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A Virus-Like Particle-Based Anti-Nerve Growth Factor Vaccine Reduces Inflammatory Hyperalgesia: Potential Long-Term Therapy for Chronic Pain

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Chronic pain resulting from inflammatory and neuropathic disorders causes considerable economic and social burden. For a substantial proportion of patients, conventional drug treatments do not provide adequate pain relief. Consequently, novel approaches to pain management, involving alternative targets and new therapeutic modalities compatible with chronic use, are being sought. Nerve growth factor (NGF) is a major mediator of chronic pain. Clinical testing of NGF antagonists is ongoing, and clinical proof of concept has been established with a neutralizing mAb. Active immunization, with the goal of inducing therapeutically effective neutralizing autoreactive Abs, is recognized as a potential treatment option for chronic diseases. We have sought to determine if such a strategy could be applied to chronic pain by targeting NGF with a virus-like particle (VLP)-based vaccine. A vaccine comprising recombinant murine NGF conjugated to VLPs from the bacteriophage Qβ (NGFQβ) was produced. Immunization of mice with NGFQβ induced anti-NGF–specific IgG Abs capable of neutralizing NGF. Titer could be sustained over 1 y by periodic immunization but declined in the absence of boosting. Vaccination with NGFQβ substantially reduced hyperalgesia in collagen-induced arthritis or postinjection of zymosan A, two models of inflammatory pain. Long-term NGFQβ immunization did not change sensory or sympathetic innervation patterns or induce cholinergic deficits in the forebrain, nor did it interfere with blood-brain barrier integrity. Thus, autovaccination targeting NGF using a VLP-based approach may represent a novel modality for the treatment of chronic pain. The Journal of Immunology, 2011, 186: 1769–1780.

Chronic pain is a highly debilitating condition with multiple etiologies and pathophysologies. It is a serious health problem that affects ~20% of European and United States adult populations (1–4). Current therapies provide adequate relief to <30% of those suffering chronic pain (2, 3, 5). The two most widely used classes of analgesic drugs, nonsteroidal anti-inflammatory drugs, and opioids, are limited in their efficacy and tolerability. Furthermore, their long-term administration is accompanied by serious side effects (1). There-

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Abbreviations used in this article: AUC, area under the curve; BBB, blood-brain barrier; CGRP, calcitonin gene-related peptide; ChAT, choline acetyltransferase; Cia, collagen-induced arthritis; CX, cortex; DBB, diagonal bands of Broca; HC, hippocampus; L/Y, lysates; MS, medial septum; NGF, nerve growth factor; NGFQβ, nerve growth factor conjugated to virus-like particles from the bacteriophage Qβ; PBS-T, PBS + 0.5% Tween; RA, rheumatoid arthritis; RT, room temperature; SCG, superior cervical ganglion; smNGF, nerve growth factor purified from murine submaxillary glands; SN, supernatants; TrkA, tropomyosin-receptor kinase; TRPV1, transient receptor potential vanilloid receptor 1; VLP, virus-like particle.

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with the high-affinity TrkA receptor (13, 14). Engagement of the TrkA receptor leads to phosphorylation of the transient receptor potential vanilloid receptor 1 (TRPV1), an ion channel with an important role in hyperalgesia (15). Phosphorylation of TRPV1 increases its basal activity and promotes trafficking and insertion of TRPV1 containing vesicles into the cell membrane. In the longer term, proteins including sodium channels, such as Nav1.8 and acid-sensing ion channel 3, brain-derived neurotrophic factor, calcitonin gene-related peptide (CGRP), and substance P and TRPV1 itself are induced (7). These proteins further facilitate activation and excitability of nociceptors and second-order neur-ons in the CNS. During tissue injury and inflammation, NGF also triggers the release of pain mediators from mast cells, such as bradykinin, 5-hydroxytryptamine, histamine, and PGE2 and NGF itself. This results in a positive-feedback loop that reinforces the sensitization of nociceptors (16).

Injection of NGF results in the development of rapid and persistent pain and hyperalgesia (12, 17, 18). Elevated NGF levels have been detected in a number of chronic pain states in humans, including arthritis, cystitis, prostatitis, and chronic headaches (19–24). Recognition of NGF as a mediator of pain and its involvement in persistent pain in adults has stimulated the development of numerous biologics and pharmaceuticals that antagonize its activity (5, 11). Neutralization of NGF by specific Abs has been shown to decrease thermal and mechanical hyperalgesia in animal models of acute and chronic pain that include: cutaneous injection of CFA (17, 25), arthritis-associated pain (26), and bone cancer (27). A phase II clinical trial with the anti-NGF mAb tanzeumab demonstrated suppression of osteoarthritic pain (28). An acceptable safety profile in >675 treated patients has seen clinical testing expand into numerous chronic pain indications and phase III trials (29).

Chronic pain in humans is generally defined as a condition that lasts for periods of 6 mo or more. Effective treatment would hence require continuous sequestration of NGF. Active vaccination to induce neutralizing anti-NGF Abs is an approach with the potential to achieve this goal. Induction of enduring neutralizing anti-NGF Abs titers could obviate the need for frequent administrations of expensive mAbs or pharmaceuticals. To test the feasibility of this concept, we generated a vaccine comprising mature NGF covalently conjugated to a virus-like particle (VLP) carrier derived from the bacteriophage Qβ. We have previously validated this approach both preclinically and clinically for other Ags (30–35).

