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Axon-induced mitogenesis of human Schwann cells involves heregulin and p185^{erbB2}

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ABSTRACT **The ability of sensory axons to stimulate Schwann cell proliferation by contact was established in the 1970s. Although the mitogen responsible for this proliferation has been localized to the axon surface and biochemically characterized, it has yet to be identified. Recently a family of proteins known as heregulins (HRGs) has been isolated, characterized, and shown to interact with a number of class 1 receptor tyrosine kinases, including the *erbB2*, *erbB3*, and *erbB4* gene products. These factors include glial growth factor, a Schwann cell mitogen. We have tested the effects of antibodies against components of this system (HRGβ1 and p185^{erbB2}) in cocultures of rat sensory neurons and human (or rat) Schwann cells to elucidate the role of these proteins in axon-induced Schwann cell proliferation. 2C4, a monoclonal antibody specific for the human p185^{erbB2} receptor tyrosine kinase, bound to the surface of human Schwann cells and reduced human Schwann cell incorporation of [^{3}H]thymidine by >90% compared with untreated controls in this coculture system. This antibody had no effect on rat Schwann cell incorporation of [^{3}H]thymidine under similar conditions. A polyclonal antibody raised against HRGβ1 reduced human and rat Schwann cell incorporation of [^{3}H]thymidine by nearly 80% and up to 49%, respectively, relative to controls. These results imply that a HRG, or a HRG-like molecule, is a component of the axonal mitogen. This mitogen is presented to Schwann cells by axons and induces proliferation through an interaction that involves p185^{erbB2} on Schwann cells.**

The phenomenon of Schwann cell (SC) proliferation driven by axonal contact was demonstrated nearly 20 years ago by *in vitro* observations using both rat (1) and chick (2) tissues. Characterization of this mitogenic activity has shown it to be associated with the surface of the axon and to be sensitive to trypsin, heat, and glutaraldehyde treatment, indicating the involvement of a protein component (3). This protein is salt extractable, indicating that it is peripherally, rather than integrally, membrane bound (4). Mitogenic activity can be detected in the solubilized fraction (130,000 × *g*, 1 hr) of the extract, and can be inhibited by low concentrations (half-maximally at 0.5–0.7 μg/ml) of heparin (4). Further, treatment of axons with heparatinase removes the mitogenic activity from the axon (5). These results indicate that the axonal mitogen is a protein or protein–proteoglycan complex peripherally associated with the axonal membrane (see ref. 6 for review).

The primary sequence for one of the best-characterized SC mitogens, glial growth factor (GGF), has been elucidated and expression plasmids containing the appropriate cDNA have allowed the production of these purified factors (7). Sequence analysis has shown that GGF is a member of a family of proteins which include GGF II (7), acetylcholine receptor-inducing activity (ARIA) (8), Neu differentiation factor (NDF) (9), and heregulin (HRG) α and β (see refs. 10 and 11 for review). Chemical crosslinking experiments (12) have shown HRG to interact with p185^{erbB2}. These proteins have also been shown to stimulate the phosphorylation of a number of class 1 receptor tyrosine kinases, including those encoded by *erbB2*, *erbB3*, and *erbB4* (7, 10, 12–14).

Studies by us (15) and by others (16) have shown other members of the HRG family (NDF, HRGβ1) to be effective mitogens for SCs. The stimulation of human SC proliferation by purified, soluble recombinant HRG can be blocked by a monoclonal antibody raised against the p185^{erbB2} receptor (2C4) (15).

Our recent observations on human SC proliferation have shown that rat sensory neurons are mitogenic for human SCs. In this study we have cocultured human (and rat) adult-derived SCs with purified rat sensory neurons in the presence or absence of antibodies against p185^{erbB2} or HRGβ1 to investigate the importance of these proteins in the axonal induction of human SC proliferation. In light of previous evidence that the axonal mitogen is related to a heparin-sulfate proteoglycan (4, 5), these observations indicate that axon-driven SC proliferation may result from a proteoglycan-bound HRG (or HRG-like protein) on the axon surface interacting with p185^{erbB2} on the SCs.

