Clin Exp Med (2013) 13:49–57
DOI 10.1007/s10238-012-0177-6

ORIGINAL ARTICLE

Properties of antibodies to a synthetic peptide representing an epitope shared by receptors of the type I cytokine family

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Received: 16 June 2011 / Accepted: 18 January 2012 / Published online: 1 February 2012

Abstract Previous works from our laboratory demonstrated that the monoclonal antibody (MAb) called R7B4 is directed to an epitope shared by various receptors corresponding to the type I cytokine receptor family, containing the common motif WSXWS or the homologous F(Y)GEFS. Later a consensus peptide significantly recognized by the MAb was identified and synthesized (sequence HGYWSEWSPE). In the present work, an homologous of the consensus sequence (HHGYWSEWSPE) was conjugated to PADRE adjuvant to produce Ab that could simulate the MAb activity, that is, acting as hormone and/or cytokine antagonists. The covalently conjugated peptide-PADRE was a better immunogen than the consensus peptide alone according to the reactivity of sera from C57BL/6 immunized mice and, besides, no Ab to PADRE were detected. Furthermore, Ab to consensus peptide elicited after peptide-PADRE inoculation into mice behaved as immunomodulatory agents, since they improved the humoral response to a foreign antigen (in this case ovalbumin). In addition, the Ab inhibited the in vitro proliferation of various cell lines, mainly cells derived from human and mouse breast cancer. Thus, immunization with the conjugate peptide-PADRE prepared under the experimental conditions described herein originated immunomodulatory Ab that, in the future, could be tested in some pathological conditions.

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Keywords Type I cytokine receptors · Monoclonal antibody · Consensus epitope · PADRE adjuvant

Introduction

We have previously shown that the monoclonal antibody (MAb) termed R7B4 blocked the human growth hormone (hGH) binding to lactogenic, somatogenic and human-specific liver receptors [1–4] as well as the binding of prolactin (PRL) and interleukins 2 (IL-2) and 6 (IL-6) to their respective cellular receptors [1, 3].

To identify the epitope recognized by the MAb R7B4, 34 synthetic decapeptides corresponding to sequences from various type I cytokine receptors containing the conserved motifs WSXWS or F(Y)GEFS [5–7] were prepared. Results indicated that 21 peptides were significantly recognized by the Ab, the most reactive sequences being those contained in PRL and diverse IL receptors. Consequently, taking into account the number of times that a given amino acid was found in the same position, the following consensus sequence was established: HGYWSEWSPE (His-Gly-Tyr-Trp-Ser-Glu-Trp-Ser-Pro-Glu) [8].

Thus, since the consensus sequence should be similar to a portion of the epitope recognized by the MAb, we expected (1) that the consensus peptide should bind to the Ab paratope inhibiting its action and (2) that polyclonal Ab elicited after immunization with the consensus peptide should imitate the activity of the MAb R7B4.

In fact, both hypothesis were proved, since the addition of the consensus peptide to the incubation medium significantly reversed MAb action on the binding of ^{125}I-hGH to its specific receptors, and antisera elicited in mice immunized with the consensus sequence inhibited ^{125}I-hGH binding to rat and rabbit receptors [8].

The aim of the present work was to amplify the immune response of mice to the consensus epitope inoculating simultaneously a strong T-cell stimulator, the synthetic peptide-PADRE, for "Pan HLA-DR binding epitope" [9–11]. Accordingly, a synthetic conjugated comprising both PADRE and a homologous of the consensus peptide sequence containing an extra His residue was prepared (peptide-PADRE). It was found that Ab to consensus peptide present in sera from C57BL/6 mice immunized with the conjugated mimicked the MAb R7B4 activity, since they strongly inhibited binding of hGH to its specific receptors present in rat or rabbit liver. Furthermore, results indicated that these Ab behave as immunomodulators of the humoral immune response against a protein antigen (Ag) and were also able to inhibit the "in vitro" proliferation of various tumor cell lines.

Materials and methods

Materials

Pituitary hGH (AFP-9755-A) was provided by the NIDDkDa's National Hormone & Pituitary Program and A.F. Parlow, Harbor-UCLA Medical Center, Torrance, CA.

Reagents and solvents used for peptide synthesis were purchased from Applied Biosystems (Foster City, CA, USA). Fmoc-protected L-amino acids with the following side-chain protections: Glu (γ-*tert*-butyl), His (trityl), Ser (*tert*-butyl), Tyr (*O*-*tert*-butyl), trifluoroacetic acid (TFA), 1,2-ethanedithiol, thioanisole, phenol and complete and incomplete Freund's adjuvants were obtained from Sigma-Aldrich Inc. (Illinois, MO, USA).

