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Production and evaluation of chicken antibodies against a synthetic peptide from glial growth factor.

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Keywords: neuregulins, glial growth factor (GGF), immunoglobulin Y (IgY), synthetic peptide.

Abstract. Neuregulins (NRG) are proteins that belong to the family of epidermal growth factors. It is well established that these factors are essential for the development and maintenance of the nervous system. Due to the difficulty of purifying enough quantities of these factors and the lack of specificity from commercially available antibodies, the aim of this work was to produce antibodies against a synthetic peptide capable to detect and identify neuregulin GGFβ isoforms. To accomplish this goal, polyclonal antibodies were raised in hens against a synthetic peptide designed from the GGFβ1 extracellular sequence. The sequence analysis was made using different epitope-predicting programs. Our results showed that the peptide sequence selected was immunogenic because it was capable of inducing a specific type B immune response in the experimental animal model. These antibodies were also capable of recognizing a recombinant GGF protein and GGF isoforms present in different samples. Our results suggest that the development of immunoglobulin Y (IgY) using synthetic peptides represents, a valuable tool for neuroscience research.

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Producción y evaluación de anticuerpos de gallinas contra un péptido sintético del factor de crecimiento glial.
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Palabras clave: neuregulinas, factor de crecimiento glial (GGF), inmunoglobulina Y (IgY), péptidos sintéticos.

Resumen. Las Neuregulinas (NRG) son proteínas que pertenecen a la familia de los factores de crecimiento epidermal. Se ha demostrado que estos factores son esenciales para el desarrollo y mantenimiento de la funcionalidad del sistema nervioso. Debido a la dificultad para purificar estas proteínas y la falta de especificidad de los anticuerpos disponibles comercialmente, el objetivo de este trabajo fue producir anticuerpos contra un péptido sintético capaz de detectar e identificar una isoforma de la Neuregulina (GGFβ). Para lograr este objetivo, se desarrollaron anticuerpos en gallinas (IgY) contra un péptido sintético diseñado a partir de la secuencia aminoacídica de la región extracelular de GGFβ, utilizando programas de predicción de epítopes. Los resultados demuestran que el péptido seleccionado fue immunogénico debido a que estimuló una respuesta inmune específica tipo B en el modelo utilizado. Estos anticuerpos fueron también capaces de reconocer una proteína recombinante e isoformas de GGF presentes en diferentes muestras biológicas. Nuestros resultados demuestran el potencial valor de las inmunoglobulinas Y (IgY) contra péptidos sintéticos como una herramienta de aplicación para la investigación en neurociencia.

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INTRODUCTION

Type 1 neuregulins (NRG-1) are a protein family of structurally related growth factors comprised of glial growth factor (GGF) (1,2), neu differentiation factor (NDF) (3), acetylcholine receptor inducing activity (ARIA) (4), heregulin (HER) (5) and sensory and motor neuron-derived factor (SMDF) (6). All of these isoforms are generated by alternative splicing from a single gene (7). All NRG-1 isoforms have an epidermal growth factor (EGF)-like sequence, which is necessary to account for NRG-1's biological activities (5, 3). Splicing also generates two variants of the C-terminal part of the EGF-like domain resulting in NRG-1α and NRG-1β, which display different affinities for NRG receptors (3, 9). These factors can be found in neurons and glial cells from central and peripheral nervous systems and play an important role in the survival, proliferation and differentiation of these cells (8-12). NRGs are also implicated in the pathogenesis of several human diseases, including schizophrenia, multiple sclerosis and breast cancer (13-15).

GGF was first described by Brockes *et al.* (1) which isolated an active component from bovine pituitary extract capable of inducing proliferation of cultured Schwann cells from sciatic nerves. It was demonstrated that the protein responsible for this proliferative effect has an approximate molecular weight of 31 kDa (16). Lately, Goodearl *et al.* (2) isolated and character-

ized three GGF polypeptides termed GGF-I, a 34-kDa species with properties similar to the previously identified molecule, GGF-II and GGF-III, of 59 kDa and 45 kDa, respectively. Other studies revealed multiple bioactivities for GGF. For example, during development, GGF is trophic for Schwann cell precursors, stimulates Schwann cells proliferation (17) and is critical for the survival of terminal Schwann cells in the developing neuromuscular junction (18). More recently, it has been proposed that GGF signaling promotes schwannoma pathogenesis (19).

