Papamattheou, M. G., **J. G. Routsias**, E. E. Karagouni, C. Sakarellos, M. Sakarellos-Daitsiotis, H. M. Moutsopoulos, A. G. Tzioufas, and E. N. Dotsika. 2004. ***¢ ůgε ε ŮŰεɢɐ Űɤ з ɇ -əŮε űɞəgŰŰŸɟ ɤ з ŮɞŬɘ Ŭ́ Ŭɟ ŬɑŰɋŰɋ ɔɘŬ Űɋз Ů Ŭɔɤ ɔɐ Űεg ɘŭɘɛŰǵ ɘɚɞɨ ï ŬзŰɘŭɘɛŰǵ ɘɚɞɨ ŭɘɚŰɨ ɞg, ɏ́ ŮɘŰŰ Ŭ́ ɧ Ŭзɞůɞ́ ɞɑɋůɋɠ ε ŮŰεз*** συμπληρωματικό επίτοπο 289-308αα της La/SSB. Clinical and experimental immunology 135:416-426.

Ο σχεδιασμός νέων εμβολίων για την πρόληψη των αυτοανόσων νοσημάτων μπορεί να βασιστεί στην κατάργηση των ανεπιθύμητων ανοσολογικών αποκρίσεων έναντι συγκεκριμένων αυτοαντιγόνων. Στη παρούσα μελέτη διαπιστώθηκε ότι η ανοσοποίηση ποντικών BALB/c με δύο γραμμικούς Τ και Β κυτταρικούς επιτόπους του ανθρώπινου αυτοαντιγόνου La/SSB (μεταξύ των περιοχών 289-308aa και 349-364aa) καθώς και με τα συμπληρωματικά τους πεπτίδια οδήγησε στην εκδήλωση χαρακτηριστικών Τ και Β κυτταρικών απαντήσεων. Η ανοσοποίηση με τον επίτοπο 289-308aa και τον συμπληρωματικό του στάθηκε ικανή να δημιουργήσει ένα ιδιοτυπικό-αντι-ιδιοτυπικό δίκτυο, με αντισώματα έναντι και των δύο επιτόπων. Ο πολλαπλασιασμός ειδικών για το πεπτίδιο Τ κυττάρων και η παραγωγή κυτταροκινών αποκάλυψαν την επαγωγή μιας απάντησης Τ βοηθητικών κυττάρων σε δύο φάσεις (Th1--> Th2 τύπου). Μετά από ανοσοποίηση και με τις δύο μορφές πεπτιδίων επικράτησε η ανοσοσφαιρίνη κλάσης IgG1, ενώ η απάντηση από IgG2b > IgG2a επιτεύχθηκε με τον συμπληρωματικό επίτοπο 349-364. Συμπερασματικά, μετά από ανοσοποίηση, τα συμπληρωματικά πεπτίδια του κύριων επιτόπων του La/SSB μιμούνται τη δράση των αυτοαντισωμάτων έναντι αυτών των επιτόπων, εγκαθιστώντας ένα ενεργό ιδιοτυπικό-αντί-ιδιοτυπικό δίκτυο.

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T cell help is required to induce idiotypic–anti-idiotypic autoantibody network after immunization with complementary epitope 289–308aa of La/SSB autoantigen in non-autoimmune mice

M. G. PAPAMATTHEOU*, J. G. ROUTSIAS†, E. E. KARAGOUNI*, C. SAKARELLOS‡, M. SAKARELLOS-DAITSIOTIS‡, H. M. MOUTSOPOULOS†, A. G. TZIOUFAS† & E. N. DOTSIKA* *Laboratory of Cellular Immunology, Hellenic Pasteur Institute, Athens, Greece, †Department of Pathophysiology, School of Medicine, University of Athens, Greece, and ‡Department of Organic Chemistry and Biochemistry, School of Physical Sciences, University of Ioannina, Greece

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SUMMARY

Immunotherapies against autoimmune diseases have been of limited success. Preventive vaccines could be developed on the basis to abrogate unwanted immune responses to defined autodeterminants. In this study it is shown that immunization of BALB/c mice with two linear T and B cell epitopes of the human La/SSB autoantigen (spanning the regions 289–308aa and 349–364aa) and their complementary forms specified by the complementary mRNA, results in characteristic B and T cell responses. Mice immunized with the 289–308aa epitope or its complementary peptide elicited specific antibodies against both epitopes. In contrast, mice immunized with the 349–364aa epitope or its complementary peptide mounted antibody titres against the immunizing peptide only. According to these data, the 289–308aa epitope and its complementary form were capable to generate an idiotypic–anti-idiotypic response, which were cross-regulated. Peptide-specific T cell proliferation and cytokine production *in vitro* revealed the induction of a two-stage T helper response (Th1→Th2 type) after immunization with either the epitope 289–308 or its complementary peptide. IgG1 was the predominant subclass after immunization with the two forms of epitopes 289–308 and 349–364, while a response of the IgG2b > IgG2a was obtained after the immunization with the complementary form of 349–364 epitope reflecting the TH2/TH1 polarization, respectively. Our data suggest that the complementary peptides of two immunodominant epitopes of human LaSSB can mimic the autoantibodies against these epitopes and establish an active idiotypic–anti-idiotypic network.

Keywords anti-idiotypic antibodies complementary peptides La/ SSB Sjögren's syndrome Th1/ Th2

INTRODUCTION

La/SSB is an autoantigen which along with two other proteins the Ro52 and the Ro60 kDa, costitutes a complex with human cytoplasmic RNAs and forms ribonucleoprotein particles (hYRNPs). Autoantibodies of this complex are found frequently in the sera of patients with primary Sjögren's syndrome (SS) and systemic lupus erythematosus (SLE) [1]. Although the aetiology of an autoimmune response against La/SSB remains largely unknown, considerable evidence suggests that autoantibody responses to La/SSB autoantigen in normal mice are antigen-driven, involving a number of B and T cell determinants [2,3]. B cell epitope mapping of human La/SSB, using overlapping synthetic peptides, showed that anti-La/SSB autoantibodies from SS and SLE recognize four major linear antigenic epitopes [4,5]. Parallel surveys of T cell mapping of the human La/SSB indicated multiple T cell epitopes, either dominant or subdominant [6–8]. Further studies showed that the 289–308aa epitope is a minor B cell/major T cell epitope [4,6,7], whereas the 349–364aa epitope is a major B cell/minor T cell epitope [4,7].