In this study, we report preclinical efficacy and preliminary safety of the vaccine NGF conjugated to VLPs from the bacteriophage Qβ (NGFQβ). Induced Abs proved to neutralize NGF and suppress hyperalgesia in different rodent models of acute and chronic inflammatory pain.

Materials and Methods

Mice

DBA1 and C57BL/6 mice were purchased from Harlan Netherlands (Horst, The Netherlands). All mice were maintained under specific pathogen-free conditions and used for experimentation according to protocols approved by the Veterinary Office of the Kanton of Zurich (Zurich, Switzerland).

Cloning, expression, and purification of murine NGF

The nucleotide sequence encoding aa 19–241 of mouse pro-NGFβ and an additional 9 aa extension at the C terminus comprising a hexahistidine-tag and GGC sequence was ligated into the eukaryotic expression vector pCB28 in frame with the signal sequence of the κ L chain of γ Ig-derived of the pSECtag2/Hygro A,B,C vector (Invitrogen, Carlsbad, CA). The resulting plasmid, NGFpCB28, encodes for the signal sequence followed by the pro-NGFβ sequence, a His6 tag, and a linker containing two glycines and a cysteine at the C terminus. HEK 293T cells were transfected with NGFpCB28, and supernatants containing processed mature His6-tagged NGF were harvested and NGF purified by Ni2+ affinity purification.

NGF sandwich ELISA

Mouse anti-mouse NGF mAb (Chemicon International, Temecula, CA) was diluted in carbonate buffer (0.1 M NaHCO3 [pH 9.6]) to a concentration of 1 μg/ml and coated overnight at 4°C on microtiter wells. After blocking, plates were incubated with 2% BSA in PBS + 0.05% Tween (PBS-T), plates were incubated for 2 h at room temperature (RT) with increasing concentrations of recombinant human NGF or NGF purified ex vivo from submaxillary glands (Abd Serotec, Düsseldorf, Germany) in PBS-T + 2% BSA. Then, plates were washed six times with PBS-T and incubated with 1:500 dilution of a sheep anti-NGF polyclonal Ab (AbD Serotec) for 1 h at RT. After another six washing steps with PBS-T, a 1:1000 dilution of HRP-labeled rabbit anti-sheep Ab (Chemicon International) was applied for 1 h at RT. After six final washes, the enzymatic reaction was started by adding 100 μl substrate solution (0.4 mg/ml 1,2 phenylene diamine dihydrochloride [Sigma–Aldrich, St. Louis, MO] and 0.01% H2O2 in 66 mM NaH2PO4 and 35 mM citric acid [pH 5]) to all wells. The color reaction was stopped with 5% H2SO4 and absorbance measured at 450 nm using an ELISA reader (Bio-Rad, Hercules, CA).

NGF receptor binding assay

The rat TrkA receptor (R&D Systems, Minneapolis, MN) was diluted in carbonate buffer (0.1 M NaHCO3 [pH 9.6]) to a concentration of 1 μg/ml and coated overnight at 4°C on microtiter wells. After blocking, plates were incubated with increasing concentrations of recombinant or ex vivo-purified NGF as described above. Receptor-bound NGF was detected with a sheep anti-NGF polyclonal Ab as described above.

To test the capacity of induced Abs to inhibit the interaction of NGF with its receptor TrkA sera of mice immunized with Qβ or NGFQβ were collected and total IgG fractions isolated from sera by protein G (Amer- sham Biosciences, Piscataway, NJ) purification. NGF was biotinylated at its free sulfhydryl groups with Maleimide-PEG11-Biotin (Pierce, Rock- ford, IL) and biotinylated NGF preincubated for 1 h at 37°C with increasing concentrations of purified Abs. The biotinylated NGF was then given to the plate coated with TrkA receptor and incubated for 2 h at RT. After six washes with PBS-T, a 1:1000 dilution of HRP-labeled streptavi-din (Jackson ImmunoResearch Laboratories, West Grove, PA) was added for 1 h at RT. After six final washing steps, the enzymatic reaction was started as described above.

To test the capacity of NGF binding to NGF covalently attached to Qβ particles, 1 μg/ml mouse anti-Qβ mAbs (Cytos Biotechnology, Schlieren, Germany) were coated onto ELISA plates as described above. After binding of different concentrations of VLPs to the immobilized Ab, exposed NGF was detected with 1 μg/ml rat TrkA containing a human IgG Fc fusion. Bound receptor was detected with a 1:1000 dilution of HRP-labeled goat anti-human IgG (Jackson ImmunoResearch Laboratories).

NGF bioactivity assay

NGF bioactivity was assessed by measuring proliferation of the erythro-blastoma cell line TF-1 (American Type Culture Collection, Manassas, VA) in response to increasing concentrations of NGF. Briefly, 104 TF-1 cells were seeded in 100 μl DMEM medium (supplemented with 10% FCS, 10 mM HEPES, 1% penicillin/streptomycin, and 1% glutamax) per well of a 96-well flat-bottom plate. Increasing concentrations of rNGF or ex vivo-purified NGF was added to the cells. After 40 h, cells were labeled with BrdU labeling reagent (Roche Diagnostic Systems, Somerville, NJ), which is incorporated into proliferating cells. Twenty-four hours later, cells were fixed and subsequently incubated with a peroxidase-conjugated anti-BrdU mAb (Roche Diagnostic Systems). After extensive washing, 100 μl tetrabenzyl-benzidine substrate solution was given to each well. The color reaction was stopped with 5% H2SO4 and absorbance measured at 450 nm. To test the in vitro neutralizing activity of Abs raised by immunization with NGFQβ, sera of mice immunized with Qβ or NGFQβ were collected and total IgG purified as described above. The capacity of purified total IgGs to neutralize the bioactivity of NGF was tested by incubating a constant concentration of 1 ng/ml NGF purified from submaxillary glands with increasing concentrations of purified total IgGs for 1 h at RT. The NGF was then added to the cells, and cell proliferation was quantified by BrdU incorporation.