METHODS

Preparation of Neurons. Cultures of rat sensory neurons were prepared as described (17). In brief, dorsal root ganglia were removed from 15-day Sprague–Dawley rat embryos, dissociated by trypsinization and trituration through a flame-narrowed borosilicate pipette, and rinsed twice in CH5 medium [Eagle's minimal essential medium (GIBCO) supplemented with 10% human placental serum, glucose (4.96 mg/ml), NGF (100 ng/ml; Boehringer Mannheim)]. All culture media contained penicillin (50 units/ml) and streptomycin (50 μg/ml). The dissociated ganglia were resuspended in CH5 culture medium at a density of 1 ganglion per drop, and one drop was placed in the center of a 95-mm^2 collagen-coated Aclar coverslip (Allied Fiber and Plastics, Pottsville, PA). Cultures were maintained in a humidified 5% CO_2 atmosphere. Treatment with the antimitotic 5-fluorodeoxyuridine (10 μM) eliminated the proliferating nonneuronal cells, leaving cultures of purified postmitotic neurons. Cultures were maintained for at least 1 week after cessation of 5-fluorodeoxyuridine treatment to remove any residual traces of this antimitotic.

Preparation of SCs. SCs were derived from adult peripheral nerves of rats and humans by a modification of published methods (18). Human nerves consisted of phrenic nerves and

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Abbreviations: SC, Schwann cell; HRG, heregulin; GGF, glial growth factor; HGF, hepatocyte growth factor; LI, labeling index.

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sections of lumbar plexus harvested from organ donors by the University of Miami Transplant Team. Rat sciatic nerves were removed aseptically from adult Sprague–Dawley rats. With fine forceps under a dissecting microscope, the nerves were dissected free of epineurium, and as much perineurium as possible. The stripped nerves were cut into 1- to 2-mm segments and ≈50 pieces were placed in a 100-mm Corning plastic tissue culture dish and maintained in 10 ml of D10 medium [Dulbecco's modified Eagle's medium (DMEM; GIBCO) with 10% fetal bovine serum and antibiotics]. Culture medium was replaced twice weekly, and nerve pieces were removed to fresh dishes when monolayers of fibroblasts began to grow on the dish surface (every 7–10 days). SCs were obtained by enzymatic dissociation of the nerve pieces. Approximately 50 pieces of nerve were incubated overnight in 1 ml of DMEM containing dispase (1.25 units/ml; Boehringer Mannheim), collagenase (0.05%; Worthington), 15% fetal bovine serum, and antibiotics. The following day the nerve was gently triturated through a flame-narrowed borosilicate pipette, washed two or three times in D10 medium, and seeded onto a 100-mm polylysine-coated plastic tissue culture dish in 10 ml of D10 medium. The next day dead cells and myelin debris were rinsed away from the adherent cells.

Preparation of Neuron–SC Cocultures and Assay for Mitogenesis. SCs were removed from culture dishes by trypsinization (0.05% trypsin and 0.02% EDTA in Hanks' balanced salt solution; GIBCO), rinsed two or three times and suspended in CH5 medium, and plated onto neuronal cultures at a density of 10^4 SCs per neuronal culture. SCs were allowed to settle overnight and the medium was changed the next day (day 1). On day 3 the medium was replaced with CH5 medium containing [*methyl*-^{3}H]thymidine (0.5 μCi/ml; 1 μCi = 37 kBq). On day 4 the cultures were rinsed, fixed, and immunostained to visualize SCs (anti-S100; Dako) and neurites (anti-neurofilament antibody SMI-31:Sternberger–Meyer, Jarrettsville, MD) and processed for autoradiography (see below). SC [^{3}H]thymidine labeling indices (LIs) were calculated by counting 10 random fields per coverslip (250–350 cells) and are expressed as (no. of [^{3}H]thymidine-positive SCs/total no. of SCs) × 100%. Only SCs in contact with SMI-31-positive processes were counted. All experiments used four or five coverslips per condition.

