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Molecular Immunology 39 (2002) 103–112

Molecular Immunology

www.elsevier.com/locate/molimm

A monoclonal antibody against NeuGc-containing gangliosides contains a regulatory idiotope involved in the interaction with B and T cells

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Received 18 July 2001; received in revised form 28 December 2001; accepted 8 February 2002

Abstract

P3 (IgM-κ) is a monoclonal antibody (mAb) reacting with *N*-glycolyl neuraminic acid (NeuGc)-containing gangliosides and sulfated glycolipids. To explore the nature of the idiotope defined by 1E10, we used a phage-displayed random peptide library. After three rounds of selection, seven different phagotopes were isolated. Noteworthy, all the sequences were found to bear the basic amino acid-rich motifs KPPR (3) or RRPR/K (4). This recursive selection of basic sequences by 1E10 mAb confirmed previous suggestions of the involvement of charged residues in the interaction between γ-type Ab2 and P3 mAb. The binding of 1E10 to phage peptides representing each group was completely inhibited by P3 mAb. In addition, other Ab2 to P3 were able to recognize these peptides. Thus, phage peptides seem to be mimotopes of the idiotope recognized by anti-idiotypic antibodies in P3. Phage motifs were represented in the lineal sequence of P3's heavy chain H-CDR3 and a 14-mer peptide representing this region was able to specifically inhibit 1E10 binding to P3. Previous studies showed that P3's idiotype was autoimmunogenic and shared by antibodies with different specificities. Now, we demonstrated that P3 mAb is able to activate a network cascade involving autologous anti-idiotypic and anti-anti-idiotypic T cells. Thus, P3's idiotype fulfill the three criteria previously established to define a "regulatory idiotype". Particularly, data presented here revealed the immunodominance of the H-CDR3 of this mAb as a T cell epitope. Thus, H-CDR3 is simultaneously involved in the interaction of P3 mAb with anti-idiotypic B and T cells, behaving as a potential regulatory idiotope.

Keywords: Monoclonal antibody; H-CDR3; *N*-Glycolyl neuraminic acid; Idiotype

1. Introduction

Gangliosides are normal differentiation antigens expressed on the membranes of most mammalian cell types (Fishman and Brady, 1976). Although the changes that occur in their expression pattern during oncogenic transformation make gangliosides attractive candidates for immunotherapy (Hakomori, 1981, 1985), their own self nature makes them poorly immunogenic. The fact that it is possible to induce antibody responses against gangliosides (Tai et al., 1988; Portoukalian et al., 1991; Helling et al., 1995; Alfonso et al., 1995), together with the findings in cancer patients of circulating antibodies specific for these self antigens and B cells producing them (Watanabe et al., 1982; Irie et al., 1982; Cohan et al., 1982; Tai et al., 1983; Furukawa et al., 1989) indicate that self-reactive anti-ganglioside B cells have not been clonally deleted, but remain functionally silent. The mechanisms controlling the immune response against gangliosides are still unknown, but regulatory elements of the immune network could be involved as has been described in the response to other carbohydrate antigens (Rademaekers et al., 2001).

P3 is a murine IgM monoclonal antibody (mAb) which specifically recognizes *N*-glycolyl neuraminic acid (NeuGc)-containing gangliosides and sulfated glycolipids (Vázquez et al., 1995a; Moreno et al., 1998). We previously suggested that P3 mAb idiotype fulfills two of the three criteria used by Bona (Bona, 1992) to define "regulatory idiotypes": (1) it is autoimmunogenic, and (2) it is shared by antibodies of different specificities. Two main observations led to those suggestions. First, P3 mAb induced an IgG anti-idiotypic (Ab2) response in syngeneic BALB/c mice, even when injected in saline, result that was not obtained when other murine IgM anti-ganglioside mAbs were used (Vázquez et al., 1998). Second, anti-anti-idiotypic (Ab3) antibodies elicited in syngeneic mice by most Ab2 mAbs

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0161-5890/02/$ – see front matter
PII: S0161-5890(02)00041-X

specific to P3 shared Ab1 idiotopes, but they had different antigenic specificities (Id+ Ag−; Vázquez et al., 1998).

The γ-type Ab2s generated against P3 have been distinguished by the quality of the Ab3 antibodies they elicited. Among them, 1E10 mAb represents the major group of Ab2 mAbs inducing Id+Ag− Ab3 responses. This Ab2 might, therefore, identify a potential regulatory idiotope on P3 mAb and one of the aims of the present work was to define the molecular nature of this idiotope. Based on the sequence analysis of P3 and γ-type Ab2 mAbs, we have previously predicted a major role of charged residues in idiotypic interactions between P3 and its Ab2s (Pérez et al., 2001). Important evidences favoring this hypothesis are provided in the present study through the screening of a phage-displayed 9aa-peptide library against 1E10 mAb. A basic amino acid-rich motif in the H-CDR3 of P3 mAb was identified as part of the idiotope recognized by 1E10 and related Ab2 mAbs.

Although the ability of P3 mAb to induce Ab2 antibodies of the IgG isotype in syngeneic model in the absence of carrier proteins or adjuvant first indicated the involvement of autologous T cells in response to this mAb (Vázquez et al., 1998), we had not assessed this issue directly. Now, we have demonstrated that P3 idiotype is able to activate autologous T cells, and therefore, fulfills the third of Bona's criteria to define a regulatory idiotype. A 14-mer peptide representing the sequence of H-CDR3 of P3 mAb induced specific in vitro T cell proliferation in animals immunized with P3, revealing a role for the H-CDR3 in the regulatory properties of P3 mAb's idiotype. Finally, we provide first evidences of the activation of an in vivo idiotypic network cascade involving not only anti-idiotypic (T2) but also anti-anti-idiotypic (T3) cells after immunization of BALB/c mice with P3 mAb.

2. Materials and methods

2.1. Animals

Normal and athymic nu/nu female BALB/c mice, 8–12 weeks old, were purchased from the Center for Laboratory Animal Production (CENPALAB, Havana, Cuba). Animals were housed and bred in a barrier-maintained room according to the guidelines stipulated by the Animal Subject Committee Review Board at the Center of Molecular Immunology (CIM). Animal studies were performed with approval from CIM's Institutional Animal Care and Use committees.