Although the mechanisms of La/SSB autoimmunity is not known, recent studies indicate that the autoreactive B cells specific for the nuclear autoantigen La/SSB exist in normal individuals and their activation depends upon T cell assistance provided by autoreactive T helper cells [9]. Two functionally distinct Th cell subpopulations, Th1 and Th2, have been identified [10]. The balance between these two Th cell populations and their modulation was shown to play a crucial role in several autoimmune diseases [11]. Furthermore, cytokines have been suggested to play an important role in the immune dysregulation observed in SLE

Correspondence: E. N. Dotsika PhD, Laboratory of Cellular Biology, Hellenic Pasteur Institute, Vas. Sofias 127, 11521 Athens, Greece.
E-mail: e.dotsika@mail.pasteur.gr

patients and murine lupus-prone strains [12–15]. The experimental disease in mice involves two stages of cytokine production: a dominant Th1-type first expressed by the production of interleukin (IL)-2 and interferon (IFN)-γ and later a Th2-type activation, as shown by the increased production of IL-4 and IL-10 [16]. The characteristic cytokine products of Th1 and Th2 cells are mutually inhibitory for the differentiation and effector functions of the reciprocal phenotype. Therefore a shift of Th1 to Th2 or vice versa may contribute to the regulation of the autoimmune response [17]. Previous studies have shown that the regulation of Th1/Th2 response could be modulated, among others, either utilizing a specific anti-idiotypic antibody [18], or through immunization with a peptide based on the complementarity-determining region (CDR-1) of an idiotypic antibody [19].

Complementary peptides specified by complementary nucleotide sequences can bind specifically to each other, apparently as a result of having complementary hydrophobicity [20–22]. It has been shown that complementary peptides bind to one another with high specificity and moderate affinity [23,24]. Experimental evidence has confirmed that the employment of different complementary peptide pairs induce the formation of interacting pairs of idiotypic and anti-idiotypic antibodies whose combining sites are complementary. These interacting anti-idiotypic (antid) antibodies reactive with idiotypes (id) of autoantibodies or autoreactive clonotypic T cells are capable of regulating the autoimmune response and represent ideal therapeutic agents for autoimmune diseases [25–28].

Our approach is targeted to the generation of interacting pairs of idiotypic and anti-idiotypic antibodies, by immunization of complementary peptides. To this end, we tested the immunogenicity of the 289–308aa and 349–364aa epitopes and their complementary forms *in vivo* by injecting them into non-autoimmune experimental animals (BALB/c mice) in formulations of four copies coupled to a non-immunogenic sequential oligopeptide carrier (SOC_4) [29]. We evaluated the ability of these peptides to induce specific antibody production and T cell proliferation to the immunizing peptide or to the other form. Determination of the isotype profile of specific antibodies and also the cytokines, IFN-γ and IL-4, secreted by antigen-stimulated T cells, were also performed. Data obtained allowed the investigation of the type of the immune response induced by complementary peptides.

MATERIALS AND METHODS

Mice

Inbred female BALB/c mice aged 8–9 weeks were used in this study. The animals were maintained under specific pathogen-free conditions, receiving a diet of commercial food pellets and water *ad libitum* obtained from our own colony in the Hellenic Pasteur Institute, Athens.

Peptide synthesis and purification

The two La/SSB B cell/T cell epitopes, A^{289}NNGNLQLRNKEVTWEVLEG^{308}, pep289–308, G^{349}SGKGKVQFQGKKTKF^{364}, pep349–364, as well as their complementary peptides S^{308}FEYFPSHFFVPELEVTIIC^{289}, cpep289–308 and K^{364}FRFLALKLYFSFTRP^{349}, cpep349–364, were synthesized by the stepwise solid phase procedure (SPPS) in their amide forms on a benzydrylamin resin according to standard methods [30]. A new helical carrier for multiple anchoring of antigenic peptides incorporating an a-aminoisobutyric residue in each repetitive moiety, Ac-(Lys-Aib-Gly)$_n$(SOC_n) was applied in this study for anchoring the epitopes by the Lys-$N^{\varepsilon}H_2$ groups [29,31]. Linear and complementary La/SSB peptide epitopes were attached in four copies and two copies each, respectively, to the new tetrameric carrier SOC_4 and used for immunizations and enzyme-linked immunoassay (ELISA) experiments. An irrelevant SOC_4-peptide sequence [(Y^{154}DQLVTRVVTHEMAHA^{169})$_2$-(G^{467}NVQAAKDGGNTAAGR^{482})$_2$]-SOC_4: *Leishmania* gp63] was also constructed and used as a control peptide. All peptides were purified by high-performance liquid chromatography (HPLC) and subjected to amino acid analysis and mass spectroscopy (MS) that confirmed their purity and identity.

La/SSB recombinant protein

La/SSB recombinant protein prepared from a La/SSB cDNA as described previously [32] and purified by poly(U)-Sepharose affinity chromatography [33].

Immunization protocol

For assessment of antibody production and specific T cell proliferation, groups of six mice were immunized intraperitoneally (i.p.) with 100 μg of each peptide conjugated to SOC_4 and emulsified in Complete Freud's Adjuvant (CFA) on day 0. Two boostings were followed of half-doses of conjugated peptides, respectively, in Incomplete Freud's Adjuvant (IFA) at days 15 and 30. The antigen dose and the number of boostings were determined in preliminary experiments. Experimental mice were bled at different post-immunization time-points and sera were stored at –20°C until testing for specific antibodies. Fifty-six days after the first immunization, three animals of each group were tested for lymphoproliferative responses of spleen cells.

For lymph node T cell priming, three BALB/c mice were immunized with 100 μg of unconjugated pep289–308 in CFA or 100 μg unconjugated cpep289–308 in CFA delivered subcutaneously (s.c.) in hind footpads and at the base of the tail on day 0. Draining inguinal and popliteal lymph nodes were removed on day 10. Control mice ($n = 6$) were immunized with the adjuvant or the adjuvant and the SOC_4 carrier in similar conditions.

Detection of peptide-specific antibodies and their subclasses

Anti-peptide antibodies were detected by a modified ELISA assay optimized for each different synthetic peptide [34]. Briefly, high-binding microtitre plates were coated overnight at 4°C with 10 μg/ml of the appropriate SOC_4-peptide solution: pep289–308 or cpep289–308 in carbonate/bicarbonate buffer pH = 9·1, pep349–364 or cpep349–364 in phosphate buffer pH = 7·1. The remaining binding sites were blocked with buffer of 2% bovine serum albumin, containing 0·1% Tween 20 in phosphate buffered saline (PBS) (BB) for 1 h at room temperature. After washing with PBS 0·05% Tween 20, the plates incubated overnight at 4°C with serial serum dilutions, starting from 1 : 250 in BB. The wells were then washed and goat antimouse IgG conjugated to alkaline phosphatase or horseradish peroxidase-conjugated rat antimouse (IgG1, IgG2a, IgG2b and IgG3) were used to detect bound specific antibodies. The plates were incubated for 1 h at room temperature, followed by washing. One hundred μl/well p-nitrophenol substrate for alkaline phosphatase (Sigma, Steinheim, Germany) or 3, 3′, 5, 5′-tetramethylbenzidine (TMB) substrate (Pierce, Rockford, IL, USA) for peroxidase were added subsequently and incubated at 37°C. The absor-

bances were measured at 405 nm or 450 nm, respectively, with reference filter at 630 nm using a microplate reader (Dynatech MR 5000, Channel Islands, UK). Data obtained were presented as $\log_{10}$ of reciprocals of end-point dilutions.