Mice

Specific pathogen-free (SPF) female C57BL/6 mice from the University of La Plata, Argentina, were used at the age of 8–10 weeks. All animals were maintained in isolators, on standard laboratory chow, under SPF conditions until the end of the experiments, and received care in compliance with international legal requirements.

Radioiodination

^{125} I-hGH was prepared following the method described by Roth [12]. Specific radioactivities ranging from 90 to 120 μCi/μg were usually achieved.

Antibodies

The preparation and characterization of anti-receptor MAb R7B4 was previously described [1]. Control immunoglobulins (control Ig) were obtained from sera of non-immunized C57BL/6 mice. Ab used in some experiments were purified by precipitation with 50% (w/v) ammonium sulfate as described previously [3, 4].

Synthesis and purification of PADRE and peptide-PADRE

The synthesis of unhomologous of the consensus sequence containing a further His residue (His-His-Gly-Tyr-Trp-Ser-Glu-Trp-Ser-Pro-Glu), PADRE adjuvant (dAla-Lys-Cha-Val-Ala-Ala-Trp-Thr-Leu-Lys-Ala-dAla, being Cha: L-cyclohexylalanine) and peptide-PADRE (dAla-Lys-Cha-Val-Ala-Ala-Trp-Thr-Leu-Lys-Ala-dAla-His-His-Gly-Tyr-Trp-Ser-Glu-Trp-Ser-Pro-Glu) was performed by solid-phase Fmoc methodology on a 0.25-mmol scale. Peptides were assembled on an automatic peptide synthesizer model 431 A (Applied Biosystems Inc. Foster City, CA, USA). Fmoc amino acids were incorporated as hydroxybenzotriazole active esters. The Fmoc protecting group was removed with 20% piperidine in *N*-methylpyrrolidone (NMP).

Peptide cleavage of the resin was achieved with trifluoroacetic acid (TFA)/ethanedithiol/water 9.5:0.25:0.25 (v/v) for 2 h at room temperature. The suspension of resin was filtered, and the crude material was precipitated by adding 15 ml of cold diethyl ether and washed three times with diethyl ether. The residual ether was removed by evaporation under reduced pressure, and the peptide was lyophilized.

PADRE peptide was purified by high-performance liquid chromatography (HPLC) on a C18 Vydac semi-preparative column, 1 × 25 cm (The Separation Group, Hesperia. CA, USA) eluted with a linear acetonitrile gradient (24–80%, in the presence of 0.1% of TFA) over 35 min at 1.5 ml/min. The main peak was collected, lyophilized and repurified on the same column.

Consensus peptide and peptide-PADRE were purified by HPLC on a C4 Vydac semi-preparative column, 1 × 25 cm (The Separation Group, Hesperia. CA, USA) eluted with an acetonitrile gradient (0–70% in 40 min at 1.5 ml/min). The main peak was collected, lyophilized and repurified on the same column.

In each case, peptide purity was verified by amino acid analysis, sequence determination and mass spectrometry performed at the LANAIS-PRO (National Protein Sequencing Facility, UBA-CONICET, Buenos Aires, Argentina).

Mice immunization

C57BL/6 mice were immunized subcutaneously using the following protocols:

Peptide-PADRE or PADRE

Four mice were inoculated on day 0, 15, 30 and 45 with 100 µl of PBS containing 300 µg of peptide-PADRE or PADRE emulsified in an equal volume of complete (day 0) or incomplete (days 15, 30 and 45) Freund's adjuvant (final volume, 200 µl). The animals were bled 15 days after the last inoculation.

Peptide-PADRE or PADRE plus administration of ovalbumin (OVA)

Four mice were immunized with 300 µg of peptide-PADRE or PADRE alone as indicated above, at days 0 and 15. Fifteen days later, the mice were inoculated with 100 µl of PBS containing 300 µg of OVA, emulsified in an equal volume of complete Freund's adjuvant. The animals were bled 20 days later. As a control, four animals were inoculated only with 100 µl of PBS containing 300 µg of OVA, emulsified in an equal volume of complete Freund's adjuvant and bled 20 days afterward.