2.2. Peptides

A peptide composed of 14 amino acids from the heavy chain CDR3 of P3 mAb (p-H3P3), SGVREGRAQAWFAY, with a purity grade of more than 95%, was purchased from the Center of Genetic Engineering and Biotechnology (CIGB, Havana, Cuba). This peptide was synthesized according to the solid-phase method and was purified using reverse phase HPLC (Vydac C18, 10 mm × 250 mm). Peptides from the H-CDR3 (p-H3B7, amino acids 95-102: RGAEATTWLAY) and the FR1/CDR1 regions (p-FR1/H1B7, amino acids 24-35: TSGYTFTEYTMH) of the irrelevant murine mAb B7 (IgG2a-κ; Macias et al., 1999), synthesized and purified by the methods mentioned earlier, was kindly provided by J. de León (MSc), at the Center of Molecular Immunology.

2.3. Antibodies

Monoclonal anti-NeuGc-containing ganglioside antibody P3 (IgM-κ) was generated by polyethylene glycol fusion of splenocytes from hyperimmune BALB/c mice with P3-X63Ag 8-653 myeloma cells (Vázquez et al., 1995a). Syngeneic anti-idiotypic mAbs 1E10, 4F2, 1D5 and 3B11, all of them of the IgG1-κ isotype, were obtained from BALB/c mice immunized with purified P3 mAb coupled to KLH (Keyhole Limpet Haemocyanin) in Freund's adjuvant by a similar fusion procedure (Vázquez et al., 1998). The following murine mAbs were employed in the study as controls: E1 (IgM-κ: anti-GM2), A3 (IgM-κ: anti-GM1/GM2/GM3; Alfonso et al., 1995), 8A4 (IgG1-κ: anti-idiotypic mAb specific to E1), or C5 (IgG1-κ: anti-colorectal antigen; Vázquez et al., 1995b). IgM mAbs were purified from ascitic fluid by gel filtration chromatography using a Sephacryl S-300 high-resolution column (Amersham Pharmacia Biotech, Uppsala, Sweden), followed by affinity chromatography with Hi-Trap IgM purification system (Amershan Pharmacia Biotech). IgG mAbs were purified by affinity chromatography using a Protein A-Sepharose column (Amersham Pharmacia Biotech). mAb $F(ab')_2$ fragments were obtained using a standard procedure previously described (Andrew and Titus, 1997). The mAb-biotin conjugates were obtained by incubation of mAb with 100 μg/ml of *N*-hydroxysuccimide biotin (Sigma, St. Louis, MO) for 4 h at room temperature. Then 20 μl of 1 M NH_4Cl per 250 μg of biotin was added for 10 min to stop the reaction. Finally, mAb solution was dialyzed extensively with phosphate-buffered saline (PBS; Bayer and Wilckek, 1980).

2.4. Immunization protocols

To evaluate the thymus-dependency of Ab2 and Ab3 syngeneic responses, normal and athymic nu/nu BALB/c mice (5–10 mice per group) were immunized four times at 2-week intervals, intraperitoneally, with 50 μg of purified P3 mAb in PBS or 1E10 mAb emulsified for the first injection in complete Freund's adjuvant (CFA; Sigma) and incomplete Freund's adjuvant (IFA) the subsequent doses. Animal serum samples were taken before and 7 days after the last dose.

To study the capacity of P3 mAb to induce autologous T cell proliferation, mice were immunized subcutaneously

at the base of their tails with 100 μg of mAb emulsified 1:1 with CFA and a week later the mice received a booster injection of 50 μg of mAb emulsified in IFA.

2.5. Selection of peptide recognized by 1E10 mAb from the pVIII-9aa displayed library

The nonamer random peptide library displayed on the surface protein (pVIII) was constructed by Felici et al. (1991). Selection of peptides from this pVIII-9aa library was performed using the biopanning technique, essentially as previously described (ParmLey and Smith, 1988). Briefly, polystyrene Petri dishes (∅, 35 mm) were coated by overnight incubation at 4 °C with 1E10 mAb at a concentration of 10 μg/ml in 50 mM sodium carbonate buffer (pH 9.6). After washing with Tris-buffered saline containing 0.05% Tween 20 (TBS-T), antibody-coated dishes were incubated with about 3×10^{10} M13KO7 UV-killed phage particles in a total volume of 1 ml of TBS-T containing 1 mg/ml bovine seroalbumin (BSA), for 4 h at 4 °C. In the first round of selection, 1.6×10^{11} ampicillin resistance transducing units (AmpR-TU) of the pVIII-9aa library were selected against 1E10 mAb. Later, the dishes were washed with TBS-T to remove free phages, whereas those bound to the antibody were eluted with 0.1N HCl, 1 mg/ml BSA, pH 2.2 (glycine-adjusted) and then neutralized with 2 M Tris, pH 9.0. The selected phages were amplified by infecting *E. coli* TG1 and 2.8×10^{11} AmpR-TU of them were reacted again with monoclonal antibody-coated Petri dishes. After three cycles of biopanning, the selected phages were subjected to immunoscreening and DNA analysis. Phage-producing colonies, each derived from a single clone were obtained by infecting 0.4 ml of an overnight culture of TG1 bacterial cells with 10^4 AmpR-TU of the affinity-selected phage mixture, superinfecting with 10^{11} kanamicine resistance transducing unit (KanR-TU) M13K07 helper phage particles. The cells were grown in Luria Bertoni (LB) medium containing 100 μg/ml Amp, 70 μg/ml Kan and 1 mM IPTG for 5 h at 37 °C with vigorous shaking. The phage suspension was centrifuged and the supernant phages precipitated with 4% PEG 8000/0.5 M NaCl.

2.6. Nucleotide sequencing of selected clones

The nucleotide sequences of the gene VIII inserts were determined by single stranded DNA dideoxy sequencing with the chain termination method (Sanger et al., 1977), using the T7 DNA polimerase according to the manufacturer's instructions (Amershan Pharmacia Biotech). The primer used was ATATATTCGGTCGCTGAGGC.

2.7. Antibody binding assays

2.7.1. Immunodot

Different concentrations of purified phages (10^{12} to 10^{16} ufc/ml) were fixed on nitrocellulose membranes. Later, the membranes were blocked with PBS containing 1% non-fatty milk for 1 h at 37 °C before biotinylated 1E10 mAb was added. After incubating at room temperature and washing three times with PBS, avidin–biotin–peroxidase complex (Dako, Glostrup, Denmark) was added for 30 min. The membranes were washed again and the reaction was developed with 3-amino-9-ethyl-carbazole (Sigma) in 80 mM citrate buffer (pH 4.5) containing 0.12% of H_2O_2.