Neurotrophic factors play an important role during the regeneration process of the nervous system. There is a fundamental difference between the central (CNS) and peripheral nervous system (PNS); the latter is often capable of repairing the damage caused by an injury, whereas the former generally fails in this attempt (20). Therefore, it is very important to elucidate the role played by neurotrophic factors in the course of these processes, especially NRGs.

Due to the difficulty of purifying enough quantities of these proteins and the lack of specificity from commercially available antibodies, the aim of this work was to produce antibodies capable of identifying different glial growth factor isoforms (GGF). In this regard, egg yolk immunoglobulin (IgY) have been used in multiple assays, specially in the detection of conserved mammalian proteins, due to the phylogenetic distance from mammals (21). Other advantages of IgY antibodies are that they do not activate mammalian complement, do not bind to rheumatoid factors or cell surface Fc receptors (21). Finally, the production of polyclonal IgY antibodies is easy and inexpensive to do (22).

For these reasons, in this work, we have generated monospecific polyclonal antibodies in chickens against a synthetic peptide from GGFβ neurotrophic factor, suitable for detection of this protein in different assays, which will help to discern the role played by this neurotrophic factor in the regeneration of the nervous system.

MATERIALS AND METHODS

Peptide design and synthesis

Glial Growth Factor isoform sequences (1, 2, 3 and 4) from Rattus norvegicus (access codes AF-194993/5/6/7) were analyzed with the "ANTHEPROT V.6.0 software (Deleage, G. Institut Biologie et Chimie des Proteines 7, Lyon, France), Hoops and Woods (23) "Predicted Antigenic Peptide" (MIF Bioinformatic Tools), and "Basic Local Alignment Search Tool (BLAST protein-protein)" programs (National Center for Biotechnology Information). Sequences were then analyzed (http://www.ncbi.nlm.nih.gov/BLAST/) to determine if there was any homology with other neuregulin isoforms or other protein sequences expressed in *Rattus norvegicus*. From these analyses, one peptide was chosen and synthesized by the multiple manual synthesis method described by Merrifield (24) and modified by Hougthen and coworkers (25), using the t-boc strategy (At the Laboratorio de Químicа de Proteínas de la Sección de Biohelmintiasis, Instituto de Medicina Tropical, Facultad de Medicina, Universidad Central de Venezuela). The chemically synthesized peptide was twelve amino acid long, having an *N*-terminal CG and a *C*-terminal GC dipeptide sequences to allow polymerization in order to increase its immunogenicity (26).

Animal immunization

Isabrown-laying hens (sixteen-weeks-old, 2 kg body weight) were immunized intramuscularly in the breast region with 125 μg of peptide emulsified with complete Freund's adjuvant (CFA) (1:1 v/v). Half of that amount of peptide was dissolved in in-

complete Freund's adjuvant (IFA) and used to boost immunization on days 15 and 30. Eggs were collected every day before and after immunization, individually identified, and stored at 4°C.

Animals were bred and maintained at the animal facility from Instituto de Estudios Avanzados-IDEA, Caracas, Venezuela, and cared for in accordance with the information contained in the guidelines formulated by the European Community for the Use of Experimental Animals (L358-86/609/EEC).

Isolation and purification of IgY

Isolation and purification of IgY from the yolk of preimmune and hyper-immunized eggs were done according to a modified Polson's method (27). Briefly, 3.5% (w/v) polyethileneglycol (PEG 6000; Scharlau Chemie SA, European Union) was added to yolk diluted in phosphate-saline buffer (PBS, 10mM pH 7.2) (1:3 w/v) under stirring. The supernatant containing IgY was collected by centrifugation at 3000xg for 20 min at 4°C, filtered through sterile gauze, followed by an IgY precipitation step with 8.5% PEG 6000. The reconstituted pellet was then precipitated with 12% PEG 6000. Purified IgY were resuspended in PBS buffer and stored at –20°C. The purity of these preparations was evaluated by SDS-PAGE (28).