Assessment of Antibody Effects on SC Proliferation. To assess the role of p185^{erbB2} in axon-induced SC mitogenesis, SCs were incubated with 0–100 nM 2C4, a monoclonal antibody raised against (and specific for) human p185^{erbB2}, for 30 min and then added to neuronal cultures. The presence of the antibody was maintained for the duration of the experiment. A control antibody (2H11; see discussion below) was used in the same manner.

To assess the role of HRGs in axon-induced SC mitogenesis, various dilutions (1:50 to 1:2500) of sheep polyclonal antibodies raised against human recombinant HRGβ1 (aa 1–244, expressed in *Escherichia coli*) were incubated with neuronal cultures for 2 hr (in one experiment) or overnight (in two experiments) before addition of SCs. The presence of the antibodies was maintained for the duration of the experiment. Sheep antibodies raised against hepatocyte growth factor/scatter factor (HGF/SF) were used in the same manner as a control (1:50 dilution).

Immunocytochemistry and Autoradiography. To demonstrate immunoreactivity of 2C4 and anti-HRGβ1 with SCs and neurons, respectively, cultures of SCs were doubly immunolabeled with 2C4 and anti-S100 and neuronal cultures were doubly immunolabeled with anti-HRGβ1 and SMI-31. Cultures were fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.2% Triton X-100 for 10 min, blocked in Leibovitz' L-15 (GIBCO) plus 10% normal goat serum for 10 min, and then incubated with primary antibodies (anti-S100, 1:100; SMI-31, 1:2000; 2C4, 100 nM; or anti-HRGβ1, 1:100) for 30 min. After rinsing, SC cultures were incubated for 30 min with fluorescein isothiocyanate-conjugated goat anti-rabbit and biotinylated anti-mouse antibodies, and neuronal cultures were incubated for 30 min with rhodamine isothiocyanate-conjugated anti-mouse and biotinylated anti-sheep antibodies (fluorescein/rhodamine-labeled antibodies, used at 1:100,

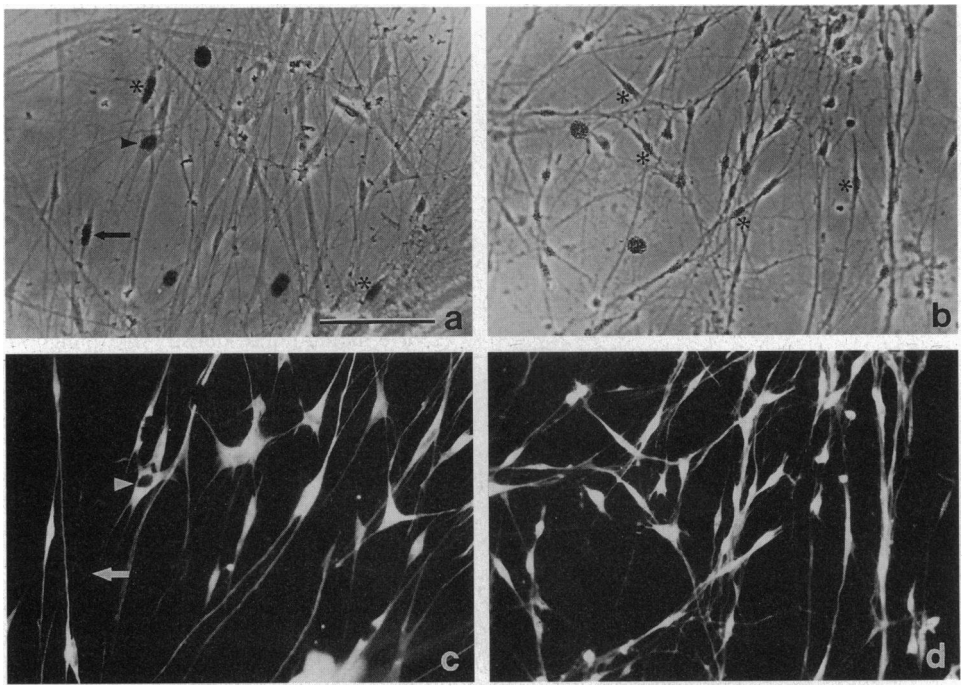

FIG. 1. SCs incorporate [^{3}H]thymidine in response to contact with rat sensory axons in culture. Phase-contrast and corresponding S100 immunofluorescent images of human (*a* and *c*) or rat (*b* and *d*) SCs cocultured with rat sensory (dorsal root ganglion) neurons are shown. Silver grains (*) can be seen over cells that have incorporated [^{3}H]thymidine. Note that morphological appearances of human cells can be deceiving: a morphologically SC-like cell (spindle-shaped with oval nucleus, arrow) is not S100 immunoreactive, whereas a morphologically fibroblast-like (flattened and multipolar with rounded nucleus, arrowhead) is S100 immunoreactive. (Bar = 100 μm.)