Production of NGFQβ conjugate vaccine

VLPs of the bacteriophage Qβ were reacted for 30 min with a 5-fold molar excess of the heterobifunctional cross-linker succinimidyl-6-(β-mal- eimidopropionamido)hexanoate (Pierce) at RT. Reaction products were dialyzed against two changes of coupling buffer (20 mM MES, 300 mM...
NaCl, 10% glycerol (pH 6) for 2 h at RT. QB-VLPs derivatized in such a way were then used for coupling to the target protein. Precoupling, purified NGF was reduced for 1 h at RT with a 5-M excess of tri(2-carboxyethyl)phosphine hydrochloride (Pierce). Reduced NGF was incubated with derivatized QB for 4 h at 4°C. To estimate the coupling efficiency, conjugated NGF-VLPs were analyzed by anti-His tag Immunoblot using anti-penta His-specific Ab (Qiagen, Valencia, CA).

Immunogenicity tests
Male DBA/1 mice were immunized s.c. with 50 μg QB VLPs coupled to NGF three times in the absence of any further adjuvant. As negative controls, mice were immunized with QB-VLPs alone. After each immunization, blood was taken. Serum was prepared by spinning the blood samples in serum tubes (Microtainer, BD Biosciences, San Jose, CA) at 10,000 × g for 10 min. Determination of NGF-specific Abs in serum samples was done by ELISA using tag- and linker-free NGF purified from submaxillary glands of male mice for coating. Briefly, NGF was diluted to a concentration of 2.5 μg/ml in carbonate buffer (0.1 M NaHCO3, [pH 9.6]) and coated overnight at 4°C on microtiter wells. After blocking with 2% BSA in PBS-T, plates were incubated for 2 h at RT with serum samples diluted in PBS-T + 2% BSA. Then plates were washed six times with PBS-T and bound Ab detected with a 1:1000 dilution of a peroxidase-conjugated goat anti mouse IgG-Fc Ab (Jackson Immunoresearch Laboratories). Titers are expressed as those serum dilutions that lead to half-maximal OD450 (OD50).

Zymosan A-induced inflammatory pain model
The ability of the NGFQβ vaccine to reduce inflammatory hypersensitivity in response to thermal and mechanical stimulation caused by the injection of the yeast extract zymosan A (Sigma-Aldrich) into the plantar side of hind paws of C57BL/6 mice was evaluated in the following way. C57BL/6 mice were immunized three times with 50 μg NGFQβ or QB alone. Seven days after the last immunization inflammatory pain was induced by injection of 20 μl 3 mg/ml solution of zymosan A in 0.9% NaCl into the plantar side of hind paws. Latency and thermal sensitivity in response to thermal and mechanical stimulation, which arises shortly postonset of the zymosan A-induced inflammation, was determined in the following way: at each time point, thermal hypersensitivity of the plantar side of both hind paws was determined in response to thermal and mechanical stimulation. For determination of thermal sensitivity, the latency of paw withdrawal poststimulation with a heat source of defined intensity (infrared beam) was measured. Latency was determined with an electronically controlled instrument (Plantart Test, Ugo Basile, Collegeville, PA). The intensity of the heat source was adjusted so that it yielded a latency of ~12–16 s in naive mice. For each hind paw and time point, six to eight measurements were taken and mean values calculated. To assess mechanical sensitivity, we determined the force that was needed to elicit a paw withdrawal in response to stimulation with dynamic von Frey Filaments (IITC Life Science, Woodland Hills, CA). All behavioral assessments were done in a blind fashion, meaning that the experimenter did not know the treatment status of the mice under investigation.

Collagen-induced arthritis model
The ability of the NGFQβ vaccine to reduce cachexia and pain in autoimmune arthritis was evaluated in a mouse model of rheumatoid arthritis (RA), so-called collagen-induced arthritis (CIA). In this model, RA was induced by intradermal injection of collagen type II (MD Biosciences, St. Paul, MN) in CFA followed by an intradermal injection of collagen type II in IFA 21 d later. The inflammation progresses steadily and culminates in ankylosis. Hypersensitivity in response to thermal stimulation was determined by measuring the force that was needed to elicit a paw withdrawal in response to stimulation with dynamic von Frey Filaments (IITC Life Science, Woodland Hills, CA). C57BL/6 mice were immunized with NGFQβ or QB alone. Brains, spinal cords, superior cervical ganglia (SCG), and hind paws were prepared from mice deeply anesthetized with nembutal (50 mg/kg, i.p.) and intracardially perfused with 4% paraformaldehyde. All tissues were postfixed in 4% paraformaldehyde, and spinal cords, ganglia, and brains were subsequently cryoprotected in 30% sucrose overnight, whereas hind paws were incubated for 4 d in 10% EDTA for decalcification as described (36).