ELISA method to test the reactivity of Ab to the various synthetic peptides

Essentially, the method described by Ball et al. [13] was used. ELISA plates (Nunc Maxi-Sorb) were coated with 2 µg of poly-L-lysine (45–50 kDa, Sigma-Aldrich Inc., Illinois, MO, USA) contained in 50 µl of 0.05 M sodium bicarbonate buffer, pH 9.6. After 1 h at room temperature and a wash with PBS, 50 µl of 1% (v/v) glutaraldehyde was added to each well and the plates washed after 15 min of incubation. The synthetic peptides were diluted to a concentration of 10 µg/ml in PBS, and 50 µl was added to the wells coated with poly-L-lysine and activated with glutaraldehyde. The plates were incubated overnight at room temperature and then washed twice with PBS. Reactive aldehyde sites were blocked by the addition of 1 M glycine, 200 µl/well, followed by 1-h room temperature incubation.

The plates were then incubated overnight at room temperature with mouse serum diluted in 0.01 M Tris, 0.13 M NaCl, pH 7.4 containing 5% of non-fat milk (TBS-M), and after washing with PBS containing 0.125 ml of Tween 20 per liter (PBS-Tween), the bound Ab were revealed with peroxidase-labeled goat IgG anti-mouse IgG (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) diluted 1:10,000 in TBS-M. As a substrate, ortho-phenylene-diamine-dihydrochloride (OPD, Sigma-Aldrich Inc., Illinois, MO, USA) with freshly added H_2O_2 was used. The reaction was stopped after 10 min by addition of 1 M H_2SO_4. The absorption was measured in an ELISA reader (Metertech Inc., Taipei, Taiwan) at 490 nm. Non-specific values of optical density were obtained in the absence of mouse serum.

Competition ELISA

This assay was used to measure the extent of inhibition produced by soluble OVA on the binding of Ab to the same insolubilized Ag. ELISA microplates (Nunc Maxi-Sorb) were coated with 100 µl of OVA at 5 µg/ml in PBS. After overnight incubation at room temperature, the plates were washed with PBS containing 0.01% Tween 20 (PBS-T) and blocked 2 h at 37°C with TBS containing 5% fetal calf serum (TBS-FCS). The plates were incubated with each serum diluted in TMS-M as to obtain an OD of 0.800–1.500 and serial dilutions of soluble OVA. Bound Ab were revealed as described above, and the OD values were expressed as percent of control.

Proliferation of different cell lines

Suspension cultures of lactogen-dependent Nb2 lymphoma cells [14] were maintained in Fischer's medium (Gibco BRL, Gaithersburg, MD) containing 50 U/ml penicillin, 50 µg/ml streptomycin, 100 µM 2-mercaptoethanol (2-ME), 10% horse serum (HS) and 10% FCS.

7TD1 cells, an IL-6-dependent hybridoma [15], were kindly provided by Drs. J. Van Snick and J-C. Renauld (Ludwig Institute, Brussels, Belgium). Cells were grown in Iscove's medium (Gibco BRL, Gaithersburg, MD) supplemented with antibiotics, 10% FCS, 2 mM glutamine, 0.24 mM asparagine, 0.55 mM arginine, 50 µM 2-ME, 0.1 mM hypoxanthine, 16 µM thymidine and 200 U/ml of mouse IL-6.

WISH cells, derived from human amnion tissue [16] H4lle cells, from a human hepatome, provided by the American Tissue Culture Collection (ATCC, USA), and MCF-7 breast cancer cell line [17] were cultured in Minimum Essential Medium (MEM, Gibco BRL, Gaithersburg, MD) supplemented with antibiotics, 10% FCS and 2 mM glutamine.

NMuMG cell line, derived from a mouse mammary gland [18], proliferated in MEM F12 medium containing antibiotics, 1 mM Hepes and 10% FCS.

A hundred microliters containing 20,000 cells of each cell line was added to 96-well microplates containing 100 µl of the indicated antiserum diluted 1:25 in the corresponding incubation medium. After 3 days of incubation, cell number was evaluated by colorimetric determination of hexosaminidase levels [19].

Microsome preparations

Livers from late pregnant Wistar rats or female rabbits (New Zealand White, 2.5 kg) were homogenized in 10 vol (v/w) of chilled 0.3 M sucrose, 1 mM PMSF, 1,000 U/L trypsin inhibitor, 5 mM Tris–HCl buffer, pH 7.4. After

centrifugation at 10,000×*g* for 20 min and then at 100,000×*g* for 1 h, each pellet was resuspended in 25 mM Tris/HCl buffer, pH 7.4.

A sample of each suspension was solubilized by heating for 30 min at 100°C in 1 M NaOH, and protein concentration was determined according to Bradford [20]. The crude microsomal suspensions (protein concentration, about 30 mg/ml) were frozen at −20°C until use.