2.7.2. Phage ELISA

An ELISA assay was used to measure the recognition of phage clones by Ab2 mAbs specific for P3 mAb. Solid phase ELISA was performed using 96-well polystyrene MaxiSorp microtiter plates (Nunc, Roskilde, Denmark) which were coated with 10^{11} phage particles per well and incubated overnight at 4 °C. After washing with PBS-T, the plates were blocked with PBS containing 1% BSA for 1 h at 37 °C and washed again. The second antibody, alkaline phosphatase-conjugated goat anti-mouse IgG, Fcγ fragment-specific (Jackson Immunoresearch Laboratories, West Grove, PA), was added and after an incubation of 1 h at 37 °C, the plates were washed and the reaction was developed with *p*-nitro-phenyl phosphate substrate (Sigma) in dietanolamine buffer (pH 9.8). The absorbance was measured at 405 nm in an ELISA reader (Organon Teknika, Salzburg, Austria). M13KO7 phage was used as negative control. Three samples of each experiment were tested and the standard deviation was less than 10% for all values. Background values of absorbance were less than 0.1.

2.7.3. ELISA assays to measure Ab2 and Ab3 responses

To measure Ab2 and Ab3 reactivity in the sera of mice immunized with P3 and 1E10 mAbs, respectively, a solid-phase ELISA was performed as previously described (Vázquez et al., 1998), using 96-well polystyrene microtiter plates (Nunc) which were coated with 10 μg/ml of purified P3 mAb or fragments of 1E10 Ab2 mAb. Alkaline phosphatase-conjugated goat anti-mouse IgG (Fcγ-specific; Jackson Immunoresearch Laboratories) was used as second antibody. E1 (IgM) and $F(ab')_2$ fragments of ior C5 (IgG1) mAbs were used as isotype matched controls.

2.7.4. ELISPOT assay for detecting anti-idiotypic-producing cells

The presence of P3-specific B cell clones in suspensions of lymph node cells (LNC) from P3 immunized animals was assessed by an ELISPOT assay previously described (Czerkinsky et al., 1983), with some modifications. Maxisorp 96-well plates (Nunc) were coated with 10 μg/ml of purified P3 mAb in carbonate buffer (pH 9.8) at 4 °C, overnight. E1 mAb was used as control antibody. After blocking with 5% BSA in PBS for 30 min at 37 °C, the wells were incubated for 6 h at 37 °C in a CO_2 incubator with various numbers of LNC in 100 μl of complete RPMI-1640 medium supplemented with 8% heat inactivated fetal calf serum (FCS, Gibco BRL, Paisley, Scotland).

Zones of solid phase-bound Ab2 secreted by individual cells were revealed as spots by the step-wise addition of alkaline phosphatase-conjugated goat anti-mouse IgG (Fcγ-specific; Jackson Immunoresearch Laboratories) diluted to 2 μg/ml and, after washing with PBS, a chromogen substrate consisting of 1 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) in 0.1 M AMP buffer (pH 10.5) containing 0.6% agarose. The plates were incubated at 4 °C overnight and the results were scored using a stereoscopic microscope.

2.7.5. Antibody binding inhibition assays

1E10 mAb (25 μg/ml) was incubated overnight at 4 °C with different concentrations of P3 mAb. Later, the mixture was added to a phage-coated ELISA microplate and incubated for 2 h at 37 °C. 1E10 mAb binding to the phages was detected by an indirect ELISA using alkaline phosphatase-conjugated goat anti-mouse IgG as second antibody, as mentioned. E1 mAb was used as a negative control. Percent specific inhibition of 1E10 mAb binding was calculated relative to buffer control.

Inhibition of 1E10 mAb binding to P3 mAb by p-H3P3 peptide was measured by ELISA. Non-saturating amounts of biotinylated 1E10 mAb were incubated overnight at 4 °C with different concentrations of p-H3P3. Then, the mixtures were added to ELISA microplate wells coated with 10 μg/ml of purified P3 mAb. After 1 h at room temperature, the plates were washed and incubated with streptavidin-conjugated horseradish peroxidase (Pharmingen, San Diego, CA) for an additional hour at 37 °C. Plates were developed with *o*-phenylendiamine citrate buffer (pH 4.5), and adsorbance was monitored at 492 nm. An irrelevant 12-mer peptide from FR1/CDR1 region of B7 mAb (p-FR1/H1B7) was used as control.

2.7.6. Proliferation assay

Draining lymph nodes (LN) from normal or athymic nu/nu BALB/c mice immunized twice with P3 mAb were collected, pooled and single cell suspensions were prepared in complete RPMI 1640 medium with 5% FCS (Gibco Life Technologies) by pressing LN to the bottom of a Petri dish with the plunger of a syringe. Non-immunized mice or animals treated only with adjuvant were used as controls. LNC were seeded into 96-well culture plates at a density of 3×10^5 per well in RPMI-1640 medium supplemented with 2.5% FCS, and incubated in the presence of different concentrations of antigens for 96 h at 37 °C in 5% CO_2/air atmosphere. The cells were then pulsed with 1 μCi [^{3}H] thymidine (Amersham Pharmacia Biotech) for the last 18 h of the culture period. Thymidine uptake was determined by scintillation counting. Concanavalin A (2 μg/ml) was used as positive control. Tests were run in triplicate. Stimulation index (SI) was calculated for each triplicate by dividing the mean radioactivity of stimulated cells with that of cells cultured with complete medium alone. SI equal or greater than 3 were considered positive. Similar experiments using LNC from naive BALB/c mice or animals that were treated only with adjuvant were also performed. E1, A3 and ior C5 mAbs, together with a synthetic peptide from the H-CDR3 of B7 mAb, were used as negative controls.