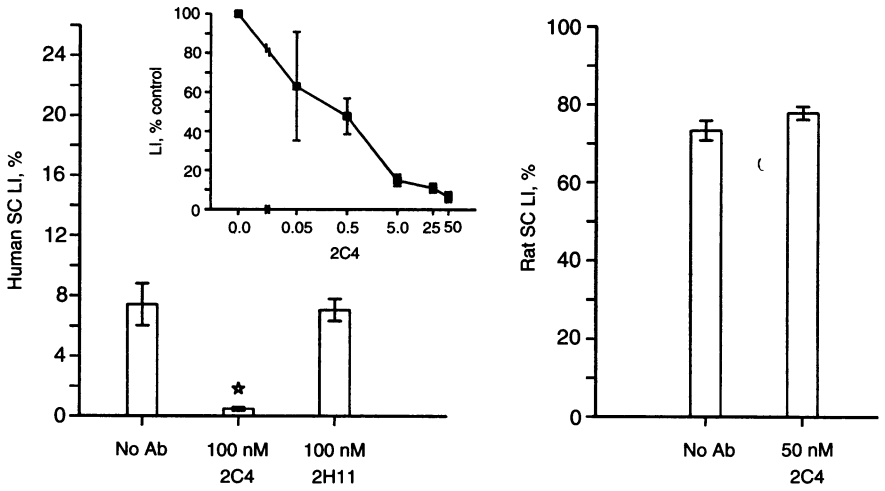

FIG. 2. (*Left*) Human SC proliferation (as assessed by [^{3}H]thymidine LIs) is reduced by the presence of the anti-p185^{erbB2} antibody 2C4. A control antibody (2H11) does not significantly affect SC proliferation. Star, $P < 0.01$; ANOVA; $n = 3$ experiments; four or five cultures per condition in each experiment. No Ab, no antibody. (*Inset*) Antibody 2C4 causes inhibition of axon-induced human SC proliferation in a dose-dependent manner; results are expressed as a percentage of control cultures without added antibody. (*Right*) Rat SCs proliferate in response to contact with rat dorsal root ganglion axons with much higher [^{3}H]thymidine LIs than comparable human SC LIs. 2C4 does not recognize the rat homologue of p185^{erbB2}, p185^{neu} (19), and therefore does not inhibit the proliferation of rat SCs. $n = 5$ cultures per group.

were from Organon Teknika–Cappel; biotinylated antibodies, used at 1:200 were from Vector Laboratories). Cultures were rinsed and incubated for 30 min with horseradish peroxidase-conjugated Vectastain ABC reagents, rinsed, and visualized with a peroxidase substrate kit (SK-4600; Vector Laboratories). Cultures were rinsed in 0.1 M phosphate buffer (pH 7.2) and mounted with Citifluor (Citifluor, London) containing 5 μM Hoechst dye 33342 (Sigma) to visualize nuclei.

For determination of SC LIs, cocultures were fixed, permeabilized, blocked, and incubated with primary antibodies (S100, SMI-31) as above. Cultures were rinsed and incubated with fluorescein-labeled goat anti-rabbit and rhodamine-labeled goat anti-mouse antibodies for 30 min. Cultures were rinsed in phosphate buffer, dipped in distilled water, dipped in 100% ethanol, allowed to dry, and mounted tissue-side-up with DPX (BDH) on glass slides. The slides were dipped in Kodak NTB-2 emulsion, dried in a humid environment, and maintained in the dark at 4°C for 5 days. Slides were reacted in Kodak D19, fixed, rinsed well in water, dried, and coverslipped with Citifluor containing Hoechst dye 33342.