Binding of hGH to rat and rabbit receptors

^{125}I-hGH (0.5–2 ng) was incubated overnight at 25°C in 0.3 ml of chilled binding medium (BM): 25 mM Tris/HCl, 10 mM MgCl2, 0.1% BSA, pH 7.4 with liver microsomes (rat, 30 µg; rabbit, 120 µg; or human, 500 µg of microsomal protein) and the indicated amounts of competitors (unlabeled hGH, Ab or peptides). As reported before [21], in order to saturate lactogenic receptors present in liver rabbit microsomes, the somatogenic specificity was achieved by incorporating 4.5 nM of ovine PRL to the incubation medium.

The reaction was stopped by the addition of 4 ml of ice-cold BM, and the membranes were sedimented by centrifugation at 800×*g* for 25 min at 4°C. Bound radioactivity was measured in an automated gamma counter. ^{125}I-hGH non-specific binding was determined in the presence of 3 µg/ml of unlabeled hGH.

Determination of mouse serum concentration of IL-2, IL-6, IL-10 and IFN γ

Plasma cytokine concentrations were calculated using de Diaclone Kits named Mouse Eli-pairs (Diaclone SAS, Besançon, France). Detection sensitivity levels of the assay methods were as follows: IL-2, 15.6–500 pg/ml; IL-6, 15.6–500 pg/ml; IL-10, 31.3–1,000 pg/ml; and IFNγ, 31.0–1,000 pg/ml.

Results

Reactivity of sera from mice immunized with peptide-PADRE

Ab to consensus peptide or PADRE were measured by ELISA as described in "Materials and methods". Results indicated that Ab from mice immunized with peptide-PADRE reacted only with the consensus peptide and no with PADRE (Fig. 1a). As expected, sera from mice inoculated with PADRE recognized only this peptide (Fig. 1b) and Ig from control animals did not show any reactivity (Fig. 1c).

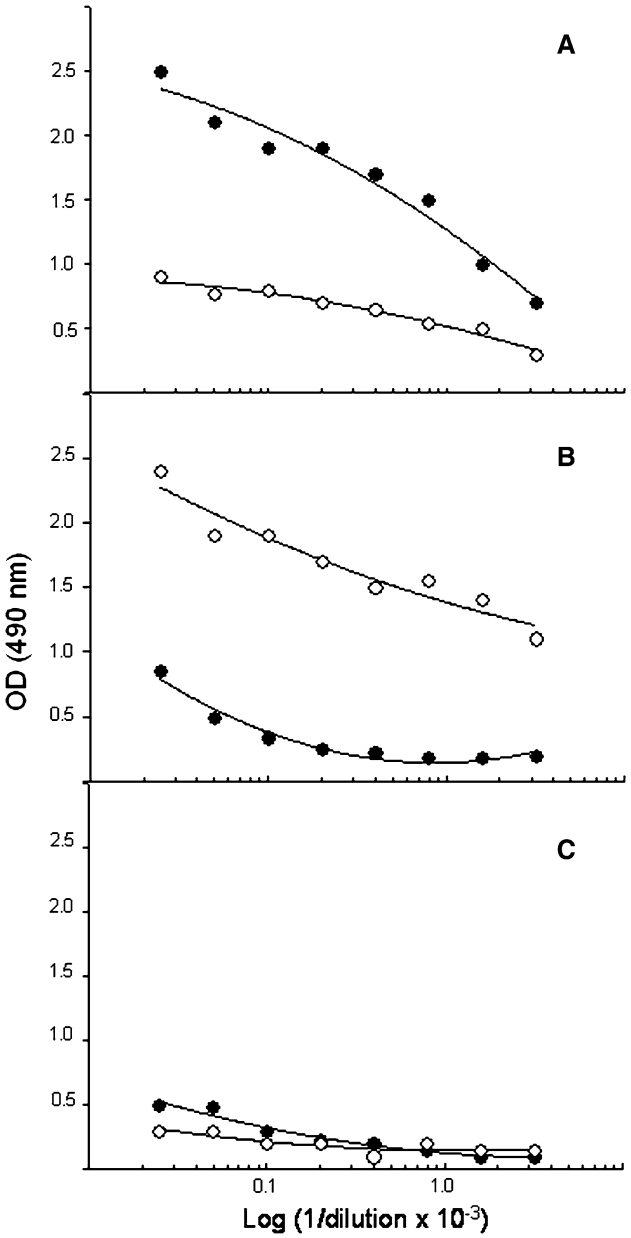

Fig. 1 Ab reactivity in sera from C57BL/6 mice immunized with peptide-PADRE or PADRE. ELISA microplates were coated with polylysine, and the synthetic consensus peptide (*filled circle*) or PADRE (*open circle*) was captured as indicated in "Materials and methods". The plates were incubated with different dilutions of a pool of antisera from four animals immunized with peptide-PADRE (**a**) or with PADRE (**b**). Normal Ig was utilized as a negative control (**c**). Bound Ab were detected with peroxidase-labeled goat anti-mouse IgG