Adherent cells were removed from LNC suspensions by incubating 6×10^6 cells/ml in Falcon polystyrene Petri dishes (Becton Dickinson Labware, Franklin Lakes, NJ) at 37 °C for 1 h. Non-adherent cells were collected, washed and counted by light microscopy. B cells were depleted by incubation of 15×10^6 LNC in a Falcon polystyrene Petri dish (Becton Dickinson Labware) coated with 10 μg/ml of goat anti-mouse F(ab′)$_2$ (Jackson Immunoresearch Laboratories) for 1 h at 37 °C. The plate was centrifuged 10 min at 1500 rpm, and the supernatant was collected. Cells were washed and the enrichment of T cell population in the cell suspension was analyzed by flow cytometry using FITC-conjugated mAbs against mouse αβTCR (pan T) and B220 molecule (pan B; Pharmingen).

3. Results

3.1. Ab2 mAb-reactive phagotopes allowed mapping of an arginine-containing motif into P3's H-CDR3

A nonamer random peptide library expressed in the N-terminus of the major coat protein (pVIII) of the M13 phage was screened against the Ab2 mAb 1E10. Enriched phage clones obtained after three rounds of biopanning were assessed by randomly isolating the clones. Subsequent screening through immunodot led to identification of 12 positive clones. The peptide sequences expressed on each individual clone were determined by sequencing the DNA encoding the inserted region into the pVIII protein.

The deduced amino acid sequences are shown in Table 1. Selected peptides, representing seven different sequences, were grouped into two classes according to their amino acid

Table 1
Sequence of peptides derived from the 9-mer phage library screened with 1E10 mAb

Clones	Sequences
Subgroup 1	
2,5,22	KTW**K**PP**R**IP
7	ERL**K**PP**R**PR
1	R**K**PP**R**PRAL
Subgroup 2	
18	KP**R**RP**R**LR
20	**R**RP**K**TYPTL
11,13,15,19	**R**RP**K**VFRQV
23	**R**RP**R**TWQPL
H-CDR3 of P3 mAb	SGV**R**EG**R**AQAWFAY

The amino acid sequences were deduced from the nucleotide sequences expressed by 12 phage clones selected by 1E10 mAb from a pVIII-9aa library after three rounds of biopanning. The clones, reported in two subgroups, carry a common motif (underlined). Positively charged amino acids spaced by two residues in the 12 peptides and in H-CDR3 of P3 mAb are marked in bold.

homology. Inside each group, highly conserved motifs were identified: K-P-P-K and K/R-R-P-K/R. Additionally, peptides from the first group contained basic residues all over, which together with those found in their motif accounted for 30–40% of the sequence. Both motifs could be condensed in a general motif K/R-P/R-P-K/R.

The sequences of 1E10-selected phagotopes were compared with those of the heavy and light chain CDRs of P3 mAb. As shown in Table 1, a region from P3's H-CDR3 (segment R_{98}–E_{99}–G_{100}–R_{100}a) was found to contain arginine residues with a pattern of spacing similar to that found in 1E10-selected phagotopes.

Phage clones 2 and 13, representing mimotopes from each subgroup, were selected for further studies. The ability of P3 mAb to inhibit the binding of 1E10 mAb to these phages was tested by ELISA. Fig. 1 shows that increasing the concentrations of P3 mAb inhibited the binding of 1E10 mAb to phage-coated wells. Over 74% inhibition of 1E10 mAb binding was achieved with 1 μg/ml of P3 mAb. The specificity was confirmed using an unrelated mAb (E1), which had no inhibitory effect on 1E10 binding to phage-expressed peptides. The ability of P3 mAb to block this binding indicated that peptides and P3 mAb bind the same or very close sites on 1E10 molecule.

In order to distinguish the anti-idiotypic antibodies generated against P3 mAb by their binding requirements, and further confirm the ability of selected phagotopes to mimic P3's idiotypic interactions, we assessed by ELISA the reactivity of 3B11, 4F2 and 1D5 Ab2 mAbs with 1E10-selected phages from each subgroup (clones 2 and 13). Fig. 2 shows that clone 2 was recognized by 1D5 and 4F2 Ab2 mAbs (A), whereas clone 13 was recognized only by 1D5, in addition to 1E10 mAb (B). 3B11 mAb did not recognize any of the phage peptides tested.

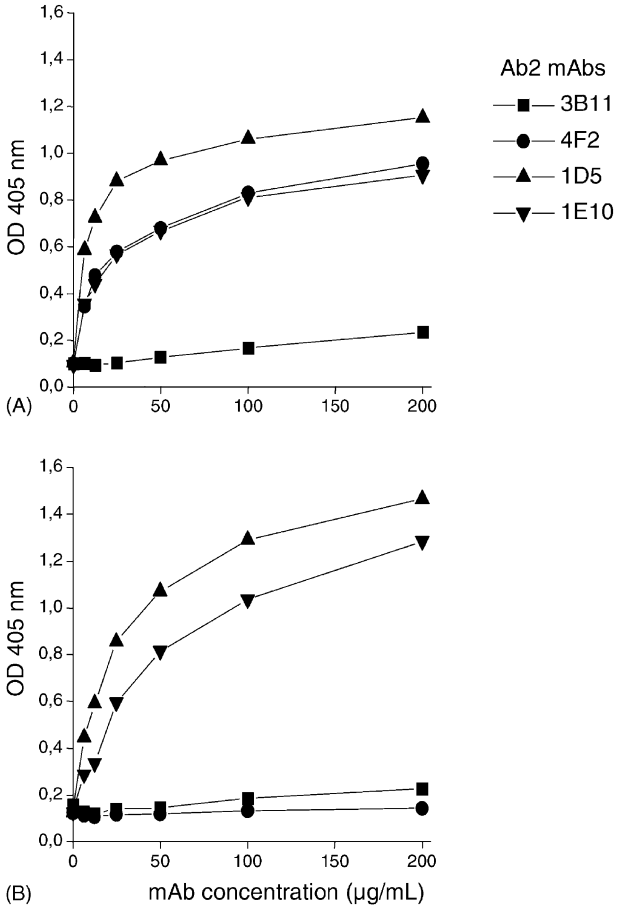

Fig. 2. Recognition of selected phage clones by different Ab2 mAbs specific to P3 mAb. Microtiter plates were coated with 10^{11} phage particle per well and the binding of Ab2 mAbs to phage clones 2 (A) and 13 (B) was detected using alkaline phosphatase-conjugated anti-mouse IgG (Fc fragment specific).