Affinity purification of antipeptide antibodies and assessment of their idiotypic–anti-idiotypic relation

Covalink ELISA plates with high covalent-binding capacity for the peptides (in the μg range) were coated with either pep289–308 or cpep289–308 (8 μg/ml, 10 wells per peptide). After incubation with sera from animals immunized with either pep289–308 (dilution: 1/100) or cpep289–308 (dilution 1/200), respectively, specific antipeptide antibodies were eluted using HCl-Gly2·8, pooled together, neutralized at pH = 7·2 and concentrated to half their original volume. Purified antipep289–308 antibodies were coated in ELISA plates. After blocking with bovine serum albumin (BSA) 2% in PBS, anticpep289–308 or normal mice IgG (20 μg/ml) were added. Following 2 h incubation, biotinylated pep289–308 was added to all wells. Subsequently the plates were incubated with streptavidin-peroxidase conjugate for 1 h at room tempreature. The colour was developed by the addition of 2, 2′-amino-bis(3-ethlbenzthiazoline-6-sulphonic acid) (ABTS) substrate and measured at 405 nm.

Homologous inhibition assays

In order to evaluate the specificity of the antipeptide antibody assays in all groups of mice, a homologous inhibition assay was performed. Briefly, sera from immunized mice at a dilution corresponding to 50% maximum binding at 1/1000 dilution were preincubated, each with the homologous soluble peptide at concentrations 0–20 μg/ml for 1 h at 37°C. The procedure was then continued as described above for antipeptide ELISA. The percentage of inhibition of the antibody binding the peptide immobilized on ELISA plates was calculated as follows: $100 \times [1 - (\text{inhibited } OD_{405nm}/\text{uninhibited } OD_{405nm})]$.

Secondary in vitro *peptide specific lymphoproliferative responses*

Lymphocytes from spleens or lymph nodes were obtained from immunized mice at predefined time points, pooled from mice within each group, and single cell suspensions (1×10^6 cells/ml) were prepared in RPMI-1640 culture medium (Seromed, Berlin, Germany). The culture medium was supplemented with 10 mM Hepes, 5×10^5 M 2-Me, 2 mM L-glutamine, 24 mM $NaHCO_3$, 100 units/ml penicillin, 100 μg/ml streptomycin and 5% heat-inactivated fetal calf serum (FCS). Cell viability was >95% as determined by trypan blue exclusion. Cells were cultured in round-bottomed 96-well microtitre plates (Costar, Cambridge, MA, USA) with different concentrations of pep289–308 or cpep289–308 in free form or culture medium alone. Cultures were incubated at 37°C in 5% CO_2 for 96 h and pulsed with 1 μCi/ml [^{3}H]-thymidine ([^{3}H]-TdR, Amersham, Co., Buckinghamshire UK) during the final 18 h. Cells were harvested and [^{3}H]-TdR incorporation was assessed by liquid scintillation counting. All cultures were performed in triplicate and results are expressed as stimulation index (SI).

Cytokine production and determination

Single lymph node cell suspensions (5×10^6 cells/ml), prepared from all groups of mice, were cultured under identical conditions as described above, in RPMI-1640 medium alone or stimulated with the linear and the complementary forms of 289–308 free peptide. The cultures were incubated for 24, 48, 72 or 96 h at 37°C in 5% CO_2 in a humidified incubator. Culture supernatants were harvested, aliquoted and stored at −70°C until assayed for specific cytokines.

Sandwich enzyme immunoassay (SEIA) was used to detect IFN-γ and IL-4 as described previously [35]. Quantification was performed using a standard dose-dependent curve and the cytokine concentrations detected in the samples as presented as pg/ml. The detection limits for the cytokines were 125 pg/ml for IFN-γ and 35 pg/ml for IL-4.

Statistical analysis

Data were expressed as the mean values with the standard deviation indicated. Using a Student's *t*-test we assessed the statistical significance of the mean values of the two groups. The probability (P) of $< 0{\cdot}05$ was considered to indicate statistical significance.

RESULTS

Priming of BALB/c mice with either pep289–308aa or cpep289–308aa induced a T cell response specific for both peptides

We investigated initially the capacity of 289–308aa and 349–364aa epitopes of the hLa/SSB autoantigen, as well as their complementary forms, to promote a primary T cell immune response in BALB/c mice. To this end, primed lymph node cells (LNCs) were stimulated *in vitro* with three antigens, homologous, complementary or unrelated peptide and ConA as potent T cell mitogen. As indicated in Fig. 1a,b, immunization with pep289–308 or cpep289–308 resulted in a dose-dependent T cell response not only to homologous peptide (6·5 and 15 SI, respectively), but also to the other form of the epitope (5·5 and 4·8 SI, respectively). The background cpm values ranged in low levels, between 350 cpm for the pep289–308 and 400 cpm for the cpep289–308 primed LNCs. The cpep289–308 was more immunogenic, as the genetic background of BALB/c (H-2^d) mice exhibited a threefold higher response to homologous peptide compared to the response obtained after pep289–308 stimulation, 15 *versus* 4·8 SI (sample/background: 6000/400 cpm *versus* 1920/400 cpm, respectively) (Fig. 1b). Stimulation of lymph node cells with the unrelated peptide did not induce a detectable immune response in either group of immunized mice. Mice who received the CFA alone were used as a negative control, whereas mitogenic responses ranged from 25 to 28 SI (data not shown). In contrast to pep289–308, immunization of the mice with the 349–364aa epitope of human La/SSB or its complementary form failed to induce a detectable lymphoproliferative response (data not shown).

Repeating immunizations with either epitopes or complementary epitopes of La/SSB-induced T cell-dependent idiotypic and anti-idiotypic antibodies

To evaluate the efficiency of the pep289–308 or cpep289–308 to induce specific B cell responses, mice were immunized with each peptide conjugated to SOC_4 oligopeptide carrier emulsified in CFA. Two boostings of the same peptide in IFA were carried out 15 and 30 days post-first immunization. Sera from individual mice were collected at day 28 and day 56 post-immunization and tested for their reactivity against all peptides in ELISA assay. As depicted in Table 1, pep289–308 or the complementary form generated at 28 days post-immunization, a strong B cell response