CGRP-positive structures were analyzed as described (37) in 40-μm-thick transverse hind paw sections taken at the level of metatarsal bones and in 14-μm-thick coronal lumbar spinal cord sections incubated overnight with a primary rabbit anti-CGRP Ab (AB15360; Chemicon International) in Tris-buffer at 4°C, followed by a secondary Alexa 488 goat anti-rabbit Ab (A11008; Molecular Probes, Eugene, OR) for 1 h RT incubation; 14-μm-thick ganglia sections and brains were stained to assess the state of the sympathetic cerebrovascular innervation. Ganglia were stained with primary rabbit anti-DH (DiaSorin, Saluggia, Italy) and secondary Alexa 488 goat anti-rabbit Ab for quantification of the total surface area. Cerebral blood vessels were stained with Alexa 488-conjugated isocyan B4, which binds to endothelial cells, and their sympathetic innervation was visualized through counterstaining with primary rabbit anti-tyrosine hydroxylase (AB152; Chemicon International) and secondary Cy3 goat anti-rabbit Abs (78136; Jackson Immunoresearch Laboratories).

Choline acetyltransferase (ChAT)-positive cholinergic neurons were counted in the mediodorsal thalamus and the lateral septum of the mice under investigation. A separate set of NGFQβ- or QB-immunized mice was used to test for a potential leakage of the blood-brain barrier (BBB). Extravasation of peripheral blood fibrinogen was used as marker of BBB impairment as described (38). Sections were prepared as described above for ChAT stainings: extravascular fibrinogen was stained in a rabbit anti-fibrinogen Ab (A0080; DakoCytomation, Carpinteria, CA) and detected as described in the ChAT staining. Mice with known BBB leakage caused by either kainate-induced temporal lobe epilepsy or by injection of 1.6 mol/l mannitol (200 μl) in the tail vein (39) were included as positive controls.

Quantification
CGRP-positive structures were quantified in the spinal dorsal horn and hind paw skin of five immunized and five control mice. Eight sections per mouse were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD), and CGRP-positive structures were either quantified automatically (spinal cord) or counted manually (hind paw skin dermis; area counted was 120 μm below epidermis covering 240 μm per section in length). The total DBH-positive area of the ganglia was quantified using ImageJ software (National Institutes of Health). ChAT immunoreactive basal forebrain neurons of medial septum and diagonal bands of Broca were counted in an area of 2 mm2 per section in four sections at 200 μm intervals as described (40) between Bregma 0.38–1.34 mm in the coronal plane. Cerebral blood vessels were stained with primary rabbit anti-CGRP Ab and detected as described (Fig. 1A). All quantifications were performed on randomized sample images arranged by an observer blinded to the treatment of the mice. Statistical analysis was done with GraphPad Prism 4 (GraphPad, San Diego, CA).

Results
Production of an NGF-VLP conjugate vaccine
Murine pro-NGF, containing a 9 aa extension at the C terminus comprising a hexahistidine-tag and GGC sequence, was expressed in HEK293T cells. The terminal cysteine was introduced into the construct to enable covalent conjugation of NGF to the surface of the VLP via the heterobifunctional chemical cross-linker succinimidyl-6-(β-maleimidopropionamido)hexanoate. Immunoblot analysis of HEK cells transiently transfected with NGFP-CB28 showed expression of the 29-kDa pro-NGF and a higher molecular mass glycosylated form (Fig. 1A). Analysis of cell-culture medium showed the presence of a 14.7-kDa band corresponding to mature NGF. A fainter 20-kDa band previously described by others for cell lines constitutively secreting NGF was also observed (41). Edman analysis of the secreted mature NGF showed the expected N-terminal sequence, which confirmed the mature protein was correctly processed (data not shown). NGF was purified from culture...
supernatants to homogeneity by immobilized metal ion affinity chromatography (Fig. 1A).

The authenticity of the purified rNGF Ag was further investigated by measuring its bioactivity and its ability to bind

conformation-dependent mAbs and the TrkA receptor. For these analyses, NGF purified from murine submaxillary glands (smNGF) was used as a standard comparator. ELISA (Fig. 1B) demonstrated that both rNGF and smNGF were equally well recognized by two neutralizing NGF Abs. Binding to TrkA was also shown to be similar for both recombinant and smNGF (Fig. 1C). rNGF-induced proliferation of TF-1 cells with 10-fold lower bioactivity than smNGF (Fig. 1D). The relatively minor differences in receptor binding and bioactivity may reflect some interference from the C-terminal linker sequence. From these studies, we concluded the rNGF to be of a quality suitable for vaccine production and immunization studies.

NGF was rendered highly repetitive by chemical conjugation to the surface of the VLP QB. Immunoblot analysis of the coupling reaction revealed bands with molecular weights corresponding to NGF with one or more QB coat protein monomers (Fig. 1E). It was estimated by densiometric analysis there were ~60 NGF molecules per VLP. Further analysis of the vaccine demonstrated that NGF coupled to the VLP retained its ability to bind the TrkA receptor (Fig. 1F). These results show that NGF maintained its conformational integrity and accessibility to the receptor binding site even when displayed on the surface of the VLP.

**Induction of NGF-neutralizing Abs by NGFQB**

Groups of mice were immunized s.c. with NGFQB without the addition of adjuvant. By day 10, after only one immunization, NGF-specific IgG Abs were induced in all mice. Titters could be boosted by subsequent immunizations. Twenty days after the third immunization (day 40), the peak titer measured was 1:50,000. Then, immunization was discontinued and from day 40 onwards, the level of NGF-specific Abs declined (Fig. 2A).