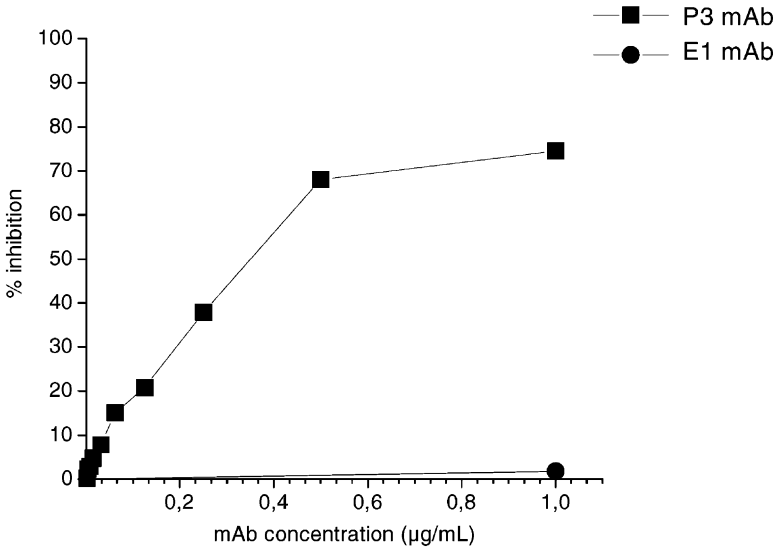

Fig. 1. Inhibition of the binding of 1E10 mAb to phage clone 13 by P3 mAb. Different concentrations of P3 mAb were incubated with 1E10 mAb (25 μg/ml) and the mixtures were added to ELISA microplate wells coated with 10^{11} phage particle per well. The irrelevant IgM mAb E1 was used as a negative control. Percentage of inhibition was calculated relative to 1E10 mAb binding in absence of inhibitors.

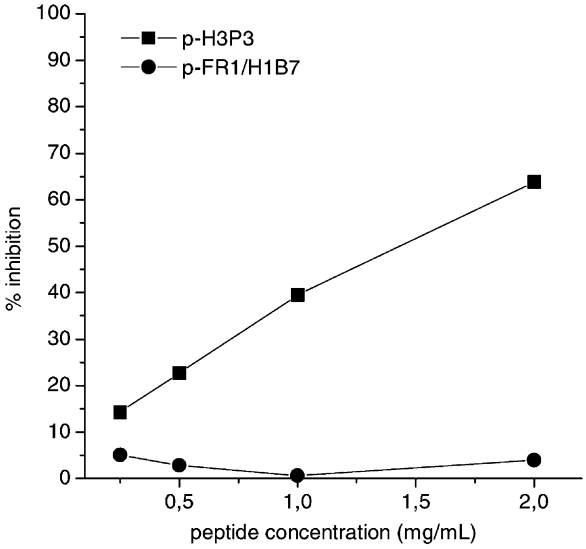

Fig. 3. Inhibition of 1E10 binding to P3-coated plates by a peptide representing the p-H3P3. Increasing concentrations of p-H3P3 were incubated with a subsaturating concentration of biotinylated 1E10 before adding to P3-coated wells. Results are expressed as the percentage of inhibition relative to the binding of 1E10 to P3 in the absence of inhibitor peptide. Peptide p-FR1/H1B7 was used as a negative control.

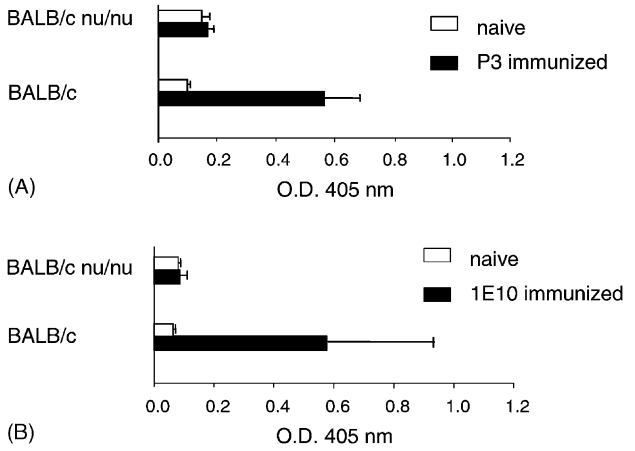

Fig. 4. Thymus-dependency of Ab2 and Ab3 responses. BALB/c mice and athymic nu/nu littermates received four injections with 50 μg of P3 in PBS at 14-day intervals. Animal serum samples were taken before and 7 days after the last dose and the total IgG Ab2 response to P3 (A) was measured by ELISA (sera diluted 1/100). (B) Similarly, Ab3 response in mice immunized with 1E10 in AF was measured by ELISA in F(ab′)2 1E10-coated plates (sera diluted 1:1000). Data represent average O.D. from 5 to 10 mice.

To evaluate the relevance of P3's H-CDR3 residues in the interaction with 1E10 mAb, a 14-mer peptide comprising the entire p-H3P3 was tested for its ability to inhibit the interaction between 1E10 and P3 mAbs. When preincubated with biotinylated 1E10, this peptide inhibited the binding of this Ab2 to P3 mAb, as measured by ELISA (Fig. 3). The inhibition was dose dependent, reaching a value close to 60% with 2 mg/ml of peptide. Under the same conditions, an irrelevant peptide used as control was unable to inhibit 1E10–P3 mAb interaction.

3.1.1. T cells are required for Ab2 and Ab3 syngeneic responses

To investigate the thymus-dependency of the anti-idiotypic response, normal and athymic nu/nu BALB/c mice were injected intraperitoneally with four doses of P3 mAb in PBS. Sera from immunized mice were assayed for IgG Ab2 reactivity to P3 mAb by ELISA. Only normal BALB/c mice elicited the Ab2 antibodies after the immunization with P3 mAb (Fig. 4A). This Ab2 response was specific for P3's idiotype because no binding of immunized animal sera against the irrelevant isotype-matched E1 mAb was detected (data not shown). Neither in the sera from P3-immunized athymic mice nor in those from naive mice, the presence of Ab2 antibodies was detected. The relevance of T cells in the generation of Ab3 response against 1E10 mAb was also tested. Sera from normal and athymic BALB/c mice immunized with four doses of 50 μg of 1E10 mAb emulsified in Freund's adjuvant were assayed for Ab3 reactivity to the F(ab′)2 fragment of 1E10 (Fig. 4B). Again, Ab3 antibodies against 1E10 mAb were observed in the sera from normal but not athymic BALB/c mice. Thus, Ab2 and Ab3 responses in this system are thymus-dependent.