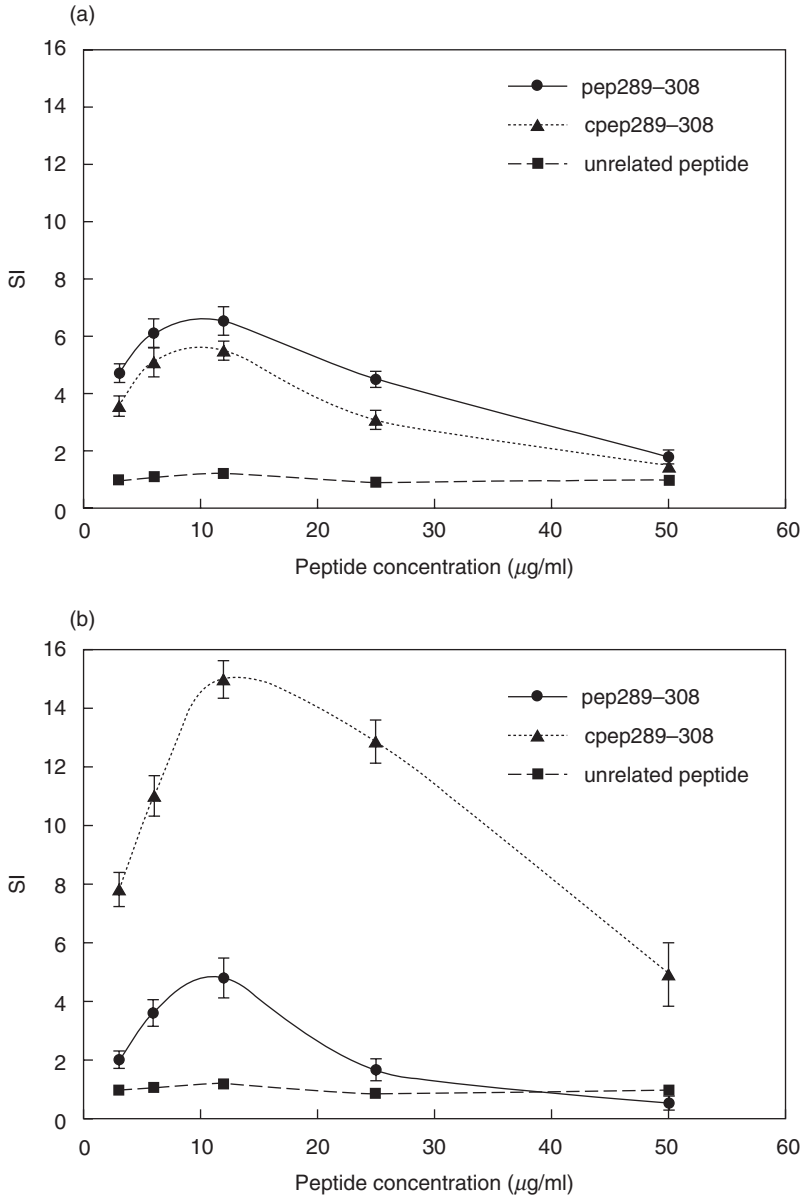

Fig. 1. Pep289–308- and cpep289–308-specific lymph node T cell responses. Pep289–308 (a) or cpep289–308 (b) primed lymph node T cells coincubated with pep289–308 (●), cpep289–308 (▲) or an unrelated peptide (■) in sequential concentrations as indicated in Materials and methods. The lymphocytes were cultured in triplicate for 96 h at 37°C. Cultures were pulsed with 1 μCi/ml [^{3}H]-TdR during the last 18 h. The results represent the [^{3}H]-TdR incorporation, expressed in SI ± s.d. from triplicate cultures of pooled cells. Each experimental group consisted of cells pooled from three mice. Results are representative from three independent experiments.

specific to the homologous peptide. Interestingly, immunization with both peptides elicited antibodies also recognizing their selected complementary form. This antibody response, elicited by complementary peptides, reflected the establishment of an idiotypic/anti-idiotypic network as described previously [36]. The idiotypic–anti-idiotypic relation of antipeptide antibodies was confirmed further by affinity purification of antipep289–308 and anticpep289–308. The isolated antibodies were applied, subsequently, in a competitive ELISA assay as described in Materials and methods. As presented in Fig. 2, the interaction of anticpep289–308 antibodies with immobilized antipep-289–308 abolished the binding of biotinylated pep289–308 peptide (76·7% inhibition), suggesting their idiotypic–anti-idiotypic relation. In contrast, normal IgG produced only a minor inhibition effect on antipep289–308/biotinylated–pep289–308 interaction (27·5% inhibition).

Idiotypic–anti-idiotypic antibody levels were maintained at a high level until the 56th day post-immunization, which may be due either to the continuous release of antigen by the adjuvant, or the continuous supply of antigenic peptides derived from the endogenous mouse La/SSB, or the V region of the antipep289–308 antibodies. The involvement of the endogenous mouse La/SSB in the idiotypic response initiated by the 289–308aa epitope may induce diversification of the response to other intramolecular determinants of the La/SSB autoantigen. However, no reactivity to pep349–364 or its complementary form was obtained. On the

Table 1. Total IgG responses to human La/SSB epitopes in immunized BALB/c mice

Immunization (SOC_4 conjugated in CFA/IFA)[1]	Total IgG antipeptide response[2] (– $\log_{10}$ of serum antibody titre)							
	28 days p.i.				56 days p.i.			
	anti-P1	anti-P2	anti-P3	anti-P4	anti-P1	anti-P2	anti-P3	anti-P4
pep289–308 P1	4·2 (1·780)	3·1 (0·300)	<1	<1	3·2 (0·350)	3·8 (0·900)	<1	<1
cpep289–308 P2	4·0 (1·110)	5·6 (2·540)	<1	<1	3·8 (0·910)	5·4 (2·220)	<1	<1
pep349–364 P3	<1	<1	4·8 (2·010)	<1	<1	<1	4·2 (1·800)	<1
cpep349–364 P4	<1	<1	<1	3·5 (0·500)	<1	<1	<1	3·2 (0·380)
Sham-immunized	<1	<1	<1	<1	<1	<1	<1	<1

[1]Mice were immunized i.p. on day 0 with 100 μg/mouse peptide conjugated to SOC_4 in CFA, and two boostings with the same peptide (50 μg/mouse i.p.) were followed on days 15 and 30. [2]Experimental mice were bled on day 28 and day 56-post immunization and sera were tested for specific antibodies using peptide-specific ELISA. The results given are the mean number of $\log_{10}$ of serum antibody titres from six individual mice from one of three independent experiments. The values in brackets correspond to the mean OD values in 1/400 serum dilution. The mean OD value for sham-immunized mice was 0·050 in the same serum dilution. p.i. = post-immunization.