Preparation of cultured sciatic nerve conditioned medium (CM)

Conditioned medium (CM) was prepared from cultured rats sciatic nerves from adult Sprague-Dawley as indicated in a previous work (29). Nerves were cultivated in groups of 8 in T-25 culture flasks containing 6 mL of serum-free, Dulbecco's modified Eagle's medium (DMEM). At the beginning of day 9, nerves were transferred to brand new flasks. After 24 h, day 9 CM was collected and fresh serum-free DMEM was added to each flask. The same procedure was repeated for days 10 and 11. CM pools from days 9 to 11 were lyophilized and dialyzed against sodium phosphate-buffered saline, pH 7.3 (PBS) plus 0.4 mM EDTA, and maintained at –70°C until used.

Schwann cell cultures

Cells were obtained from adult Sprague-Dawley rat sciatic nerves by the method of Rutkowski *et al.* (30) with several modifications (31). Briefly, nerves were removed, stripped of epineurium, cut into thin slices from adult nerves and cultured in DMEM medium with 10% fetal bovine serum (FBS) for 14 days, changing the medium every 3 days. On day 14, medium was changed by a similar one containing collagenase (0.241 mg/mL) and dispase (1.94 mg/mL). After 24 hours, nerve pieces were mechanically dissociated and centrifuged at 1,200 rpm for 5 min. Aliquots of cell suspensions (0.5 mL) were plated in T-25 culture flasks previously treated with type I rat tail collagen (0.5% in acetic acid 1N). After 24 hours, 52.6 μL/mL Cytidine (cytosine-riboside, Sigma), were added to each flask and left for 24 hours. Cultures were then washed with DMEM-FBS to eliminate Cytidine and then kept in 5 mL of DMEM-FBS. This medium was changed every 3 days until day 9, when cultures were put in 5 mL serum-free DMEM for 3 more days. Finally, Schwann cells were maintained in DMEM culture medium containing 10% FBS and 2 mM Glutamine for three days prior to use. Cells were subjected to lysis buffer (50 mM Tris pH 7.4; 150 mM NaCl; 1% NP-40; 1mM EDTA) containing protease inhibitors (100 μM Antipapain; 5 μg/mL leupeptin; 5 μg/ml pepstatin). Lysates were sonicated and centrifuged at 10.000×g for 10 min at 4°C. Supernatants were collected and proteins were quantified using the Bradford protein assay (Bio-Rad, Hercules, CA).

ELISA measurements

Titer of antibodies to peptide constructs were measured by enzyme linked immunosorbent assays (ELISA) according to Voller *et al.* (32). Briefly, microtiter plates (Greiner Immulon® 2HB) were coated with 5 μg of peptides in PBS buffer (pH 7.4). Plates were incubated overnight at 4°C and then, blocked with 3% BSA in PBS buffer (pH 7.4) for 2 h. Purified IgY diluted to 1:100 was added in duplicates and incubated at 37°C for 90 min. Plates were washed three times with 0.05% PBS-Tween 20 (PBS-T) buffer between each incubation step. Detection was developed with HRP-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO) or HRP-conjugated rabbit anti-chicken IgY (Pierce Biotechnology, Rockford, IL) diluted 1:20.000, followed by 0.01% H_2O_2 with o-phenylenediamine (Pierce Biotechnology, Rockford, IL) in phosphate-citrate buffer (0.1 M pH 5.0). Absorbance was read at 440 nm on a Synergy® HT reader (Bio-Tek, Vermont, USA).

Multiple antigen blot assay (MABA)

Peptides immunogenicity was evaluated by dot blot assays (MABA) (33). Briefly, peptides were immobilized onto a nitrocellulose membrane, using an acrylic template (Miniblotter® 28 S-L Immunetics Inc., Cambridge, MA). Two millimeter width nitrocellulose strips, containing small squares from the total number of peptides for each of the molecules investigated (GGF, SMDF and NDF), were exposed to IgY antibody diluted 1:100. Thereafter, a second antibody (HRP-conjugated rabbit anti-chicken IgY (Pierce Biotechnology, Rockford, IL) was added and developed by a chemiluminescent substrate (Luminol® Amershan, Life Sciences, UK). Positive reactions to the antigen were recorded as small black squares in a film (Hyper film®-ECL, Amershan).