3.1.2. Immunization with P3 mAb induced anti-idiotypic and anti-anti-idiotypic T cell response in BALB/c mice

The ability of P3 mAb to induce specific T cell proliferative response was evaluated in syngeneic mice. LNC from BALB/c mice immunized twice with P3 mAb emulsified in Freund's adjuvant significantly ($P < 0.05$) proliferated in vitro in the presence of this Ab1 mAb. Proliferation was dose-dependent and specific, because it was not obtained after incubation of LNC with the isotype-matched control E1 mAb (Fig. 5A). In addition, proliferation was not observed in LNC from naive BALB/c mice (Fig. 5B), mice injected only with adjuvant (data not shown) or P3-immunized BALB/c athymic nu/nu mice (Fig. 5B). A 14-mer peptide comprising the p-H3P3 stimulated in vitro proliferation of LNC from BALB/c mice immunized with P3 mAb (Fig. 5C). No cell proliferation was observed after culture of LNC in the presence of a control synthetic peptide representing the H-CDR3 of B7 mAb.

To determine which cell populations were involved in LNC proliferation against P3 mAb and p-H3P3, we performed selective depletion of B cells by panning on goat anti-mouse IgGs-coated petri dishes and after elimination of adherent cells on Falcon polystyrene petri dishes. Depletion of B cells from LNC suspensions (T cells >94%, B cells <4%, Fig. 6A) did not lead to significant reduction of the in vitro proliferation of the remaining cells to P3 mAb or p-H3P3 (Fig. 6B). In contrast, the proliferative capacity of LNC was completely abrogated by elimination of adherent cells from previously B cell-depleted suspensions.

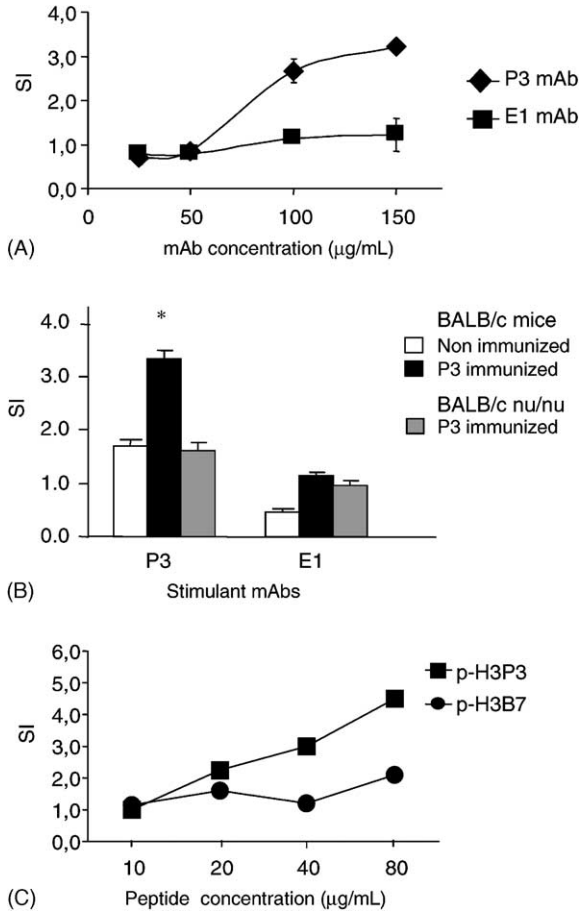

Fig. 5. Induction of idiotype-specific LNC proliferation by P3 mAb. (A) LNC from BALB/c mice (five mice) immunized with two doses of P3 mAb emulsified in Freund's adjuvant were stimulated in vitro with different concentrations of either P3 mAb or the isotype-matched control mAb E1. (B) Thymus- and P3 immunization-dependency of the LNC proliferative capacity induced by P3 mAb was assessed in BALB/c athymic nu/nu mice immunized as in (A) and in naive BALB/c mice. (C) In vitro activation by p-H3P3 of LNC from mice immunized with P3 mAb as described before, were tested for proliferation responses to different concentrations of p-H3P3 and the negative control peptide representing an unrelated H-CDR3 (p-H3B7, see Section 2; $^{*}P < 0.05$, Mann–Whitney test).

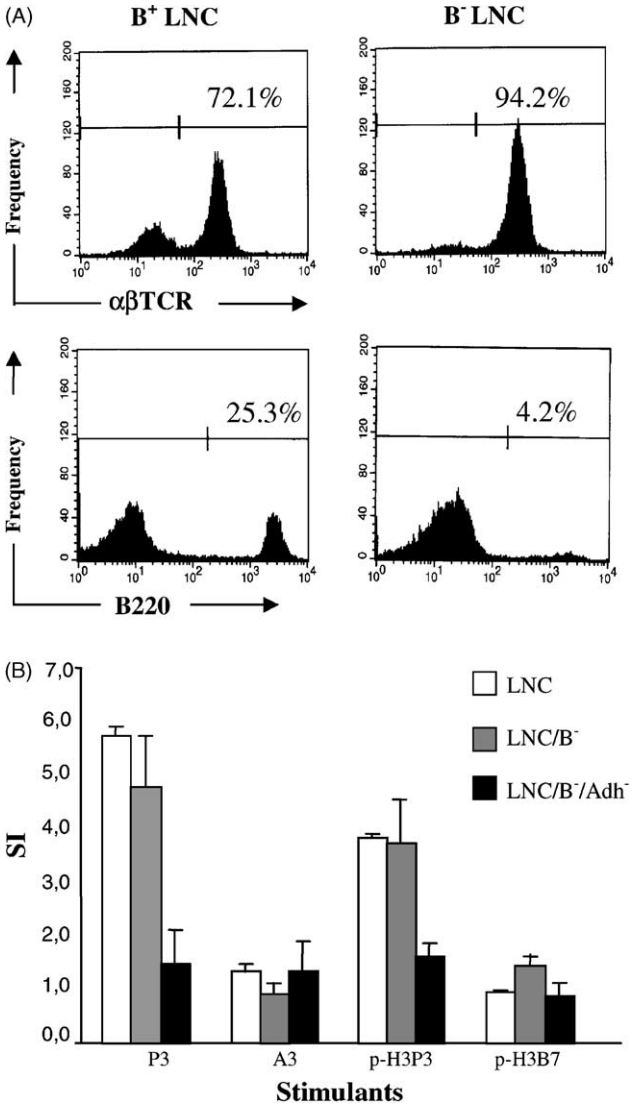

Fig. 6. In vitro proliferation of LNC from P3-immunized animals to P3 and p-H3P3 peptide is dependent on adherent cells but remains unaffected after B cell depletion. LNC were depleted of B cells by panning on anti-murine F(ab′)2 fragment-coated plates and the effectiveness of depletion was assessed by FACS (A). The remaining cells (LNC/B^{-}) were further deprived of adherent cells after incubation on plastic Petri dishes. Total LNC, LNC/B^{-} and LNC/B^{-}/Adh^{-} cells were stimulated in the presence of P3, a control mAb (A3), p-H3P3, or control peptide p-H3B7 (B). [^{3}H]-Thymidine incorporation was measured in a 4-day culture assay.