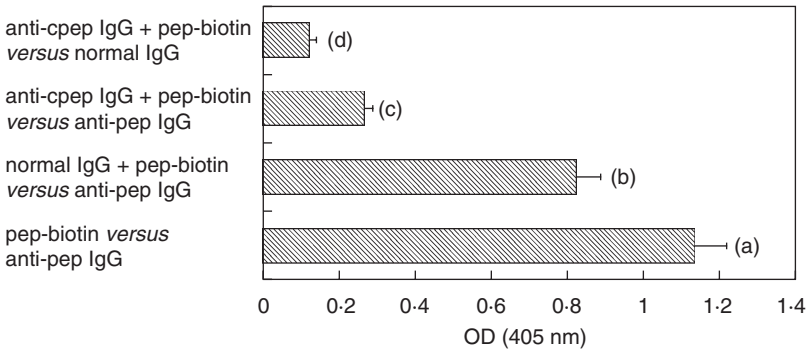

Fig. 2. Inhibition of the antipep289–308 IgG–biotin/pep289–308 interaction by anticpep289–308 purified antibodies. ELISA plates were coated with antipep289–308 purified IgG or normal IgG. Anti-pep289–308-coated wells were incubated next with either blocking buffer (BB) (BSA 2% in PBS) (a), normal IgG (b) or purified anticpep289–308-specific antibodies (c), while in normal IgG coated wells, purified anticpep289–308-specific antibodies were added (d). Subsequently, the ELISA plates were incubated sequentially with biotinylated pep289–308 and streptavidin-peroxidase conjugate. The results represent the mean OD value from triplicate wells of each sample. Results are representative of three independent experiments.

other hand, mice immunized with pep349–364 or cpep349–364 produced only a specific idiotypic response which remained strong from day 28 to day 56 post-immunization. Sera from sham-immunized mice received either CFA/IFA alone or the SOC_4 carrier did not recognize any of the two epitopes of the La/SSB or their complementary peptides.

In order to demonstrate the antibody specificity, homologous inhibition ELISA assays was performed. As illustrated in Fig. 3, the homologous inhibition of sera from mice immunized with pep289–308 and cpep289–308 led to 64% and 98% inhibition, respectively, while homologous inhibition using pep349–364 and cpep349–364 resulted correspondingly in 83% and 86% inhibition.

In the next set of experiments we sought to demonstrate the T cell contribution in the regulation of the idiotypic–anti-idiotypic network. Immunization with pep289–308 or cpep289–308 induced a specific spleen T cell response to the homologous peptide, resulting in 4 SI (sample/background: 3200/800 cpm) and 10 SI (sample/background: 11 000/1100 cpm), respectively. These T cells

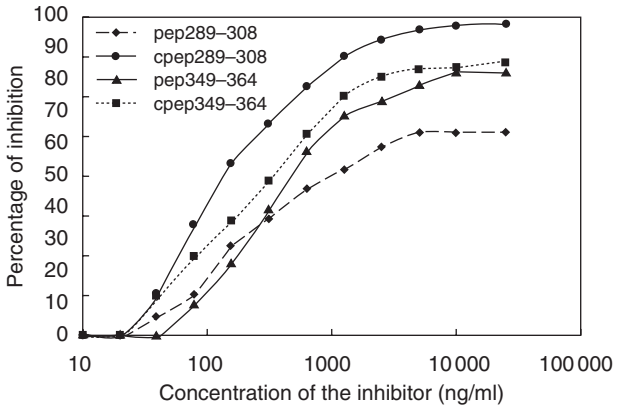

Fig. 3. Homologous inhibition of sera antipeptide antibodies. Sera from all groups of mice preincubated with sequential concentrations of the immunizing peptide (0–20 μg/ml) and tested in specific ELISA assays for the homologous peptide.

provide the necessary help to B cells for producing idiotypic antibodies. In parallel, these splenocytes also recognized the corresponding peptide with inverted hydrophobicity to peptide used for immunization (Fig. 4). Stimulation of these splenocytes with pep349–364, cpep349–364 or unrelated peptides did not induce a detectable immune T cell response. Lymphocytes from non-immunized or CFA-alone immunized mice did not respond to either peptide *in vitro*. The same series of experiments was performed with pep349–364 and cpep349–364-sensitized spleen cells, but no reactivity was obtained to either La/SSB epitope or its complementary form (data not shown).

Qualitative differences in isotype profile of idiotypic–anti-idiotypic antibody responses

In order to analyse the type of the idiotypic–anti-idiotypic antibody response elicited by the two forms of 289–308 epitope, as well as the 349–364aa epitope of the human La/SSB autoantigen, in immunized BALB/c mice, analysis of the isotype profile was performed 56 days post-immunization. The distribution of idiotypic isotype profile to pep289–308 revealed the predominance of the IgG1 and IgG2b subclasses, 60% and 30% of total IgG, respectively (Fig. 5a). The amounts of IgG2a and IgG3 isotypes were negligible. On the other hand, the anti-idiotypic antibodies