SDS-PAGE and immunoblotting

Recognition of GGF proteins in lysates from Schwann cells, conditioned medium from sciatic nerves (CM) and GGF recombinant protein (extracellular domain from GGF β1a, kindly supplied by Dr. J.C. Martinez), were carried out by Western blot according to Towbin´s method (34). Samples previously heated for 3 min at 100°C in SDS-PAGE sample buffer (1% SDS, 1% β-mercaptoethanol, 0.001% bromophenol blue, 50 mM TRIS-HCl, pH 6.8) were separated by a 15% SDS-PAGE gel under reducing and dissociating conditions (Laemmli, 1970). Proteins were electroblotted onto nitrocellulose membranes in 25mM Trizma base, 192 mM glycine and 20% methanol, pH 8.3, using a Mini Trans-Blot system (Bio-Rad, Hercules, CA) for 1 h at 100 V. After blotting, membranes were blocked overnight at 4°C with blocking solution (5% nonfat milk in PBS buffer pH 7.4, containing 0.1% Tween 20). Blots were then incubated at 4°C for 1 h with anti-peptide antibody diluted in PBS-Tween with 1% non-fat dry milk. After extensive washing, blots were incubated for 1 h with horseradish peroxidase-conjugated rabbit anti-chicken IgY (Pierce Biotechnology, Rockford, IL) diluted 1:2,000 in PBS-Tween 20. Finally, immunoreactivity was identified by chemiluminescence (ECL Western Blotting Analysis System, Amersham, U.K).

Competitive Western blot with synthetic peptides

To demonstrate the specificity of GGF recognition, anti-peptide antibodies (diluted 1:100 in blocking solution) were preincubated for 30 min with various concentrations of synthetic peptide (between 30 μg/mL and 100 μg/mL) (26). Then, strips of nitrocellulose with Schwann cells lysates, conditionated medium from sciatic nerve (CM) and GGF recombinant protein

were incubated with these depleted anti-peptide antibodies as previously described. Strips were washed and incubated with HRP-conjugated rabbit anti-chicken IgY (Pierce Biotechnology, Rockford, IL) diluted 1:2,000 in PBS-Tween 20. Finally, immunoreactivity was identified by chemiluminescence (ECL Western Blotting Analysis System, Amersham, U.K). The disappearance of a band in the Western blot was considered to be a specific competitive assay.

RESULTS

Sequence analysis and identification of epitopes

We analyzed the complete sequences of rat GGF isoforms with bioinformatic programs (see Experimental Procedure) with the purpose of determining potential antigenic determinants (B epitopes) that could induce immune responses in experimental animals. For epitope selection, the following characteristics were taken into account: i) antigenicity, ii) hydrophilicity, iii) accessibility by solvents and iv), sequences not shared by other NRG isoforms, especially SMDF and NDF. The region comprised between amino acids 160 and 171 (KKEVSRVLCKRC) (MW:1448.8 Da.) from rat GGF β1 amino terminal region (Kringle domain) was selected. A BLAST search reported 100% homology to only three proteins, all corresponding to GGF2. This sequence is shared by all rat GGF and human GGF2 isoforms (7), and is not present in rat or human NDF and SMDF neuregulin 1 isoforms.

Purity of IgY anti-GGF

Evaluation of purified IgY antibodies by SDS-PAGE analyses showed, in addition to a few weak bands, two protein bands with relative molecular masses of 68 and 25 kDa that correspond to heavy and light chains from Y immunoglobulin, coinciding with the commercial control (Fig. 1).