LNC from BALB/c mice immunized twice with P3 mAb in Freund's adjuvant also proliferated in a dose-dependent manner to 1E10 Ab2 mAb. No proliferation was detected when cells were stimulated with the irrelevant isotype-matched mAb ior c5 (Fig. 7A). Proliferation induced by 1E10 was also dependent on priming with P3 mAb, because LNC from animals not immunized with the Ab1 mAb, but only treated with the adjuvant, did not proliferate in vitro against 1E10 Ab2 mAb (Fig. 7B). No cell proliferation was observed when LNC from P3-immunized athymic mice were used in the experiments, indicating the thymus-dependency of this phenomenon. In vitro LNC proliferative response against 1E10 was not affected by B cells depletion, whereas adherent cells absorption completely abrogated LNC proliferation (Fig. 7C). Taking together all these results, both T and adherent cells seem to be required for proliferation against P3 and 1E10 mAbs of LNC from P3 mAb-primed BALB/c mice.

Since anti-anti-idiotypic T cells induced by immunization with P3 mAb could have resulted from priming by endogenous Ab2 elicited during the response to this Ab1, we examined whether Ab2-producing B cells were detectable at day 10 when LNC were obtained for proliferation assays. As it is shown in Fig. 8, B cells producing Ab2 IgG specific for P3's idiotype were detected by ELISPOT assay in LNC from mice immunized with this Ab1.

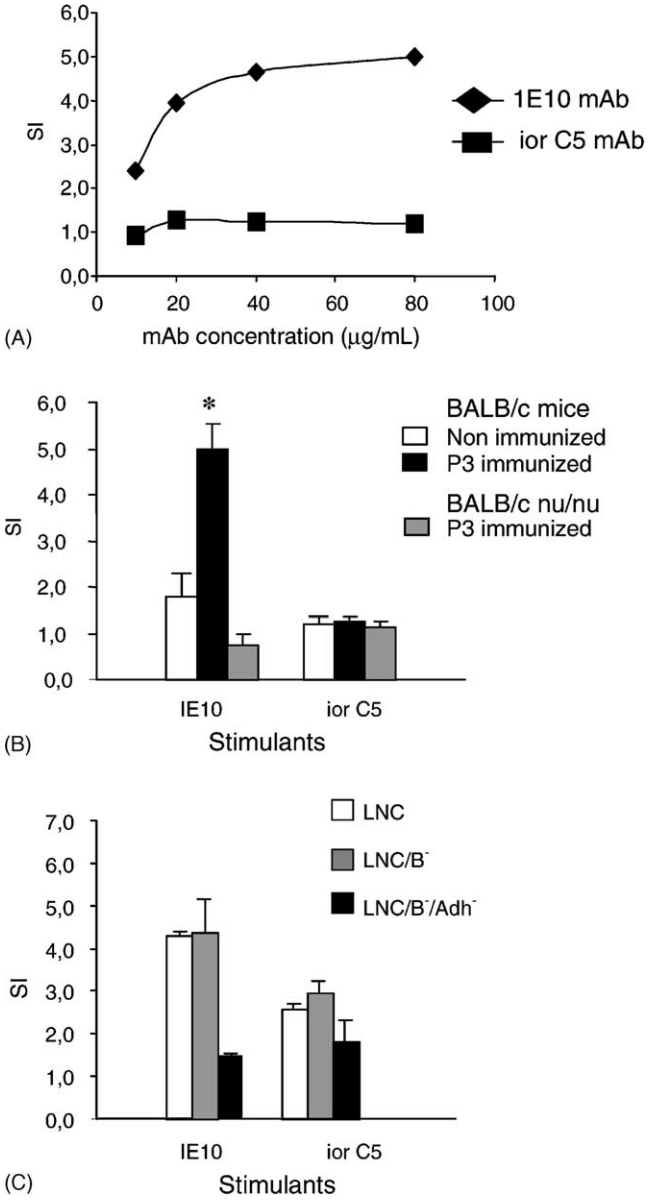

Fig. 7. Anti-anti-idiotype T cell response in BALB/c mice immunized with P3 mAb. (A) LNC from BALB/c mice immunized twice with 100 μg of P3 mAb (Ab1) emulsified in CFA followed by a booster injection 7 days later with 50 μg of the antibody emulsified in IFA specifically proliferate in a 4-day culture assay to 1E10 Ab2 mAb, ior C5 mAb was used as isotype-matched control. (B) BALB/c mice and athymic nu/nu littermates were immunized as described and proliferation in the presence of 1E10 Ab2 and isotype-matched control ior C5 was assessed. LNC from naive BALB/c mice were used as control. (* $P < 0.05$, Mann–Whitney U-test). (C) In vitro proliferation of LNC from P3-immunized animals to 1E10 is dependent on adherent cells but remains unaffected after B cell depletion (for details, see legend to Fig. 6).

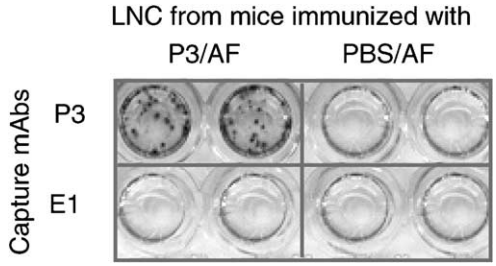

Fig. 8. Specific clonal expansion of Ab2 producing B cells after P3 mAb (Ab1) immunization. The presence of P3 specific B cells was assessed by ELISPOT. LNC from BALB/c mice immunized with P3 mAb (Ab1) emulsified in Freund's adjuvant (P3/AF), or PBS (PBS/AF), were plated on wells coated with P3 mAb or isotype-matched control E1 mAb. Alkaline phosphatase conjugated anti-mouse IgG (Fc-specific) antibody was used to detect Ab2 producing clones. The reactivity was developed with a 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate solution.