To investigate whether the Abs induced by NGFQB were neutralizing, an ELISA-based receptor-inhibition assay was performed. As shown in Fig. 2B, protein G-purified IgG from mice immunized with NGFQB inhibited binding of NGF to TrkA, whereas IgG from mice immunized with QB alone did not. The neutralizing effect of the Abs was also demonstrated in a cell proliferation assay. Abs purified from NGFQB-immunized mice inhibited proliferation of NGF-responsive TF-1 cells, whereas IgG from control mice failed to do so (Fig. 2C). From these results, we concluded that NGFQB is able to overcome immune tolerance to NGF and induce NGF-specific Abs. Moreover, these Abs were able to efficiently neutralize the action of NGF in vitro.

**Suppression of zymosan A-induced inflammatory pain**

To investigate the ability of immunization against NGF to suppress pain in vivo, we tested NGFQB in a rodent model of inflammatory hyperalgesia. Zymosan A injected into the plantar side of the hind paws of rodents causes local inflammation that fully develops within 6–8 h then slowly subsides over a period of 1 wk. Inflammation causes mice to exhibit increased sensitivity to thermal stimulation of the inflamed paw (thermal hyperalgesia) and reduced tolerance to punctuate mechanical stimulation (mechanical sensitization).

Groups of mice were immunized three times with either QB or NGFQB. Measurement of NGF-specific Abs 1 wk after the last immunization showed titers of ~1:30,000. At this time point, inflammation was induced in the left hind paws by injection of zymosan A. Throughout the course of the experiment, the extent of inflammation was determined by measuring paw volume. Similar levels of inflammation were recorded for both control and test groups (data not shown). Sensitivity to mechanical and thermal stimulation was measured at various intervals after zymosan...
A injection with dynamic von Frey aesthesiometer and a Hargreaves plantar test apparatus, respectively.

Within 7 h, during the acute phase of inflammation, the mechanical force tolerated by the inflamed paws rapidly declined from ∼4 g to 1 g (Fig. 3A). During this time, there was only a minor difference in tolerance to stimulation between control and test groups. However, from 24 h onwards, a difference in tolerance to the applied mechanical force was measured. NGFQβ-immunized animals withstood significantly higher applied force than animals immunized with Qβ (area under the curve [AUC] 7–156 h; p < 0.0001). This effect was observed until day 7, but was no longer evident by day 14, at which time the zymosan A-induced inflammation had receded. Indeed, the results of three independent experiments showed statistically significant reductions in mechanical allodynia of up to 60% between days 1 and 7 (Fig. 3B).

As shown in Fig. 3C, reduced hyperalgesia to thermal stimulation was also observed for NGFQβ-immunized mice. The reduction in sensitivity occurred with similar kinetics to those observed for mechanical stimulation (AUC 7–96 h; p < 0.05). Again, assessment of independent experiments showed an average reduction in thermal hyperalgesia of the inflamed foot of up to 60% in the days following onset of inflammation (Fig. 3D).

Suppression of inflammatory pain in CIA

A second inflammatory pain model, arthritis hyperalgesia arising during collagen-induced arthritis, was used to further test the analgesic effects of NGFQβ. Groups of mice were immunized three times with either Qβ or NGFQβ. Once NGF-specific Ab titers had reached levels of ∼25,000, arthritis was induced by sequential injections of collagen in CFA followed by IFA. Animals were monitored for the development of arthritis and clinical scores assigned to each hind and fore paw. Sensitivity to thermal stimulation was measured at various time intervals for a period of 60 d in hind paws.

Fig. 4A shows that average clinical scores and disease progression in hind paws were similar for both test and control groups. Disease incidence was also comparable (data not shown). However, a marked difference in sensitivity to thermal stimulation was noted (Fig. 4B). After onset of arthritis, a progressive decrease in latency from ∼12 s (day 31) to 7 s (day 74) was measured in arthritic hind paws of animals from the control group. In contrast, during the same period, the average latency in arthritic paws of NGFQβ-immunized animals remained essentially unchanged (AUC 31–74 d; p < 0.01). To further examine the dependency of hyperalgesia on disease progression and the potential effect of NGFQβ vaccination, all latency values recorded for individual hind paws throughout the course of the experiment were grouped according to the clinical arthritis score of the corresponding paw at each time point (Fig. 4C). In the CIA model, it is common for one or more limbs not to develop inflammation in a proportion of animals. The paws from both control and NGFQβ immunized animals that failed to develop clinical signs of CIA showed no statistically significant difference in response to thermal stimulation. For paws of control animals, an increase in clinical score was associated with heightened sensitivity to thermal stimulus. Paws with clinical scores ranging from 0.5–1.0, 1.5–2.0, and 2.5–3.0 showed decreases in latency of 20%, 35%, and 45%, respectively. For NGFQβ-immunized animals, an increase in thermal hyperalgesia was only observed for paws with the highest clinical scores (2.5–3.0). The decrease in latency of 21% was similar to that observed in the paws of control animals, with scores ranging from 0.5–1.0.

Suppression of weight loss in CIA

NGF has also been implicated as a mediator of weight loss (26). Progressive unintentional weight loss, also known as cachexia, can be a clinical consequence of a chronic systemic inflammation, such as RA (42). Indeed, inhibition of NGF by anti-NGF mAbs has been reported to decrease rheumatoid cachexia in rats (26). Hence, we investigated the ability of immunization with NGFQβ to influence body weight in the CIA model. Animals were immunized and CIA induced as described above. Fig. 5A shows Qβ- and NGFQβ-immunized mice developed arthritis of a similar magnitude and over a similar time course. However, a statistically significant difference in average body weight between control and NGFQβ-treated mice was observed postonset of disease (Fig. 5B). Control mice failed to gain weight, whereas those treated with NGFQβ continued to gain weight during the course of the disease. This result suggests, as observed for anti-NGF mAb-treated rats, mice immunized with NGFQβ were similarly protected from cachexia.