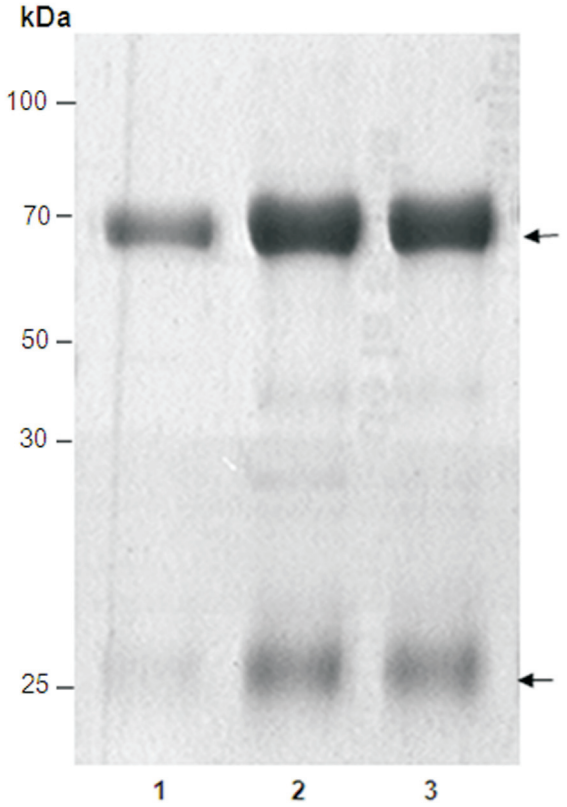

Fig 1. Analysis of IgY antibodies purified from egg yolks by SDS-PAGE. Purified IgY antibodies were run in a 10% SDS-PAGE gel and stained with Coomasie brilliant blue R250. Lane 1: 6 μg of commercial purified IgY (Thermo Scientific). Lane 2: 6 μg of IgY anti-GGF purified from chicken 1. Lane 3: 6 μg of IgY anti-GGF purified from chicken 2. Position of molecular markers are indicated in kDa on the left side.

Immunogenicity of synthetic peptide constructs

To determine the immunogenicity of the peptide derived from GGFβ1, polyclonal antibodies obtained from chickens immunized with the synthetic peptide were tested by ELISA assays. Experimental animals produced specific antibodies against the GGF peptide. Antibodies responses were evaluated weekly in order to determine variations in antibody production. In chicken 1, the peak of immunoreactivity

was reached on day 35. On the other hand, in chicken 2, the response was lower and showed two peaks on days 14 and 42 (Fig. 2). Moreover, chicken 1, immunized with the GGF peptide construct induced a specific response against the homologue peptide by MABA assays (Fig. 3) and did not recognize other peptides which sequences are specific for other neuregulin isoforms (SMDF and NDF).

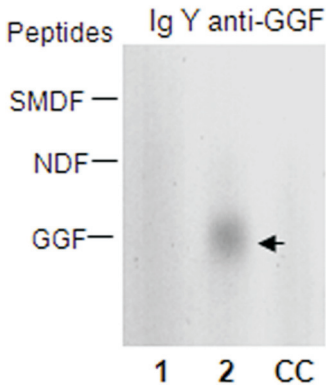

Fig. 3. Immunogenicity of GGF peptide and cross reactivity of IgY anti-GGF evaluated by MABA. 5 μg/mL of GGF peptide were immobilized onto a nitrocellulose membrane as described in Materials and Methods. Anti-GGF IgY antibody was diluted 1:100 and HRP-conjugated anti-IgY antibody at 1/20,000. Strip 1: pre-immune IgY antibody. Strip 2: anti-GGF IgY antibody. Strip CC: conjugate control.

Immunoreactivity of anti-peptide antibodies evaluated by Western blot

To determine whether IgY polyclonal antibodies were able to recognize GGF isoforms in biological samples, Western blot assays were carried out using different sources of GGF proteins like CM, Schwann cells lysates and GGF recombinant protein as a positive control (Figs. 4 and 5). Fig. 4 shows that the anti-GGF IgY antibody was able to recognize the recombinant protein and this recognition was specifically blocked by previous incubation with the peptide.