Effects of Ab to the consensus peptide on hGH binding to liver-specific receptors

It was previously demonstrated that the consensus peptide was recognized by MAb R7B4 and that Ab to this sequence simulate the MAb activity [8]. In this way, the same sera

Table 1 Effect of Ab raised in mice immunized with peptide-PADRE or PADRE on the ^{125}I-hGH binding to rat and rabbit liver receptors

Receptor source	Bound radioactivity (cpm)					
	^{125}I-hGH	MAb R7B4	Ab to peptide-PADRE		Ab to PADRE	
			Serum dilution		Serum dilution	
Rat	51,247 ± 1,421	34,450 ± 530**	1/25	40,180 ± 1,430*	1/25	47,230 ± 840
			1/50	43,520 ± 540*	1/50	50,370 ± 500
			1/100	48,510 ± 1,900	1/100	53,090 ± 110
Rabbit	33,780 ± 600	18,140 ± 340**	1/25	25,770 ± 890**	1/25	29,680 ± 490*
			1/50	27,250 ± 480**	1/50	32,580 ± 360
			1/100	30,400 ± 1,040	1/100	33,100 ± 1,600

The tracer (approximately 200,000 cpm) was incubated overnight at 25°C with rat (30 μg of protein) or rabbit (120 μg of protein) liver microsomes in the presence or absence of 50 μg/ml of purified MAb R7B4 and the indicated dilutions of sera to peptide-PADRE or to PADRE. Bound radioactivity is a mean of three determinations and is expressed as specific binding ±SD. Statistical significance in comparison with control value (bound radioactivity in the absence of Ab) by Student's *t* test is indicated by $*P < 0.01$ and $**P < 0.001$. Values obtained with 50 μg/ml of purified control Ig were as follows: rat, 52,800 ± 1,700 cpm; rabbit, 31,550 ± 790 cpm)

used in Fig. 1 were tested for their ability to inhibit hGH binding to lactogenic and somatogenic receptors present in rat and rabbit liver, respectively. Results indicated that Ab elicited in mice immunized with peptide-PADRE did impair ^{125}I-hGH binding to its specific receptors, whereas Ab to PADRE did not affect the hormone binding (Table 1).

Effect of Ab to peptide-PADRE on the humoral immune response to ovalbumin (OVA)

To explore the effect of Ab to peptide-PADRE on the humoral immune response to a protein antigen, B6 mice were first immunized either with peptide-PADRE or with PADRE and then inoculated with OVA. As a control, another group of mice was immunized only with the protein (see "Materials and methods" for details).

ELISA indicated that, as expected, anti-OVA Ab titers were higher in mice previously immunized with the adjuvant PADRE than in the animals inoculated only with OVA (Fig. 2). However, results showed that the highest titers of Ab to OVA were found in mice immunized with peptide-PADRE, suggesting that the synthetic conjugate of consensus peptide with PADRE was the most effective modulator of the humoral response to the protein Ag (Fig. 2).

Nature of the epitopes recognized by the Ab anti-OVA

Ab directed to hidden antigenic determinants or cryptotopes are disadvantageous because they do not recognize the native conformation that usually exposes a soluble protein Ag. By contrast, Ab to native or natural epitopes should effectively neutralize the Ag to be eliminated [22, 23].