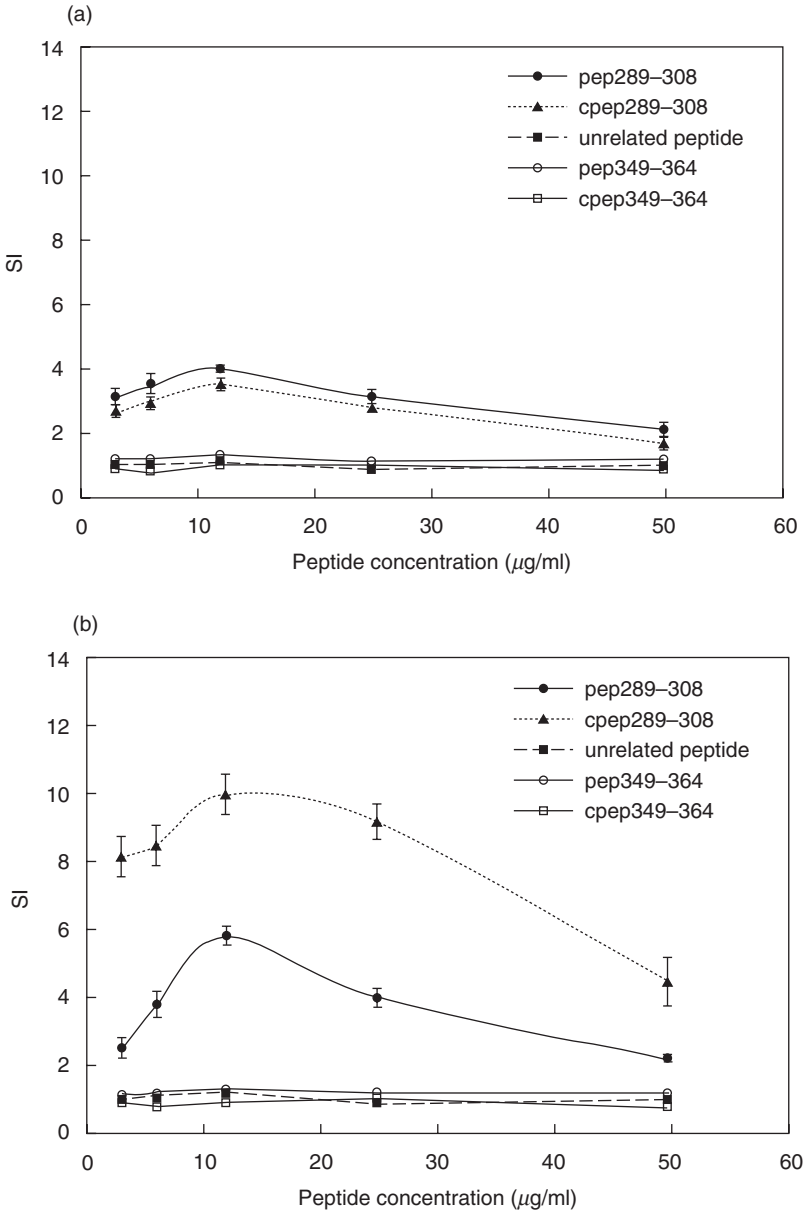

Fig. 4. Peptide-specific lymphoproliferative responses of spleen cells. Pep289–308-sensitized spleen cells (a) or cpep289–308-sensitized spleen cells (b), coincubated *in vitro* with pep289–308 (●), cpep289–308 (▲), pep349–364 (○), cpep349–364 (□) or an unrelated peptide (■) in sequential concentrations as indicated in Materials and methods. The lymphocytes were cultured in triplicate for 96 h at 37°C. Cultures were pulsed with 1 μCi/ml [^{3}H]-TdR during the last 18 h. The results represent the [^{3}H]-TdR incorporation, expressed in SI ± s.d. from triplicate cultures of pooled cells. Each experimental group consisted of cells pooled from three mice. Results are representative of three independent experiments.

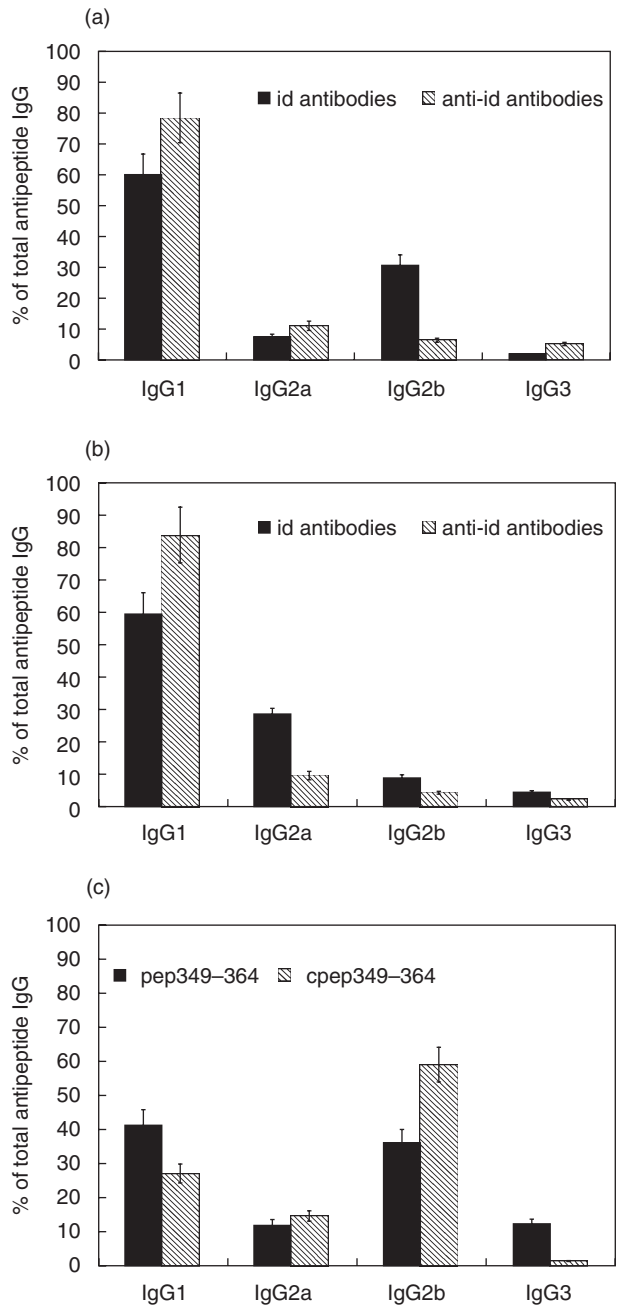

Fig. 5. Isotype profile of specific antipeptide antibodies. BALB/c mice were immunized with 100 μg/mouse pep289–308 (a), cpep289–308 (b), pep349–364 or cpep349–364 (c) conjugated to SOC_4 and emulsified in equal volume of CFA. This was followed by two boostings with an interval of 2 weeks with a half-dose of the same peptide, emulsified in an equal volume of IFA. Fifty-six days after immunization, serum samples were collected and tested in specific ELISA for determining the idiotypic and anti-idiotypic antibody IgG subclasses. The results given are the mean value from six individual mice. Preimmunized mice were used as controls (data not shown).

elicited by the pep289–308 immunization also displayed the strong dominance of the IgG1 subclass (78%) with insignificant amounts of IgG2a, IgG2b and IgG3 (11%, 6%, 5%, respectively) (Fig. 5a).

Notably, idiotypic antibody response to cpep289–308 was also characterized by the predominance of the IgG1 (58% of the total IgG) isotype with a remarkable quantity of IgG2a (27%), whereas the ratio of the other IgG subclasses was found to be IgG2b > IgG3. Antiidiotypic antibodies are mainly of IgG1 isotype (84%), while the other IgG subclasses were almost negligible (Fig. 5b).

Immunization with the pep349–364 of the hLa/SSB autoantigen resulted in a characteristic IgG subclass distribution of IgG1 > IgG2a > IgG2b ≥ IgG3-specific antibodies for the immunizing peptide. In contrast, immunization of mice with cpep349–364 induced mainly the generation of specific anticpep349–364 antibodies of the IgG2b isotype, with a small amount of IgG2a and IgG1 (Fig. 5c).

Cytokine production by specific T cells involved in the regulation of the idiotypic–anti-idiotypic antibody response to hLa/SSB peptides

The induction of precursor T helper cells, which control the production of autoantibodies, is related to their polarization on the basis of their cytokine production. To establish a correlation of the above data with a diverse secretion of cytokines by T cells from mice immunized with pep289–308 or cpep289–308, analysis of two representative cytokines, IL-4 and IFN-γ secreted upon peptide stimulation *in vitro* was performed. Spleen cells from mice immunized, as stated earlier, with pep289–308 produced significant amounts of IL-4 and a notable quantity of IFN-γ after *in vitro* stimulation with the homologous peptide. In contrast, stimulation with cpep289–308 induced increased production only of IL-4 (Fig. 6a). Additionally, spleen cells from mice immunized with cpep289–308 after *in vitro* stimulation with the homologous peptide produced remarkable quantities of IFN-γ and IL-4, while stimulation of these cells with pep289–308 induced the secretion of higher amounts only of IL-4 (Fig. 6b).