A Western blot using CM showed that the anti-GGF IgY antibody recognized three bands with relative molecular masses of 65-70, 54, and 34 kDa (Fig 5A, lane 2). These signals were markedly inhibited when the anti-GGF IgY antibody was pre-incubated with the corresponding homologous peptide (Fig. 5A, lane 3) although the strong recognition of the 65-70 kDa protein

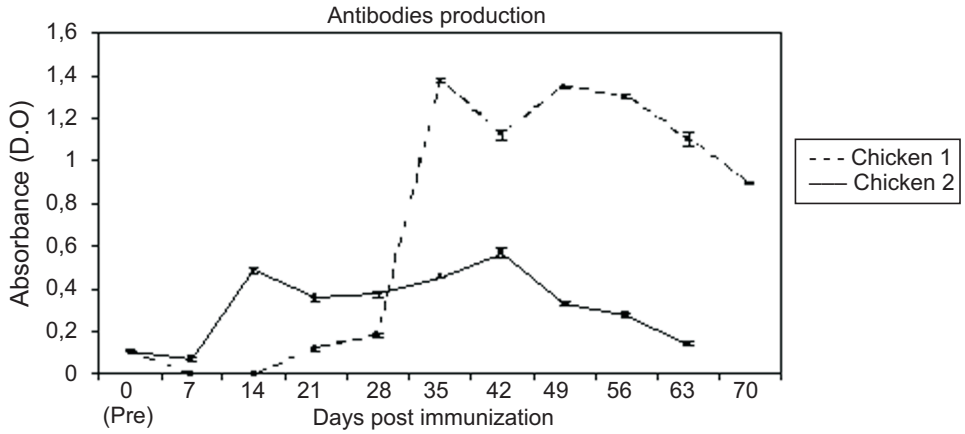

Fig. 2. Antibodies responses from chickens immunized with GGF synthetic peptide determined by ELISA. Animals were immunized with the GGF peptide as described in Materials and Methods. The ELISA plate was coated with 5 μg/well of peptide and purified IgY antibodies were added at 1:100 dilution. Binding was visualized by incubation with HRP-conjugated rabbit anti-chicken IgY diluted 1:20,000. The trends showed a weekly increase in the antibody immunoreactivity against the peptide. Measurements represent the mean of triplicate assays.

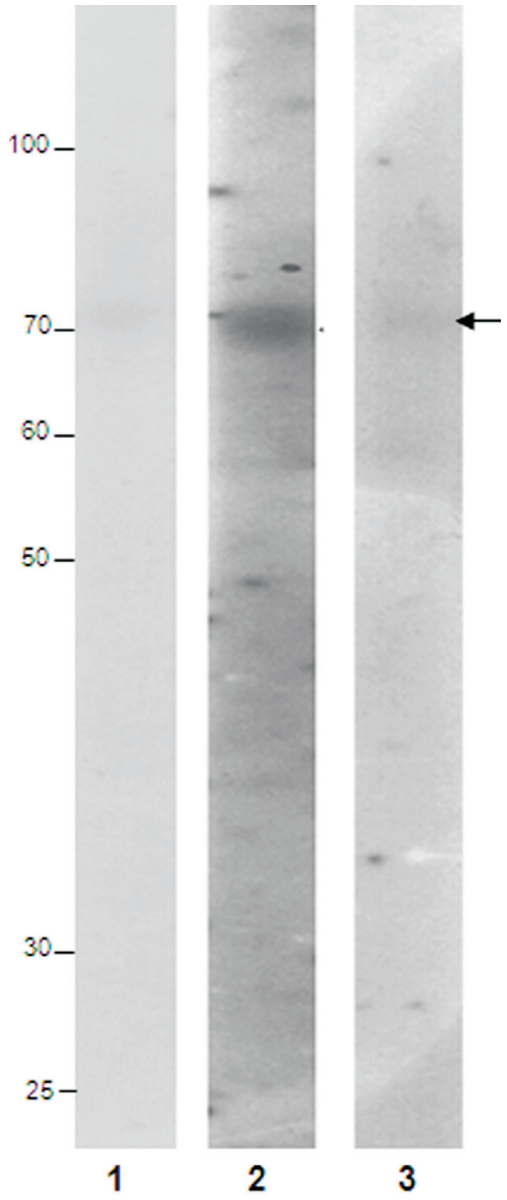

Fig 4. Reactivity of IgY anti-peptide antibodies against GGF recombinant protein. Purified GGF recombinant protein (1 μg) was run on a SDS-PAGE gel, transfered onto a nitrocellulose membrane and probed with preimmune and anti-GGF IgY antibodies (both diluted 1/200). Lane 1: preimmune IgY antibody. Lane 2: anti-GGF IgY antibody. Lane 3: anti-GGF IgY antibody pre-incubated with 70 μg/mL of homologous peptide. Arrow indicates the recombinant GGF band. Relative masses of protein standards are shown in kDa on the left side.