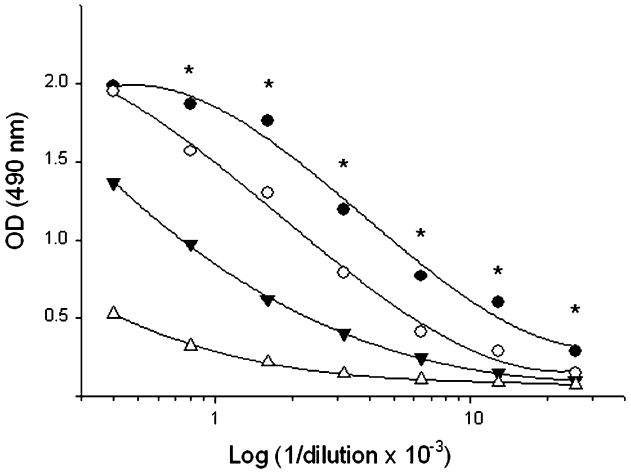

Fig. 2 Ab anti-OVA in sera from C57BL/6 mice previously immunized with peptide-PADRE or PADRE. ELISA microplates were coated with OVA as described in "Materials and methods". The plates were incubated with different dilutions of antisera from animals immunized with peptide-PADRE (*filled circle*) or PADRE (*open circle*) and then inoculated with OVA as explained in "Materials and methods". Sera from animals only inoculated with OVA (*inverted triangle*) as well as control Ig (*open triangle*) are also shown. Bound Ab were detected with peroxidase-labeled goat anti-mouse IgG. Results are means of three determinations ± SD. Statistical significance of the values obtained with antisera to conjugated peptide in comparison with antisera to PADRE by Student's *t* test is indicated by $*P < 0{,}001$. *The error bars* are included in the experimental dots

To evaluate the extent of Ab to cryptic versus native OVA epitopes, we performed competition ELISA experiments, that is, we measured the inhibition produced by a soluble Ag on the binding of Ab to the same insolubilized Ag. As reported previously [22, 23], the Ab remaining bound to insolubilized Ag in the presence of a high concentration of the same soluble Ag must be directed against cryptic epitopes. Thus, under our experimental conditions, the curve reached the plateau when 12 μg/ml of OVA was

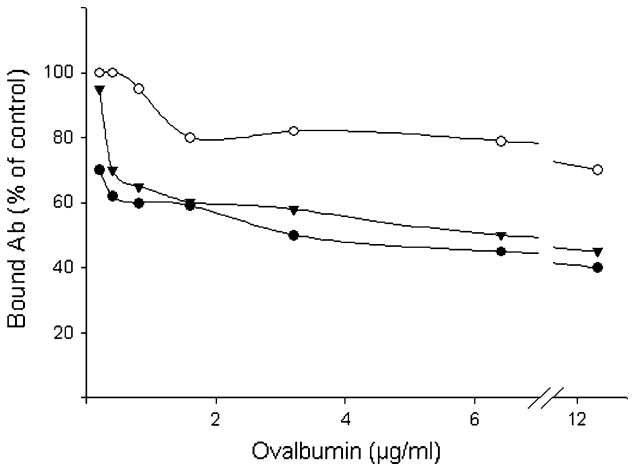

Fig. 3 Detection of Ab to native and cryptic OVA epitopes. ELISA microplates were coated with OVA as described in "Materials and methods". The plates were incubated with pooled antisera to peptide-PADRE (*filled circle*), to PADRE (*open circle*) and to OVA (*inverted triangle*) in a dilution to obtain an optic density value of approximately 1.0 (100% of binding), and different concentrations of soluble OVA were added. Bound Ab were detected with peroxidase-labeled goat anti-mouse IgG

Table 2 Calculation of the relative percentage of Ab anti-OVA native epitopes in sera from C57BL/6 mice immunized as indicated

Immunization	Dilution[a]	% of Ab anti-native epitopes[b]	Relative anti-native OVA epitopes[c]
Peptide-PADRE	5,000	50	2,500
PADRE	2,500	30	750
OVA	1,000	50	500

[a] Dilution of serum to obtain an OD = 1.0 (see Fig. 2)

[b] Percent of Ab to native OVA epitopes calculated from data presented in Fig. 3

[c] Extent of Ab to native OVA epitopes in each experimental condition: (a × b)/100

used as competitor (Fig. 3). In each case, the position of the plateau indicated the percent of Ab directed to native OVA epitopes, the rest being those Ab still bound to the insolubilized OVA and so directed to its cryptic antigenic determinants. Accordingly, results from Fig. 3 indicated that Ab to native OVA in control mice or in those previously immunized with peptide-PADRE were about 50%. By contrast, values of 30% of Ab anti-native OVA epitopes were found in mice inoculated with PADRE before immunization with OVA (Fig. 3).

Table 2 associates results from Figs. 2 and 3 to estimate the degree of Ab to native or cryptic epitopes in each experimental condition. Thus, taking into consideration both Ab titers (Fig. 2) and extent of Ab to native epitopes (Fig. 3), results showed that mice immunized with peptide-PADRE elicited the best Ab anti-OVA, because they were more abundant and directed mainly to native protein epitopes (Table 2).

Cytokine levels in plasma from mice immunized with peptide-PADRE

As described in "Materials and methods", commercial kits were used to measure the plasmatic concentration of various cytokines, that is., IL-2, IL-6, IL-10 and γIFN. Results indicated that γIFN concentration was 0.5 ng/ml in mice immunized with peptide-PADRE, whereas levels of IL-2, IL-6 or IL-10 were undetectable. No cytokine was detected in sera from control (PADRE-treated or non-treated) mice (data not shown).