Kinetics of Ab response and long-term effects of vaccination

An important safety requirement for therapeutic vaccines targeting endogenous molecules is that the Ab response they induce be reversible. To establish this, mice were immunized three times with NGFQβ over a period of 4 wk and the subsequent anti-NGF Ab response measured for ∼11 mo (Fig. 6A). Over the course of the experiment, anti-NGF titers declined in all animals to ∼10% of peak levels measured immediately postimmunization. The decline...
in Ab titer was at first rapid with a $t_{1/2}$ of $\sim 35$ d, then reached a slower phase with a $t_{1/2}$ of $\sim 160$ d. Although not expected for immunological reasons, we examined whether it was possible for endogenous NGF to boost the anti-NGF Ab response induced by vaccination with NGFQ\textsubscript{b}. Anti-NGF Abs were measured in NGFQ\textsubscript{b}-immunized mice postinjection of either buffer, NGFQ\textsubscript{b}, rNGF, or CFA, the latter being a potent inducer of inflammation that is known to induce NGF production in vivo (17, 25). Fig. 6\textit{B} shows anti-NGF Abs could be boosted by administration of NGFQ\textsubscript{b}, but not by administration of rNGF or CFA.

The effect of maintaining high levels of neutralizing Abs over an extended period of time on the normal perception of mechanical pressure and thermal stimulus was investigated. Mice were immunized three times biweekly with Q\textsubscript{b} or NGFQ\textsubscript{b} followed by further immunizations every 4–6 wk. In this fashion, anti-NGF Ab titers in the range of 20,000–40,000 were stably maintained for $\sim 1$ y (Fig. 6\textit{C}). At day 286, the peripheral baseline response of animals to thermal and mechanical stimulation was assessed. Fig. 6\textit{D} shows there was no significant difference in pain-related behavior after mechanical or thermal stimulation for control and test animals. Importantly, during this long-term study, there were no deaths and no adverse clinical signs noted. Moreover, there was no statistically significant difference in body weight and weight gain between control and NGFQ\textsubscript{b}-immunized mice (data not shown).

Influence of NGFQ\textsubscript{b} vaccination on sensory neuron innervation

Previous reports on mice expressing anti-NGF Abs throughout embryonic development and adulthood from transgenes have raised concerns that long-term sequestration of NGF could lead to a loss of CGRP-positive sensory fibers and interfere with the integrity of the BBB (43, 44). The latter could allow penetration of anti-NGF Abs into the CNS, which could potentially result in a loss of cholinergic forebrain neurons. To address this, we compared CGRP-positive sensory nerve-fiber innervations of the spinal cord and skin of long-term NGFQ\textsubscript{b}-immunized mice with control animals. In addition, we analyzed BBB integrity, sympathetic neurons of the SCG and cholinergic neurons in the basal forebrain.

Mice were immunized every 2–4 wk for a period of $\sim 5$ mo with NGFQ\textsubscript{b} or Q\textsubscript{b} VLP alone and anti-NGF titers measured. Four weeks after the last immunization, mice were sacrificed and analyzed. There was no statistically significant difference in the number of CGRP-positive nerve fibers in the dorsal horn outer
There was no significant correlation between the number of CGRP-positive structures and anti-NGF titers ($r = -0.074; p = 0.906$, ANOVA). CGRP-positive nerve fibers in hind paw skin were similarly unaffected by immunization (Fig. 7C, 7D). There were $6.18 \pm 0.61$ CGRP-positive dermis fibers in NGFQβ-immunized mice compared with $5.8 \pm 0.76$ in control mice ($p = 0.71$). Again, there was no significant correlation of anti-NGF titers with the number of CGRP-positive fibers ($r = -0.58; p = 0.301$).

**Influence of NGFQβ vaccination on BBB and basal forebrain cholinergic neurons**

The intact BBB does not allow high molecular mass blood components, such as fibrinogen, or IgG to pass into the brain. Therefore, the integrity of the BBB was assessed by staining the brains of NGFQβ-immunized and control (Qβ-immunized) mice for fibrinogen (Fig. 8A–C) and mouse IgG (not shown). Neither fibrinogen nor mouse IgG could be detected in brains of either NGFQβ- or Qβ-immunized mice ($n = 4$ per group). In line with these results, brains of immunized mice did not show elevated CD68 immunoreactivity confirming the absence of elevated microglial activation, an early marker of BBB dysfunction (45). To validate our experiments, we included diseased mice with a known defect in the BBB and analyzed their brains. We investigated six mice with kainate-induced temporal lobe epilepsy (Fig. 8C) and two mice injected with 1.6 mol/l mannitol (200 μl) into the tail vein to permeabilize the BBB (39) (data not shown). As expected, all these mice showed intense intracerebral fibrinogen labeling throughout the cortex and hippocampus.

We further compared the sizes of superior cervical ganglia, which harbor the somata of the sympathetic neurons innervating the cerebral blood vessels to maintain BBB integrity, and verified that cerebral blood vessels retained their innervation by sympathetic nerve fibers. Both groups of mice showed similar sizes of SCG (Supplemental Fig. 1). The intactness of the sympathetic innervation of the cerebral blood vessels by the sympathetic nerve fibers was confirmed by double labeling of cerebral blood vessel endothelial cells with IB4 and sympathetic nerve endings with tyrosine hydroxylase. Cerebral blood vessels of both groups of mice showed comparable patterns of innervations by tyrosine hydroxylase-positive fibers (Supplemental Fig. 2).