In the case of the 349–364aa epitope of the hLa/SSB autoantigen that, in our hands, did not succeed in triggering a T cell response, cytokine screening was performed in the sera of mice immunized with pep349–364 or cpep349–364 56 days post-immunization. Immunization with pep349–364 elicited the simultaneous production of IL-4 and IFN-γ (2000 μg/ml and 1750 μg/ml, respectively), whereas cpep349–364 induced the generation of a significant amount of IFN-γ (2500 μg/ml *versus* 500 μg/ml IL-4). The systemic detection of cytokines in the sera of immunized mice showed the induction of differential profiles of T cell subsets.

DISCUSSION

The idiotypic–anti-idiotypic network seems to play an important role in the initiation, maintenance and regulation of the autoimmune response [37–41]. Complementary peptides, derived by complementary (non-coding) DNA sequences to cDNA encoding for the epitopes appear to be capable of inducing the production of anti-idiotypic antibodies, directed towards antibodies targeting the epitopes (idiotypic antibodies). This immunoregulatory function has been demonstrated in several experimental mouse models of autoimmunity [27,42,43]. Our study provided further insights into the mechanism(s) which underlie the regulation of the response to human La/SSB epitopes in experimental non-autoimmune mouse models.

The main findings of the present report are that immunization with either the linear B and T cell epitopes of human La/SSB

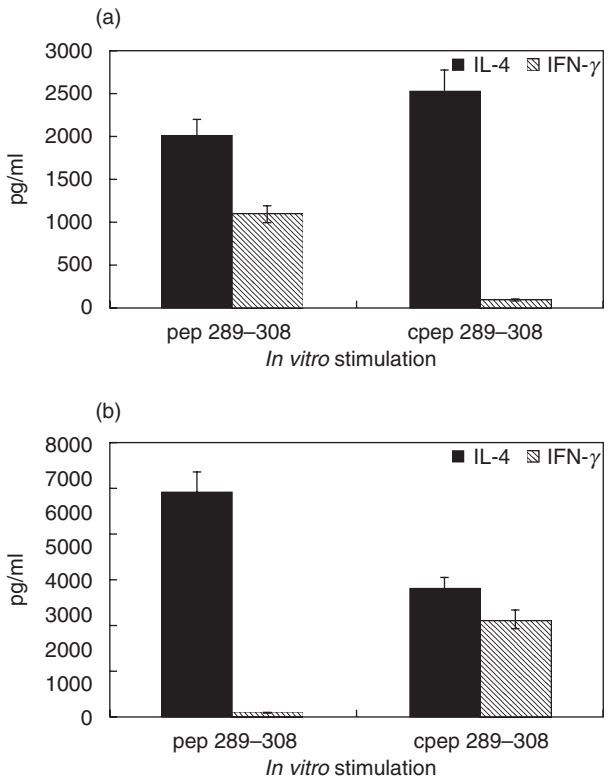

Fig. 6. Production of Th1/Th2 cytokines (IFN-γ, IL-4, respectively) in the supernatants of spleen cell cultures. Pep289–308-sensitized spleen cells (a) or cpep289–308-sensitized spleen cells (b), coincubated with pep289–308, cpep289–308 (12 μg/ml) in 24-well, flat-bottomed plates at 37°C in 5% CO_2. The supernatants were collected at designated time-points and tested in sandwich ELISA for the detection of IFN-γ and IL-4. Concentrations of IFN-γ (□) and IL-4 (■) were determined using a standard curve based on known quantities of mouse recombinant IFN-γ and IL-4. Results are representative of three independent experiments.

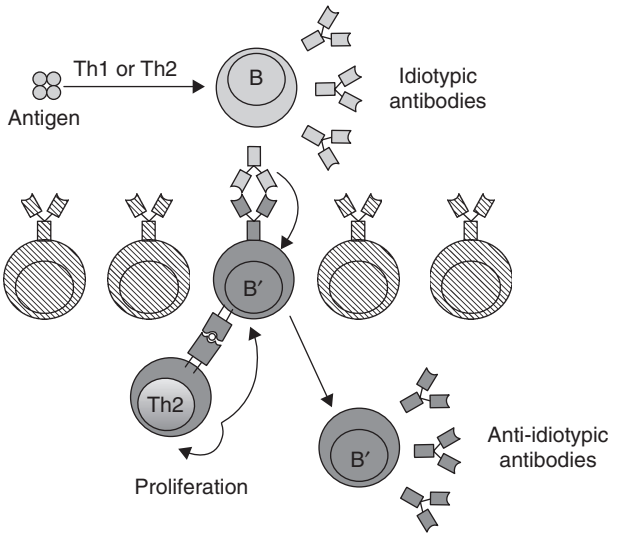

Fig. 7. Schematic illustration of the interaction of idiotypic antibodies with surface Ig of naive B lymphocytes with anti-idiotypic specificity (B′ cell).

autoantigen, pep289–308 [5,8], or its complementary form, cpep289–308, can induce the simultaneous establishment of B and T cell responses against both peptides in BALB/c mice. Furthermore, the development of the id/anti-id network in experimental animals after peptide immunization was associated with a peptide-specific pattern of IgG subclass distribution and cytokine production. In contrast, immunization with the B cell immunodominant determinant of La/SSB autoantigen, pep349–364, or its complementary form, cpep349–364, induced a strong B cell response, targeting only the immunizing peptide, with a characteristic IgG subclass distribution and cytokine pattern.

The observation that immunization of non-autoimmune BALB/c mice with the two linear epitopes of La/SSB autoantigen resulted in the induction of a strong idiotypic antibody response after 28 and 56 days p.i. is consistent with previous reports [6,8]. These studies support that immunization of non-autoimmune mice with multimer synthetic peptides derived from the mouse and human La/SSB autoantigen sequences leads to the production of polyclonal idiotypic antibody responses. Furthermore, it was found that immunization with either form of 289–308aa epitope elicited the production of both antipep289–308 and anticpep289–308 antibodies, indicating the development of an active idiotypic–anti-idiotypic network in both immunizations. This finding is in agreement with the molecular recognition theory, which suggests that immunization with a peptide corresponding to a given epitope can elicit the production of both antipeptide and anticomplementary peptide antibodies that recognize each other in an id–anti-id manner [44,45]. In fact, the affinity purification and the assesment of the idiotypic–anti-idiotypic interaction of antipeptide and anticomplementary peptide antibodies by a competitive ELISA assay demonstrated their id–anti-id mutual recognition. In addition, this id–anti-id relation has been demonstrated already by our group for antipep289–308 and anticpep289–308 antibodies in sera from patients with Sjögren's syndrome and systemic lupus erythematosus [46].