Effect of Ab to peptide-PADRE on cell proliferation

We tested the effect of Ab to peptide-PADRE on the proliferation of six cell lines, derived from humans (WISH, H4lle and MCF-7 cells), rats (Nb2-cells) or mice (7TD1 and NMuMG cells). Because serum samples were scarce, the Ab effect was first tested on MCF-7 cells in order to choose the best dilution to be used. Since data from Table 3 indicated that serum diluted 1:50 specifically inhibited cell growth, the screening of the various cell lines was performed using that serum dilution. Results showed that sera from animals immunized with the synthetic conjugate were able to impair the growth of Nb2, H4lle, MCF-7 and NMuMG cells, while no effect was shown with WISH and 7TD1 cell lines (Fig. 4). In no case, normal sera (from non-treated mice) showed any effect (Fig. 4).

Discussion

The aim of this work was to explore the possible immunomodulatory activity of Ab to the consensus peptide representing the MAb R7B4 target on receptors for type I cytokine family. Since the MAb humanization is a very difficult task, this approach could help to prepare a more useful and simple putative therapeutic agent. To improve the immunogenic activity of the consensus peptide, we added an extra His residue at the *N*-terminal portion and then the peptide was conjugated with a strong T-cell adjuvant, the peptide called PADRE [10, 11]. Results indicated that sera from mice immunized with peptide-PADRE contained high Ab titers directed solely to the consensus peptide and that these Ab inhibited hGH binding to rat and rabbit liver receptors, demonstrating that the polyclonal antisera simulated the MAb R7B4 activity.

Cytokines from the type I family, including PRL and GH, have immunomodulatory properties, activating either lymphocytes or macrophages [24–26]. Thus, the rational of

Table 3 Effect of Ab to peptide-PADRE or PADRE on MCF-7 cell line proliferation

Optical density					
Ab to peptide-PADRE		Ab to PADRE		Control Ig	
Serum dilution		Serum dilution		Serum dilution	
1/25	0.74 ± 0.05*	1/25	0.92 ± 0.04*	1/25	1.03 ± 0.10
1/50	0.76 ± 0.02*	1/50	0.92 ± 0.06	1/50	1.05 ± 0.20
1/100	1.03 ± 0.06	1/100	1.07 ± 0.10	1/100	1.10 ± 0.10

Approximately 20,000 cells/well were incubated for 96 h at 37°C with the indicated dilutions of antisera to peptide-PADRE, to PADRE or control Ig. (pool of four mice in each case). Cell growth was determined using the hexosaminidase method [19]. Results are means of three determinations ± SD. Values of optical density in the absence of any competitor were 1.30 ± 0.06 and 0.70 ± 0.04 in the presence of ammonium sulfate-purified MAb R7B4 (50 μg/ml). Statistical significance in comparison with values of 100% of cell proliferation by Student's *t* test is indicated by $*P < 0.001$