RESULTS

Human SC Proliferation in Response to Axonal Contact. Human SCs cultured in contact with rat sensory neurons consistently incorporated [^{3}H]thymidine (indicative of DNA synthesis and proliferation), resulting in LIs of 6–15% after 24 hr of [^{3}H]thymidine exposure (Figs. 1 and 2; see also Fig. 5). These LIs are similar to previously observed results in this coculture system (T.K.M., unpublished work). Work by Sobue *et al.* (19) indicates that human SCs proliferate in response to contact with neuronal membrane preparations with much higher LIs (26–59%) than reported here. These numbers may have been artificially high because of difficulty in distinguishing between human SCs and human fibroblasts without specific immunological markers (see Fig. 1). LIs of 6–15% are also considerably lower than LIs exhibited by adult-derived rat SCs under the same conditions (18) (Fig. 2); it seems reasonable to ascribe this difference to relative incompatibilities in the cross-species axon–SC interactions. Limited studies employing human neurons, however, indicate that human SC LIs remain in the 6–15% range when human neurons are substituted for rat neurons (T.K.M., unpublished work). Further, rat SCs are equally prolific on rat and human sensory neurons, indicating that the lower LIs seen with human SCs may be a property intrinsic to the human SCs themselves.

Effects of Anti-p185^{erbB2}. Human SCs, but not human fibroblasts, reacted with the 2C4 antibody (Fig. 3). Rat DRG axons did not react with the 2C4 antibody (data not shown). Preincubation of human SCs with 2C4 reduced the axon-driven mitogenic response in a dose-dependent manner (>90% at 100 nM 2C4). Unpublished observations (L. Bald, B. Fendly, and M.X.S.) indicate that 2C4 does not bind the *erbB3* or *erbB4* gene products, suggesting that the involvement of p185^{erb2} is critical in transducing the mitogenic signal (Fig. 2). A control antibody (2H11) that also recognizes p185^{erbB2} but does not

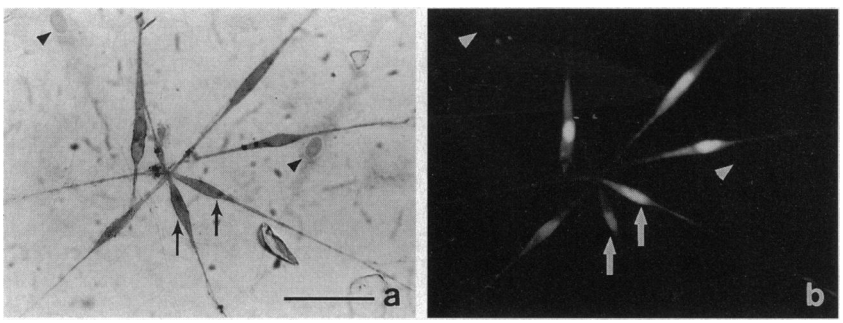

FIG. 3. Human SCs are immunoreactive with the antibody (anti-p185^{erbB2}) 2C4. Phase-contrast and immunoperoxidase staining (*a*) shows 2C4-positive cells (arrows). These cells are confirmed as SCs by immunofluorescent labeling for S100 (*b*). Fibroblasts (arrowheads) do not react with 2C4 or anti-S100. (Bar = 50 μm.)