The antigen-driven antibody production in BALB/c mice and the establishment of the idiotypic–anti-idiotypic network in 289–308aa immunization prompted us to investigate the role of T cells in the development and regulation of the immune response. T cell responses in primed lymph node cells as well as in sensitized spleen cells revealed that pep289–308 and cpep289–308 are T cell epitopes. Both peptides exhibited T cell reactivity not only after stimulation with the immunizing peptide but also with the complementary form of the 289–308aa epitope. One possible explanation for this observation is a potential cross-reactivity between the pep289–308 and its complementary peptide at the T cell level. This assumption, however, cannot explain the observed two stages production of autoantibodies and cytokines. An alternative extrapolation of our findings is associated with recent studies supporting that the initiation and maintenance of the idiotypic–anti-idiotypic network is based on the mutual interaction of T helper cells with B cells presenting idiopeptides derived from the variable regions of the idiotypic antibodies. The strong priming of the T cells by pep289–308 or cpep289–308 was characterized by the copious peptide-specific IL-2 production which occurred when pep289–308 or cpep289–308-primed LNCs restimulated *in vitro* with each peptide (data not shown). Pep349–364, characterized already as a major B cell epitope [4] or its complementary peptide, cpep349–364, failed to induce a detectable T cell response, reflecting most probably the absence of a T cell determinant

within the moiety of the peptide in the context of the specific genetic background of BALB/c (H-2^d) mice. On the contrary, pep289–308 and its complementary peptide exhibited the most significant B and T cell response in BALB/c mice in comparison with the other epitope, indicating an enhanced T and B cell epitope nature in H-2^d haplotype mice. It has been shown that immunization with synthetic peptides resulted in various data depending on the experimental animal haplotype [47].

The cytokine profile was defined after their measurement in supernatants of spleen cells for pep289–308- and cpep289–308-immunized mice. IL-4 and IFN-γ were evaluated in the supernatants of spleen cells obtained from either pep289–308- or cpep289–308-immunized animals after *in vitro* stimulation with the homologous peptide. The idiotypic response was characterized by a simultaneous increase of IL-4 and IFN-γ, suggesting that a dual Th1/Th2-type activation occurred. On the other hand, in all immunization experiments the anti-idiotypic response proved to be of the Th2 type, as attested by the abundant production of IL-4 and the absence of IFN-γ upon *in vitro* stimulation with the complementary form of the immunizing peptide.

In the present study, the IgG subclasses profile was evaluated in pep289–308- and cpep289–308-immunized animals. It was shown that the idiotypic antibodies, independently of the immunizing peptide, had a polyclonal pattern of IgG1 and IgG2 subclasses. In contrast, antibodies of IgG1 type predominated in anti-idiotypic response, regardless of the immunizing peptide. Previous studies have shown that the Th2 cytokine, IL-4, induces IgG1 isotype-switching, whereas the Th1 cytokine, IFN-γ, is the stimulator for class-switching in favour of IgG2a and IgG2b [17,48,49]. This is in accordance with our results, where both IgG1 and IgG2a/b antibodies detected in the Th1/Th2 idiotypic response in contrast to IgG1 subclass antibodies dominated the anti-idiotypic Th2 response. Nevertheless, a distinct IgG subclass distribution characterized the idiotypic response for each immunizing peptide, with IgG1/IgG2a and IgG1/IgG2b types to predominate in antipep289–308 and anticpep289–308 response, respectively.

The IgG subclass profile was also evaluated in pep349–364 and cpep349–364 immunizations where a strong antibody response, targeting only the immunizing peptide, was observed. Therefore, immunization of BALB/c mice with pep349–364 elicited the establishment of a dual Th2 and Th1 type of response. This was determined by the cytokine pattern (IL-4 and IFN-γ) and the isotype distribution (IgG1 and IgG2b subclasses) obtained in sera of mice 56 days post-immunization. To the contrary, cpep349–364 immunization generated massive production of IFN-γ in sera of mice, which in turn stimulated class-switching to the IgG2b isotype.

As presented in this study, an anti-idiotypic response to both pep289–308 and cpep289–308 peptides is a Th2-dominated immune response, although the idiotypic response against the same peptides is of mixed Th1/Th2 type. These results can be interpreted by the following mechanism (depicted in Fig. 7): first, immunization with the antigenic peptide (in the presence of Freund's adjuvant) leads to the secretion of idiotypic IgG antibody by activated B cells (plasma cells) with antipeptide specificity. The activation of these B lymphocytes requires the assistance of Th1 and/or Th2 T helper cells, depending on the nature and concentration of the immunogen, the genetic background of the animals, the route of immunization and the presence of adjuvants [11,50]. As the idiotypic (antipeptide) antibody produced, it interacts selectively with the surface Ig of naive B lymphocytes with anti-idiotypic specificity (B′ cell in Fig. 7). According to the molecular recognition theory, this population of B cells bears antigen receptors with anticomplementary peptide specificity [44,45], and thereby can internalize and process the idiotypic antibodies. As a result, complementary peptide-like idiopeptides are presented preferentially to Th2 cells [51,52]. The B′ cells receive, in turn, Th2 help, resulting in their clonal expansion, differentiation to plasma cells and subsequent secretion of anticomplementary peptide antibodies. In a similar way, the same mechanism is applicable in the case of cpep immunizations with the difference that the anticpep response becomes idiotypic (Th1/Th2) and the antipep response is anti-idiotypic (Th2-dominated).

The two-stage cytokine production (Th1→Th2 type), observed in our immunization experiments, is reminiscent of the cytokine production profile reported for experimental lupus erythematosus [16,53,54]. In this experimental model of SLE, the induction of the disease is achieved by activation of an idiotypic network with antibodies bearing or targeting the anti-DNA 16/6 idiotype [55–57]. In addition, the activation of the 16/6 idiotypic network is accompanied by the production of anti-La/SSB antibodies, sharing common characteristics with the human anti-La/SSB antibodies [37]. Furthermore, the same idiotypic network can be activated and experimental lupus can be induced upon immunization with a monoclonal anti-La/SSB antibody [37]. It is worth mentioning that both the two-stage cytokine production (Th1→Th2) and the targeting of the cpep289–308 peptide by anti-idiotypic anti-La/SSB antibodies are characteristics also observed in human SS and SLE diseases [46,58,59].

From the above discussion, our data demonstrated the two-stage activation of the idiotypic network (Th1→Th2) upon immunization with either the La/SSB B cell/T cell epitope, pep289–308, or its complementary peptide, cpep289–308, but not upon immunization with the major B cell epitope of La/SSB pep349–364 and its complementary counterpart cpep349–364. A potential mechanism explaining the series of cytokine production in our system and the necessity for incorporation of both B cell and T cell epitope characteristics in the same idiopeptide mimetic for activation of the idiotypic network is also presented. Furthermore, the cpep289–308 peptide, resembling idiopeptide structures on both murine and human anti-La/SSB antibodies, might prove useful for immunoregulation of anti-La/SSB response in a similar manner to that presented recently for modulation of experimental lupus erythematosus with idiopeptides belonging to 16/6 anti-DNA antibodies [19,60,61].

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