NGF is an important survival factor for basal forebrain cholinergic neurons during development; thus, anti-NGF induced by vaccination with NGFQβ could affect the survival of these cells. We analyzed the numbers of cholinergic neurons in two areas of the basal forebrain, the medial septum, and the diagonal bands of Broca (Fig. 8D, 8E). Cholinergic neurons were identified by the presence of ChAT by neurostereological quantification in NGFQβ-immunized and control mice ($n = 5$ each). We counted on average $155 \pm 15$ ChAT-positive neurons per 2 mm$^2$ and section in control mice versus $144 \pm 14$ in NGFQβ-immunized mice ($p = 0.61$, unpaired $t$ test; $n = 5$ each) (Fig. 8F). There was no correlation of the number of ChAT-positive neurons with anti-NGF titers ($r = -0.12; p = 0.848$).

**Discussion**

One of the most challenging problems in modern medicine is the treatment of patients with chronic pain. Chronic pain is said to be the most costly health problem in the United States. In Europe, it accounts for nearly 500 million working days lost each year and costs the European economy ~35 billion Euro annually (3). Almost one in five people in Europe and the United States suffer from chronic pain with an average duration of 7 y. Twenty percent of chronic pain patients suffer for >20 y. In two thirds of these subjects, pain is inadequately controlled with current drugs (3).
Hence, the development of innovative analgesic drugs with novel mechanisms of action, and compatible with long-term use, represents one of the major goals for the pharmaceutical industry. Toward this end, an anti-NGF vaccine comprising recombinant, mature NGF covalently coupled to the surface of VLPs derived from the bacteriophage Qβ was produced. Such conjugate VLP-
based vaccines incorporate many of the immunological properties of viruses and serve as excellent immunogens that are able to overcome B cell tolerance to self-Ags without the requirement for strong adjuvants (46). Using this approach to vaccine design, we have previously demonstrated that levels of neutralizing Ab responses against self-proteins can be induced that are high enough to show therapeutic effectiveness in mice (31–35). We have also demonstrated that efficacy established in animal models can be translated into efficacy in humans (47, 48). An important safety feature and advantage of VLP-based vaccines is that autoantibodies can be induced without the addition of powerful adjuvants. The use of such adjuvants brings with it the risk of breaking T cell tolerance and potentially inducing autoreactive pathogenic T cells and/or irreversible Ab responses (49).

In the current study, we observed that immunization with NGFQβ, in the absence of added adjuvant, induced high titers of NGF-specific Abs in all treated animals. Ab levels were increased by additional administration of the vaccine and could be maintained at high levels for at least 1 y by periodic boosting. In the absence of boosting, titres waned by 90% within a year. The kinetics of the Ab response are similar to those reported previously (35). Importantly, neither endogenously produced NGF nor rNGF administered s.c. had an influence on the Ab response. The inability of NGF alone to boost the Ab response in the absence of linked T cell help provided by the VLP indicates NGF-specific T cells were not induced, another important safety feature for this type of vaccine.

An in vitro assessment of the quality of the Abs induced by immunization with NGFQβ demonstrated they blocked the binding of NGF to its cognate receptor TrkA in a solid-phase assay and neutralized NGF in a cellular proliferation assay. Importantly, the neutralizing ability of the Abs was also observed in vivo, where it was shown that immunization had an analgesic effect in two models of inflammatory pain.

Immunization of mice with NGFQβ reduced inflammatory hyperalgesia induced by s.c. injection of zymosan A, a model previously used by others to evaluate analgesic drugs (50). Statistically significant reductions in sensitivity to mechanical and thermal stimuli were observed during the 7-d course of the inflammation. The magnitude of the reduction is comparable to that reported with an anti-TrkA receptor mAb tested in a formalin-evoked pain licking responses in mice (51). It was noted that in

FIGURE 7. Innervation of lumbar spinal dorsal horn and hind paw skin by CGRP-positive sensory nerve fibers. CGRP-positive terminals in the lumbar spinal cord dorsal horn of Qβ- (A) and NGFQβ-immunized mice (B). Scale bar, 1 μm. Insets are lower magnification (scale bar, 50 μm). Transverse skin sections obtained from the metatarsal level of the hind paw dermis of Qβ-immunized control mice (C) and of NGFQβ-immunized mice (D). Scale bar, 5 μm. Arrows indicate CGRP-positive fibers. CGRP-positive cell bodies (arrowheads) are probably keratinocytes, cells in sweat glands, or hair follicles. E, Quantitative analyses. Left side shows number of CGRP-positive structures per coronal section in the dorsal horn of one side of the spinal cord averaged from eight sections per mouse. Right side shows average number of CGRP-positive fibers per transverse skin section. Each symbol represents a separate mouse. Horizontal lines indicate mean values.
the first 7 h after zymosan A injection, neutralization of NGF had little influence on pain perception. This result is perhaps not surprising because it has been reported that NGF mRNA only becomes markedly increased 2 h postonset of inflammation (23). In the very early stages of inflammation, sensitization to mechanical and thermal stimulation could predominately be due to release of pain mediators other than NGF.