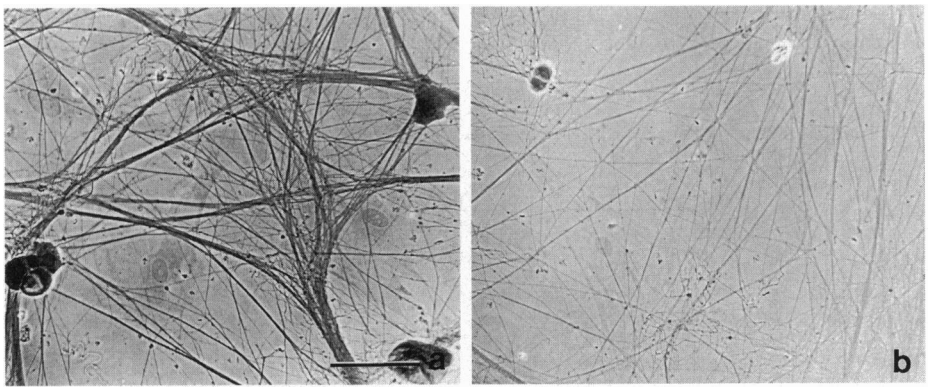

FIG. 4. (*a*) Phase-contrast photomicrograph of immunoperoxidase-processed neuronal culture shows that rat dorsal root ganglion neurons and axons are immunoreactive with anti-HRGβ1 antibody. (*b*) Control culture treated identically, except for the omission of the anti-HRGβ1 antibody, does not develop the reaction product. (Bar = 100 μm.)

block HRG-induced responses (12, 15) failed to block the axon-induced responses (Fig. 2). An experiment conducted with rat SCs and the 2C4 antibody showed no appreciable effect on axon-induced rat SC mitogenesis (Fig. 2). It was not surprising that 2C4 inhibited only human SC proliferation. A panel of antibodies raised against $p185^{erbB2}$, of which 2C4 and 2H11 are part, showed no cross reactivity with Rat-1 cells, which express the rodent homologue to $p185^{erbB2}$, $p185^{neu}$ (20). The $p185^{neu}$ message and receptor have been shown to be present in cultured rat SCs (21, 22).

Effects of Anti-HRGβ1. Rat DRG axons were immunoreactive with the anti-HRGβ1 antibody (Fig. 4); neither human SCs nor human fibroblasts reacted with the anti-HRGβ1 antibody. Preincubation of neuronal cultures with the anti-HRGβ1 antibody reduced human SC LIs by 79%; the effect could be titrated out by lowering the concentration of the antibody in the culture medium (Fig. 5). The anti-HRGβ1 antibody appears to act by masking the mitogenic signal at the level of the axon and would therefore be expected to have effects on the proliferation of both rat and human SCs. Indeed, anti-HRGβ1 caused a 49% reduction in LIs of rat SCs (Fig. 5). A control antibody raised against HGF did not have a significant effect on LIs of either rat or human SCs tested in this coculture system.

DISCUSSION

The data suggest that the axonal mitogen utilizes the same receptor on the SC that mediates HRG-induced mitogenesis. It might be argued that the axon-associated mitogen interacts with the $p185^{erbB2}$ receptor in a manner similar or identical to HRG, because antibody 2C4 recognizes $p185^{erbB2}$ and blocks both HRG-induced (15) and axon-induced proliferation, while antibody 2H11 also recognizes the receptor but does not block the effects of either mitogen. Several recent studies have suggested that heterodimerization of $p185^{erbB2}$ with *erbB3* (13, 23) or possibly *erbB4* (14) gene products is required for the formation of a functionally active HRG receptor. Sliwkowski *et al.* (12) have demonstrated that 2C4 can inhibit crosslinking between radiolabeled HRG and COS-7 cells transfected with *erbB2* and *erbB3*, but not with *erbB3* alone, indicating the specificity of 2C4 for $p185^{erbB2}$. These results do not rule out the possibility of *erbB3* and/or *erbB4* being involved in the mediation of the heregulin or axonal mitogen signaling, but implicate $p185^{erbB2}$ as being a critical factor.

Alternatively spliced forms of HRG/GGF have been shown to be expressed by neurons and the time course for this expression is temporally correlated with SC proliferation during development (7, 8, 24, 25). A number of pro-HRG isoforms are predicted to be synthesized as transmembrane precursors (7, 9–11). Although these pro forms may eventually be secreted or cleaved from the membrane, the soluble products can bind to heparin and may be further sequestered in the extracellular matrix (8–10). If the axonal mitogen produced by the neuron was released from the cell and bound to its external surface by a membrane-associated proteoglycan, this would explain the observed (5) ability of heparatinase treatment to remove the axon's ability to induce SC proliferation.