4. Discussion

The phage library technology has became a powerful tool for the epitope mapping of antibodies (Scott and Smith, 1990; Cortese et al., 1995). The selected peptides, so-called mimotopes, mimic the binding characteristics of the natural epitope. The screening of the 9mer-pVIII phage displayed peptide library with the anti-idiotype antibody 1E10 exclusively selected clones bearing the sequences K-P-P-R or R-R-P-R/K, which could be represented by the general motif K/R-P/R-P-K/R. This recursive selection of positively-charged peptides by 1E10 mAb agrees with previous findings in the sequence analysis of P3's Ab2 mAbs (Pérez et al., 2001). Sequenced Ab2 antibodies showed a general excess in H-CDRs' acidic residues, including a notorious acidic motif (E/D-D-D/Y-Y-D) in their H-CDR3 s. Complementary charged amino acid residues in the H-CDRs of P3 have been found. Thus, selected phage-displayed peptides seem to be mimotopes of the original idiotope recognized by 1E10 in P3 mAb. According to the sequence alignment, this idiotope could encompass a region from the H-CDR3 of P3. Two arginine residues found at positions 98 and 100a in this CDR seem to be the most likely candidates for the interaction with 1E10. The relevance of this region was also suggested by experiments showing the ability of the H-CDR3 synthetic peptide to inhibit the binding between P3 and 1E10 mAbs. Although the overall homology observed between mimotope sequences and H-CDR3 was low and limited only to basic residues in the motif R-X-X-R it should be noticed that 1E10 selection of phage peptides seemed to be guided almost exclusively by those conserved basic residues. However, we cannot rule out the possibility of a conformational idiotope including additionally the Arg31 residue at the heavy chain CDR1 of P3 mAb (Pérez et al., 2001).

Ab2 mAbs specific to P3 antibody showed different reactivities against peptides 2 and 13, representing each group of phage mimotopes selected by 1E10 mAb. 1D5 mAb showed the same recognition pattern as 1E10, whereas 4F2 recognized only peptide 2 bearing the motif KPPR. Due to their similar binding requirements, it seems likely that 1E10, 1D5 and 4F2 define the same or a similar idiotope in P3 mAb. On the other hand, Ab2 3B11 did not show any reactivity

against 1E10-selected peptides. This result suggests the possibility of different amino acid residues recognized by these two Ab2s, even when the presence of a negative-charged motif in their H-CDR3 strongly indicated their interaction with positive-charged residues from P3's CDRs (Pérez et al., 2001). In contrast with 1E10 mAb, 3B11 is an extensively mutated mAb that seems to define a private idiotope, most likely due to the fine fitting of the Ab1–Ab2 interaction. Among Ab2 mAbs to P3, only 3B11 was unable to induce Id+ Ab3 antibodies in syngeneic model (Vázquez et al., 1998). A correlation seems to exist between the recognition of 1E10-selected mimotopes by P3's Ab2 and their ability to induce Id+ Ab3 responses in syngeneic mice.

In a preliminary study, we concluded that P3 mAb bears an idiotype that fulfill two of Bona's criteria to define a "regulatory idiotype" (Vázquez et al., 1998). Now, we have demonstrated that P3 mAb idiotype fulfills the third criterion, i.e. it can activate autologous T cells. In the present study, we have shown the thymus-dependency of the IgG Ab2 response elicited in syngeneic mice after the injection of P3 mAb in saline. Even more, we demonstrated the capacity of this mAb to induce a specific and dose-dependent proliferation of LNC from syngeneic mice. This in vitro proliferation was observed only after immunization of BALB/c mice with P3, indicating that cells responding to this mAb were not naturally activated. Proliferation of LNC was not affected at least in vitro by B cell depletion, whereas a complete abrogation was observed following depletion of adherent cells. The later result points out the capacity of P3 mAb to induce T cell activation dependent on professional antigen presenting cells. The T cell nature of the proliferating cells was further demonstrated due to the incapacity of LNC from P3-immunized athymic BALB/c mice to proliferate in vitro when challenged with this Ab1.

LNC from BALB/c mice that were immunized with P3 also proliferated in the presence of peptide p-H3P3, suggesting that this mAb is processed to present at least this immunodominant peptide on the surface of APC. Earlier, we have suggested the relevance of the H-CDR3 to the immunogenicity of a germline antibody like P3 (Pérez et al., 2001). Although it has been indicated by other groups a T cell repertoire tolerance to germline-encoded antibody V-region diversity (Eyerman et al., 1996; Wysocki et al., 1998), the singularity of the N nucleotide-rich H-CDR3 of P3 mAb could allow T cells specific to this region to escape thymic deletion.

Together, these results reveal the importance of the H-CDR3 in the interaction of P3 mAb with anti-idiotypic antibodies inducing Id+ Ab3 and also with T cells. This idiopeptide could be defined as a "regulatory idiotope" due to its simultaneous involvement in the interaction of P3 with anti-idiotypic B and T cells, a property shared by previously reported idiopeptides (Ebling et al., 1993; Singh et al., 1995, 1996; Ward et al., 1998).

Finally, this study provides for the first time evidences of the induction of an in vivo idiotypic cascade involving anti-idiotypic and anti-anti-idiotypic T cells by an IgM germline antibody directed to widespread gangliosides. The relevance of this idiotypic cascade in the control of antibody response against these antigens still remains unknown, but the Ab1-enforced T cell proliferation to Ab2 antibodies suggests the existence of a naturally occurring B–T cell idiotypic network based on structural motifs like those ones described in the present manuscript, providing further rationale to the concept of "regulatory idiotope". The importance of the presence of B cells producing Ab2 antibodies for the in vivo activation of anti-anti-idiotypic T cells needs to be addressed in the near future.

Acknowledgements

This work was supported by the Government of Cuba and by ELEA S.A.C.I.F. y A. Laboratory, Buenos Aires, Argentina.

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