The ability of NGFQβ to alleviate pain for an extended period of time during chronic inflammation was assessed in mice subjected to CIA. This is a recognized model of chronic inflammatory pain in which progressive inflammation and hyperalgesia develop in affected limbs for at least 6 wk. Three months postimmunization with NGFQβ, the average sensitivity to thermal stimulation in arthritic mice had gone back to the levels recorded before disease induction. In contrast, nonvaccinated animals developed hyperalgesia; sensitivity to thermal stimulation was increased by ∼45%. Only for NGFQβ-immunized animals with the most severe inflammation (scores of 2.5–3) was sensitivity increased, but the hyperalgesia was ∼50% reduced relative to nonvaccinated animals. Similarly, effective suppression of autoimmune arthritic pain in rats has been reported with anti-NGF mAbs (26).

Importantly, in both models, we demonstrated that neutralization of NGF did not affect responses in healthy mice when their paws were exposed to pressure or heat. Rather, only mechanical sensitization or thermal hyperalgesia associated with inflammation was affected. Furthermore, the presence of persistently high neutralizing NGF-specific Abs in healthy animals repeatedly vaccinated with NGFQβ for a period of 10 mo did not alter baseline sensitivity toward mechanical and thermal stimulation. This is in accordance with our results from the CGRP-immunoreactivity study clearly indicating that sensory innervation in hind paw skin and lumbar spinal dorsal horn is preserved post NGF immunization.

Studies (43, 44) on mice expressing monoclonal anti-NGF Abs from transgenes have raised concerns about the safety of long-term anti-NGF treatment. These studies reported disruption of the BBB after loss of sympathetic superior cervical ganglion neurons, which are required to maintain BBB integrity. They also showed a subsequent loss of cholinergic basal forebrain neurons. In the NGFQβ-immunized mice studied in this paper, we did not obtain evidence for a compromised BBB. Furthermore, sympathetic innervation of cerebral blood vessels and the size of the SCGs also did not differ between the two groups of mice. An absence of fibrinogen deposits in the brains of NGFQβ-immunized mice demonstrated the BBB was not adversely affected. The number of cholinergic neurons in the basal forebrain was also unaffected. An obvious difference with our system and that used in the previous

**FIGURE 8.** BBB and cholinergic forebrain neuron integrity. A–C, Immunoperoxidase staining of fibrinogen in cortex (CX) and hippocampus (HC). Scale bar, 500 μm. Neither Qβ-immunized control mice (A) nor NGFQβ-immunized (B) mice show any fibrinogen-immunoreactivity in the cortex or the hippocampus, whereas strong fibrinogen immunoreactivity was found in both structures in mice with a compromised BBB due to temporal lobe epilepsy (C). Cholinergic forebrain neurons labeled with anti-ChAT Abs in the medial septum and the diagonal bands of Broca (DBB) of Qβ-immunized control mice (D) and of NGFQβ-immunized mice (E). Scale bar, 500 μm. F, Quantification of cholinergic neurons in the basal forebrain is indicated in D. Each symbol represents a separate mouse. Horizontal lines indicate mean values. MS, medial septum.
transgenic approach is that anti-NGF mAbs expressed as transgenes are present throughout embryonic development may thus interfere with the developmental functions of NGF.

Our results are in accord with preclinical studies investigating anti-NGF mAbs for the treatment of pain (26, 27, 52). In rodents and primates, no denervation or changes in sensory or sympathetic innervation density or structural toxicities in the nervous system have been reported (28). Moreover, the presence of natural anti-NGF autoantibodies in adult humans is not associated with neurologic deficits (53–58). Nevertheless, the effects of long-term neutralization of NGF must to be carefully considered. Clinical studies conducted with tanezumab will help establish the safety profile for long-term neutralization of NGF and may thus provide a framework to guide the development of an anti-NGF vaccine.

Vaccination represents a new approach to the relatively novel passive immunization therapies currently being developed for the treatment of chronic pain. Whether or not vaccination targeting NGF could be a safe and effective contribution to pain management remains to be determined and would require further preclinical testing and extensive clinical development. Nevertheless, the potential advantages of such an approach may warrant such investment. One advantage of autovaccination could be favorable pharmacodynamics. The longevity of vaccine-induced neutralizing anti-NGF Abs we have observed in mice could result in long-lived therapeutic benefit by alleviating pain for several months. Indeed, immunization once or twice per year may be sufficient to maintain Ab titers at levels high enough for sustained efficacy. Another significant advantage would be cost of goods for which a vaccine would provide clear cost savings over expensive mAb therapies. Thus, active autovaccination may have the potential to offer a treatment option for a debilitating condition that affects the quality of life of millions of people.

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Supplementary Figure 1.

Size of the superior cervical ganglion in the two groups of mice. (A,B) comparison of the total area by DBH-immunoreactive tissue of Qβ-immunized (A) and QβNGF-immunized (B) mice. Scale bar 100 um. (C,D) Quantitative analyses. (C) Average size of the superior cervical ganglia compared between the two groups. Sizes are averaged from the left and right ganglion per mouse, each symbol represents one mouse. Straight lines indicate mean values. Qβ mice had 465500 ± 13910 (mean ± SEM, n=5) while QβNGF had (545500 ± 30770) with non significant P = 0.1530. (D) anti-NGF titer correlation investigation. No significant correlation between the size of the ganglion and the anti-NGF titers (r=0.215, P = 0.7278).
Supplementary Figure 2

Sympathetic innervation of IB4-positive (green color) cerebral vessels by tyrosine hydroxylase-positive (red color) nerve fibers. (A, B) Cortical innervation of mock-immunized Qβ mice (A) and NGF-immunized QβNGF mice (B), scale bar 15 μm. White squares represent single blood vessels with innervation as in (C, D). (C, D) Magnified innervation of Qβ (C) and QβNGF (D) mice, scale bar 10 μm.