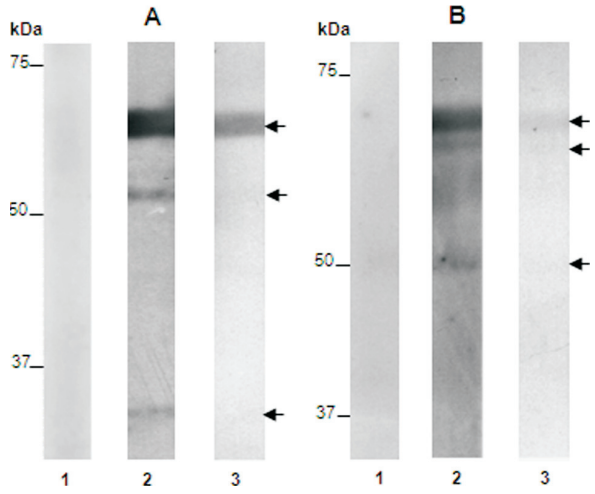

Fig 5. Reactivity of anti-GGF IgY antibodies against sciatic nerve conditioned medium (CM) (A) and Schwann cells lysates (B). Samples (10 μg) were run on a SDS-PAGE gel, transfered onto a nitrocellulose membrane and probed with IgY antibodies. Lane 1: preimmune IgY antibody. Lane 2: anti-GGF IgY antibody. Lane 3: anti-GGF IgY antibody pre-incubated with 70 μg/mL of homologous peptide. Arrows indicate specific polypeptides bands recognized by sera. Position of molecular markers are indicated in kDa on the left side.

did not disappeared completely. On the other hand, antibodies developed in chicken displayed reactivity to 65-70, 63 and 54 kDa proteins present in Schwann cell lysates (Fig. 5B, lane 2). This recognition was markedly inhibited when the antibody was preincubated with the homologous peptide (Fig. 5B, lane 3).

DISCUSSION

In order to understand the role played by neurotrophic factors like NRGs in the course of nerve regeneration processes, it is essential to have tools capable of demonstrating the presence of these factors in different samples. However, the high homology between NRG1 isoforms, especially between NDF and GGF, makes impossible the

use of purified proteins or partial fragments to produce antibodies to differentiate them. This homology explains the low specificity obtained from mammalian commercial IgG antibodies used for detecting NRGs.

Specific antibodies are important tools for detecting proteins in biological samples and can be used in a vast array of methodological applications, such as Western blot, ELISA, immunohistochemistry, and cell sorting assays. The use of chicken antibodies (IgY) in several research fields has increased significantly in recent years, mainly because producing antibodies in eggs is both cost effective and minimizes animal welfare concerns (22). This methodology has several advantages and follows "The principles of Human Experimental Technique" which are summarized in three words: reduce, replace and refine (35). Additionally, chickens are useful animals for developing specific antibodies against mammalian conserved proteins due to the phylogenetic distance between birds and mammals (21).

In this study, we have generated monospecific polyclonal antibodies in chickens against a synthetic peptide from GGFβ neurotrophic factor, suitable for detection of this protein in different assays. The selected GGFβ peptide was 12 amino-acids long, with added N-terminal cysteine-glycine and C-terminal glicine-cysteine sequences to allow for polymerization by disulphide bond formation. In this way, an epitopic polypeptide of high molecular weight could be produced avoiding the use of carriers. In general, the titer of antibodies obtained with monomeric peptides is usually poor, which is why polymerizable peptides are used. The polypeptide sequence showed a high epitope prediction score according to bioinformatic analysis tools, and could induce a strong and specific immune response in chicken models. ELISA and MABA results indicated that antibodies showed correspondence between the predictive study and the immunogenicity obtained in hens. Moreover, anti-GGF IgYs were able to recognize a recombinant purified protein while showing no cross reactivity with peptides corresponding to other neuregulin isoforms (SMDF and NDF).