It is puzzling that the anti-HRG antibodies were more effective in inhibiting proliferation of human SCs than rat SCs. Inasmuch as this polyclonal antiserum was raised against a human form of the protein, it is not surprising that it would be difficult to predict exactly how it reacts with rat HRGs. It should also be noted that mitogens present in the serum-

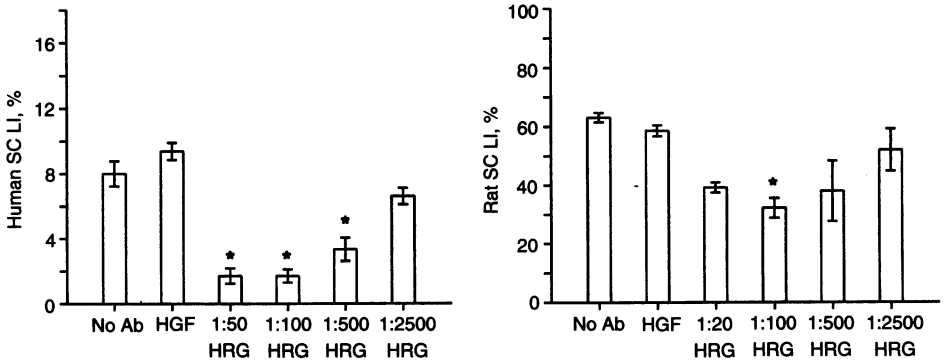

FIG. 5. (*Left*) Human SC proliferation (as assessed by [^{3}H]thymidine LIs) is reduced by the presence of anti-HRGβ1 (HRG) antibody in a dose-dependent manner. Control anti-HGF polyclonal antibodies do not significantly affect SC LIs. Star, $P < 0.01$; ANOVA, $n = 3$ experiments; four cultures per condition in each experiment. No Ab, no antibody. (*Right*) Rat SC LIs are reduced by the presence of anti-HRGβ1 antibody. No effect is seen in the presence of control anti-HGF antibodies. Star, $P < 0.05$; ANOVA, $n = 3$–4 cultures per condition.

containing culture medium may have differential effects on rat and human SCs. Growth factors present in serum, such as platelet-derived growth factor and transforming growth factor β1 can elicit proliferation in rat SCs (reviewed in ref. 26). These mitogens would most likely not be affected by anti-HRGβ1 antibodies.

Our ability to obtain certain of these results only by the use of human SCs underscores the need for the use of human tissues in studies to complement investigations utilizing animal models. With the advancement of the description of the human genome many proteins will first be sequenced in the human before animal homologues can be found. If human tissues are not used in experimental paradigms many of these proteins/factors may not be useful due to incompatibility with tissues derived from animal models.

Overexpression of $p185^{erbB2}$ has been correlated with poor prognosis in mammary, ovarian, lung, gastric, and other tumors (reviewed in refs. 27 and 28). Tumors of the peripheral nervous system are often manifested by the uncontrolled growth of SCs. Experimental induction of schwannomas in the trigeminal nerve by exposure to ethylnitrosourea invariably involved mutations in the *erbB2* (*neu*) gene (29). Neurofibromas have been shown to contain a SC mitogenic activity similar to the axonal mitogen (30), and acoustic neuromas have been shown to contain a SC mitogenic activity similar to GGF, on the basis of HPLC (31). Further, Kimura *et al.* (32) have reported the isolation from a schwannoma-derived cell line of a 31- to 35-kDa, epidermal growth factor-like, heparin-binding molecule with mitogenic activity for SCs, astrocytes, and fibroblasts. These results suggest that the underlying etiology of some SC tumors may be the abnormal expression of normal SC mitogens or the receptors through which these mitogens exert their effects.

Similar concentrations of anti-$p185^{erbB2}$ (4D5) have been shown to block proliferation of certain breast and ovarian tumor cell lines (27). Antibodies (4D5) to the $p185^{erbB2}$ receptor are currently in clinical trials (28) for the treatment of breast cancer because they have been shown to effectively inhibit the proliferation of certain breast tumor cells. Similar approaches may also prove useful in controlling proliferation of SCs in pathological conditions of the peripheral nervous system.

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