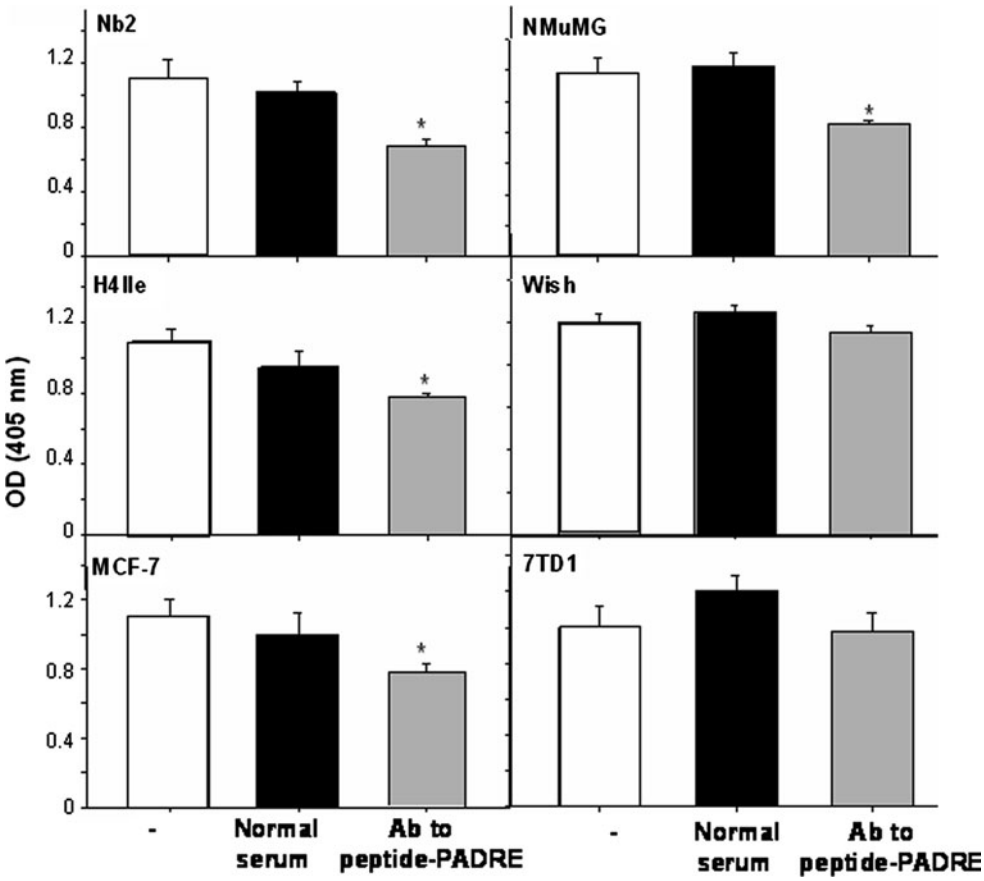

Fig. 4 Effect of Ab to peptide-PADRE on cell proliferation. As described in "Materials and methods", 20,000 cells/well were incubated for 96 h at 37°C with 100 μl of 1/50 dilution of normal serum or antisera (pool of four mice) to peptide-PADRE. Percentage of cell growth in the presence of ammonium sulfate-purified MAb R7B4 (50 μg/ml) was as follows: Nb2-cells 65%, NmumG 80%, H4IIe 88%, WISH 100%, MCF 7 67% and 7TD1 100%. Cell growth was determined using the hexosaminidase method [19]. Results are means of three determinations ± SD. Statistical significance in comparison with values of 100% of cell proliferation by Student's *t* test is indicated by $*P < 0.001$

this work was to assume that Ab to the consensus peptide will be directed to cytokine receptors and could have immunomodulatory properties since they should inhibit cytokine activities. To test this possibility, mice previously immunized with the peptide-PADRE conjugate were then inoculated with OVA. Results indicated that Ab titers to OVA were higher in treated animals than in controls and that most Ab were directed to native OVA epitopes. These results suggest that Ab directed to peptide-PADRE improved both the amount and the quality of the humoral response to the protein Ag.

Surprisingly, it was found that γIFN concentration rose in sera from mice immunized with peptide-PADRE, indicating a strong effect of the immunogen on the cells producing the cytokine, mainly T helper 1 lymphocytes (Th1). γIFN is one of the most important endogenous mediators of immunity and inflammation and plays a key role in host defense against intracellular pathogens [27]. Since γIFN functions may be mediated by cross-regulation of cellular responses to other cytokines [27], the humoral immunomodulatory activity of Ab to peptide-PADRE toward OVA may be related to the release of this cytokine.

It is well-known that cell proliferation depends of numerous factors, cytokines among them [28, 29]. Since MAb R7B4 recognizes various cytokines receptors, including PRL and GH receptors, Ab to the conjugate peptide-PADRE were tested in their ability to act on the proliferation of different cell lines. Results indicated that a significant inhibition of cell growth was obtained when Ab were included in Nb2, NMuMG, H411e and MCF-7 cell cultures, whereas no activity was observed on WISH and 7TD1 cell lines.

Since there is increasing evidence that PRL and GH act as growth promoters of breast tumors, competitive PRL and/or GH receptors antagonists were designed in order to block receptor activation [30, 31]. Our results showed that Ab to peptide-PADRE were able to inhibit both MCF-7 and NMuMG cell lines, derived from human breast cancer and mouse mammary gland, respectively, suggesting that immunization with the conjugate could be a good candidate to be tested as inhibitor of breast tumor growth.

As reported in Hamill et al. [32], there is considerable activity currently focused on the development of immunomodulators as treatments for inflammatory conditions and as vaccine adjuvants. The properties of the synthetic construction peptide-PADRE described in this work suggest that it is a first approximation to achieve that goal.

Acknowledgment The authors are indebted to Dr. Pierre L. Masson (de Duve Institute, Brussels, Belgium) for helpful discussions and critical revision of the manuscript. This work was supported by grants from CONICET and Universidad de Buenos Aires.

Conflict of interest The authors declare no conflict of interest.

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