expressing the complementary idiotype was coupled to red blood cells. However, using this method it was reported that Pn-immunized mice produced certain number of T15-specific AFC on day 6 after the challenge[21] while others[2] failed to observe this phenomenon. It was possible that, in this case, chemical coupling to red blood cells may modify their susceptibility to antibody-mediated lysis. In our experiments we employed the ELISPOT assay and the specificity control we performed showed us the reliability of this method.[19]

In contrast to BALB/c mice, 3-month-old D1.LP mice were able to produce a significant amount of specific anti-Id AFC on day 6 after Pn-immunization; the magnitude of the anti-Id response further increased on day 9. The PC-specific response in BALB/c and D1.LP mice differed in regard to either its magnitude and idiotypic repertoire.[11,12] Almost all PC-antibody produced by BALB/c mice were T15$^+$ while only a minority of those produced by D1.LP were T15$^+$. In addition, it was reported that BALB/c mice have detectable levels of T15$^+$ immunoglobulin in normal preimmune sera, presumably due to an environmental stimulation.[22]

We speculated that the concentration of T15 in these animals may maintain a state of tolerance in T15-reactive lymphocytes. On the other hand, D1.LP mice may not acquire tolerance to the T15 idiotype because the concentration of T15$^+$ antibody is too low. Consistent with the tolerance

Table 4. Anti-T15 response in idiotypically-suppressed BALB/c mice—primary immunization (ELISPOT plaques/spleen)

No.	Day	PC*	Anti-TEPC-15†	Anti-MOPC-315†	*P*‡
2	0	35 ± 10	35 ± 15	25 ± 12	—
4	3	4000 ± 650	25 ± 10	30 ± 14	—
5	6	38 500 ± 6000	540 ± 90	30 ± 15	0·04
5	9	30 000 ± 11 000	950 ± 140	25 ± 18	0·025
3	15	25 000 ± 8500	1100 ± 200	40 ± 10	0·03

* Anti-PC and anti-T15 responses in 3-month-old BALB/c mice, neonatally injected with anti-T15 mAb, upon Pn primary immunization. The responses were evaluated, by ELISPOT assay, at different times after the antigenic stimulation (day 0 = not immunized). The values represent the number of κ-chain PC-specific AFC. Means were from number (No.) of mice/group (± SE).

† Anti-idiotypic (T15) response on filters coated with either TEPC-15 myeloma protein (T15id$^+$, IgA/κ) or MOPC-315 myeloma protein (T15id$^-$, IgA/κ). The values represent the number of AFC of IgG isotype.

‡ Probability (*P*) values were calculated by Wilcoxon Rank test. The *P* values were omitted when not significant. The *P* values refer to anti-T15 and anti-MOPC-315 responses only.

Table 5. Influence of T cells on anti-Id response *in vitro*

Responder cells*	T cells added†	PC-specific AFC‡	anti-TEPC-15	*P*§
B	none (no antigen)	8 ± 2	10 ± 3	—
B	none	50 ± 12	7 ± 4	
B	T1 (1×10^5)	90 ± 30	8 ± 4	
B	TS (1×10^5)	110 ± 20	16 ± 4	—
B	T1 (2×10^5)	170 ± 12	9 ± 3	
B	TS (2×10^5)	185 ± 25	25 ± 4	0·045
B	T1 (3×10^5)	250 ± 30	12 ± 5	
B	TS (3×10^5)	230 ± 15	37 ± 6	0·04

* B cells were isolated and pooled from groups (six or seven mice/group) of BALB/c primed mice and cultured for 6 days in presence or absence (where indicated) of antigen.

† T cells from six BALB/c mice (T1) and five idiotypically-suppressed (TS) donors that had received a single injection of Pn vaccine were pooled and then added to the B-cell culture at various numbers.

‡ Kappa-chain PC-specific and IgG T15-specific responses were enumerated by ELISPOT assay after 6 days culture. The values of PC-specific antibody represent the antibody response per 10^6 B cells. The values of anti-T15 antibody response represent the antibody response per 5×10^6 B cells. Specificity control for anti-T15 response was performed measuring AFC on dishes coated with MOPC-315 myeloma protein ($T15id^-$, IgA/κ) and the number of AFC did not exceed the background level (data not shown).

§ Probability (*P*) values were calculated by the Mann–Whitney *U* test and were omitted when not significant.

hypothesis is the notion that it is much more difficult to induce, in BALB/c mice, a specific response to TEPC-15 myeloma protein than to other PC-reactive proteins.[23] Thus, we reasoned that manipulation of the idiotypic repertoire may have a direct influence on the anti-Id response.

The idiotype-specific suppression induced by neonatal injection of anti-T15 mAb enabled us to modify the idiotypic repertoire of the anti-PC response in BALB/c mice. According to our working hypothesis Id-suppressed mice were able to produce specific anti-T15 antibody upon Pn primary immunization showing a profile of the anti-Id response comparable to that of singly immunized D1.LP mice. We interpreted these data as showing that high levels of idiotype-positive antibody may regulate the complementary anti-Id response.

Table 6. Influence of T-cell subsets on anti-Id response *in vitro*

Responder cells*	T cells added†	PC-specific AFC‡	anti-TEPC-15	*P*§
B	none (no antigen)	8 ± 2	12 ± 5	
B	none	50 ± 12	9 ± 4	—
B	TS/a (1×10^5)	130 ± 35	18 ± 3	
B	TS/b (1×10^5)	65 ± 30	9 ± 6	—
B	TS/a (2×10^5)	235 ± 60	27 ± 5	
B	TS/b (2×10^5)	60 ± 21	11 ± 4	0·04
B	TS/a (3×10^5)	280 ± 70	35 ± 8	
B	TS/b (3×10^5)	65 ± 8	10 ± 3	0·035

* B cells were isolated and pooled from groups (six or seven mice/group) of young (B-y) primed mice and cultured for 5 days in presence or absence (where indicated) of antigen.

† CD4 (TS/a) or CD8 (TS/b) from five Id-suppressed donors that had received a single injection of Pn vaccine were pooled and then added to the B-cell culture at various numbers.

‡ Kappa-chain PC-specific and IgG anti-T15-specific antibody responses were enumerated by ELISPOT assay after 6 days culture. The values represent the antibody response per 10^6 B cells. The values of anti-T15 antibody response represent the antibody response per 5×10^6 B cells. Specificity control for anti-Id response was performed as described in legend Table 5.

§ Probability (*P*) values were calculated by the Mann–Whitney *U* test and were omitted when not significant.

Further study indicated that splenic CD4 cells from Id-suppressed mice may rescue the anti-Id response in B cells cultures from donors normally (*in vivo*) non-producing anti-Id antibody.

The auto-anti-Id response is known to be strictly dependent on the presence of functional T cells.[24,25] At this time the mechanism(s) of T-cell involvement in the generation of anti-Id response remains unknown. It may be possible that during the primary response in BALB/c mice T15-reactive clones are present in a state of tolerance that does not allow the maturation of B cells to antibody-producing cells. The B-cell anergy, however, may be reversible by signals from T cells; so the unresponsiveness of BALB/c mice to T15 idiotype may be related to a tolerance at the level of T15-specific T helper cells. This hypothesis may be in line with a tolerance model which has been proposed.[26]

Our results indicated that the idiotypic response varies according to the genetic make-up of the host and that the expression of a certain idiotype may regulate the production of the complementary anti-Id antibody. Furthermore, the tolerance to highly-expressed self-Id appeared to be a T-cell-dependent mechanism.

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