At first, GGFs were identified as a group of three members of potent Schwann cell mitogens (1, 2). Then, cloning of GGF cDNAs demonstrated that these proteins are translated from alternatively spliced mRNAs transcribed from a single gene (7). In addition, they showed that only one of the cloned neuregulins from pituitary glands corresponds to a GGF isoform (7). Brockes *et al.* (1), also using pituitary glands, demonstrated that under no reducing condition, GGF is a dimer with an apparent molecular weight of 56 kDa. Our IgY antibody detected a specific band of 60 kDa corresponding to GGF II as reported by Goodearl *et al.* (2) (see Fig. 5). The other bands probably are the result of GGF II proteolytic processing or other alternative spliced forms.

There are few authors that reported specific molecular weights for NRGs without mentioning NRG isoform types, probably because the lack of specificity from commercial antibodies. However, Francis *et al.* (8) reported the presence of eleven NRG isoforms in cultured astrocytes from neonatal rats, ranging from 16 to 105 kDa, which include proteins of 73, 65, 51, 44, and 33 kDa. The latter shows that there is a wide variability of NRG isoforms, mainly because the gene alternative splicing and, perhaps, as a result of differential processing and glycosylation of the same proteins. This fact has already been reported for NDF, a NRG isoform, by Burgess *et al.* (36), who explained that there are multiple intermediaries of the same neurotrophic factor (ProNDF) that might be related to its dis-

tribution and function within the cells. In our case, we could be detecting a wide variety of modified or intermediates GGF forms.

Malavé *et al.* (37), reported the presence of several bands when the CM was developed with an anti-panNRG (raised against a peptide from the EGF-like domain common to all neuregulin-1 isoforms; Carroll *et al.* (38), ranging from 80 to 29 kDa. The anti-GGF IgY antibody recognized three proteins in the CM, which were also been detected by the anti-pan NRG previously. Sciatic nerve conditioned medium has been shown to contain secreted neuregulins. Villegas *et al.* (29, 39) reported a neurotrophic activity related to the presence of neuregulins (54 kDa) in an adult rat sciatic nerve conditioned medium (CM). Additionally, we have demonstrated that CM is capable of inducing survival and proliferation of CM-treated PC12 cells (40). More recently, Ma *et al.* (41) showed that heparin-binding forms of NRG1 (type I/II), secreted by the axons are required for early Schwann cell survival and differentiation. However, no reports have been published so far identifying GGF as part of any nerve conditioned medium beyond any doubt.

When the anti-GGF IgY antibody was evaluated against Schwann cell lysates, proteins of 65-70, 63 and 50 kDa were detected. These bands were also detected by Raabe *et al.* (10, 11) in Schwann cell lysates, but he also found other bands of 80, 35 and 32 kDa. This difference was due to the use of antibodies that also recognized the common EGF-like domain. It has been demonstrated that GGF expression by Schwann cells increases during Wallerian degeneration (38, 42), suggesting a potential autocrine signaling mechanism. Stonecypher *et al.* (43) suggested that GGF NRG-1 isoform act as secreted factors and accumulate at high concentrations in structures such as Schwann cell basement membranes. Thompson and coworkers (44), found mRNAs from NDF, SMDF and GGF (NRG-1 isoforms) in sciatic nerves as well as in cultured glial cells (Schwann, astrocytes, olfactory unsheathing cells) and in tissue from the olfactory bulb and brain (cerebellum and cortex). These reports support the idea that Schwann cells are capable of producing several NRG forms, GGF being one of them.

In summary, the present study demonstrates that the antibodies raised were specific and capable of recognizing GGF in conditioned medium (CM) and Schwann cells lysates, indicating the potential usefulness of the IgY antibody for the study of this neurotrophic factor. This work also demonstrates that the development of IgY antibodies using synthetic peptide technology is a useful tool for studying closely related factors that may be involved in the regeneration of the nervous system. The anti-GGF IgY antibody development will apply in our investigation to discern the role played by this neurotrophic factor in neuronal regeneration.

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