Cultured sympathetic neurons synthesize and release the cytokine interleukin 1 β

(substance P/autonomic neuron/Schwann cell)

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Contributed by M. V. L. Bennett, August 3, 1992

ABSTRACT Autonomic neurons help to regulate immune responses, and there are reciprocal interactions between the nervous and immune systems. This study seeks to define some of the molecular mechanisms that may underlie such interactions. Immunoblot analysis indicated that cultured sympathetic neurons synthesize and release the cytokine interleukin 1 β (IL-1 β). In addition, RNA blot analysis of cultured sympathetic neurons demonstrated that the neurons contain mRNA encoding IL-1 β . It was previously shown that explant cultures of sympathetic ganglia and dissociated cocultures of neurons with ganglionic nonneuronal cells synthesize substance P, whereas *in situ* levels of substance P and its mRNA are low. An antagonist at the interleukin 1 receptor markedly depressed this increase in substance P in cultures, suggesting that endogenous IL-1 β mediates the synthetic response, at least in part. Because pure neuronal cultures do not contain substance P and neurons synthesize and release IL-1 β , the actions of the cytokine require the presence of ganglion nonneuronal cells. These observations suggest a role for autonomic neurons in influencing immune responses by synthesizing and secreting at least two known immunoregulators, the cytokine IL-1 β and the neuropeptide substance P.

The cytokine interleukin 1 β (IL-1 β) mediates some of the immune responses to antigens, malignant cells, inflammatory stimuli, and tissue injury (1). It is synthesized by a number of cells including keratinocytes, synovial fibroblasts, lymphocytes, activated macrophages and monocytes, astrocytes, and microglia (2). The cytokine acts in early immune responses as a chemotactant for lymphocytes and as a signal for eosinophil and basophil degranulation (for review, see refs. 1–3). IL-1 β also stimulates fibroblast proliferation and the synthesis of growth factors, such as nerve growth factor and platelet-derived growth factor, suggesting a function in cellular and tissue-repair processes (2–4). IL-1 β mRNA and receptor binding have been found in rodent hippocampus, cerebellum, and striate cortex, leading some to postulate a neurotrophic or neurotransmitter role for the cytokine (5, 6).

The autonomic nervous system contributes to homeostasis by regulating the function of most major organ systems. Despite increasing evidence for interactions between the nervous and immune systems, the mechanisms that mediate the relationship between autonomic and immune responses are unclear. Both the peptide transmitter, substance P, and IL-1 β have been identified with various aspects of these interactions. For example, substance P and IL-1 β are associated *in vivo* with the pathophysiology of rheumatoid arthritis (3). They are also associated with the *in vitro* stimulation of prostaglandin E₂ production by cultured synoviocytes, via different receptor systems (3).

Substance P and its mRNA are present at low levels in normal sympathetic ganglia *in situ* (7–11). Explantation of the rat superior cervical ganglion (SCG) significantly elevates levels of substance P, and treatment of the explants with IL-1 β further increases peptide expression (7–9). Pure neuronal cultures do not synthesize substance P, whereas neurons cocultured with ganglion nonneuronal cells do so, indicating involvement of nonneuronal cells in the response (7, 9, 10). Treatment of cocultures with IL-1 β increases substance P expression but does not cause substance P expression in pure neuronal cultures. Earlier studies had shown that denervation of or injury to the SCG also induced substance P synthesis (11). Therefore, we asked whether endogenous IL-1 β is involved in regulation of substance P in the SCG and what cell types within the ganglion might produce the cytokine. In this study we report that sympathetic neurons synthesize and release IL-1 β and that an antagonist at the interleukin 1 receptor reduces substance P synthesis by SCG neurons in culture. Taken together, these findings suggest that SCG neurons, and other sympathetic neurons as well, may participate in autonomic responses to injury.

METHODS

Tissue Culture. Neonatal (postnatal day 1–3) rat SCG were grown as intact ganglia in explant culture as described (7) in Ham's nutrient F-12 supplemented with 10% fetal bovine serum (GIBCO), murine nerve growth factor (100 ng/ml), penicillin (50 units/ml), and streptomycin sulfate (10 μ g/ml). Some cultures were treated with interleukin 1 receptor antagonist (IL-1ra; 500 ng/ml; Synergen, Boulder, CO; refs. 12 and 13), human IL-1 β (1 ng/ml; Genentech), or both from the time of dissection until harvested for peptide determination at 48 hr in culture.

SCG neurons were grown in dissociated culture as described elsewhere (7, 10) in media containing Ham's nutrient F-12 supplemented with 10% fetal bovine serum, nerve growth factor at 100 ng/ml, penicillin, and streptomycin sulfate. Pure neuronal cultures were obtained by treatment with cytosine arabinoside (1 μ M) on day 2 of culture, which eliminated ganglion nonneuronal cells and resulted in cultures that were >99% pure as determined by morphological and immunostaining criteria (10). Cultures not treated with cytosine arabinoside (cocultures) contained both neurons and ganglion nonneuronal cells. Neuron numbers in either pure or coculture conditions ranged from 10,000 to 12,000 neurons per 35-mm culture dish. Some cultures were

Abbreviations: IL-1 β , interleukin 1 β ; SCG, superior cervical ganglion or ganglia; IL-1ra, interleukin 1 receptor antagonist.

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treated with IL-1ra (500 ng/ml), murine IL-1 β (1 ng/ml; R & D Systems, Minneapolis), or both on days 2, 5, and 8 and harvested on day 10 as described by Freidin and Kessler (7).

For immunoblot experiments, the cultures were switched from serum-containing to serum-free N2 (14) medium 36 hr after plating. On day 8 of culture, the culture medium was removed and dried under vacuum, and the cells were harvested for substance P determination as described (7).

Immunoblot Analysis. The dried media samples were dissolved in 1 $\times$ Laemmli buffer containing 2% SDS, 62.5 mM Tris-HCl (pH 6.8), 5% 2-mercaptoethanol, 10% (vol/vol) glycerol, and 0.1% bromophenol blue, and aliquots were taken for protein determination (Bio-Rad protein assay). The protein samples (15 μ g) and murine IL-1 β (1 ng; R & D Systems) were separated on a discontinuous Tris/glycine gel and subsequently transferred to nitrocellulose (Amersham) by semi-dry electrophoresis. The blots were then processed for antibody detection with an antibody directed against murine IL-1 β (1 μ g/ml; R & D Systems) and visualized using the enhanced chemoluminescence (ECL) immunoblot detection kit (Amersham), as per manufacturer's instructions.

RNA Blot Analysis. Total RNA was harvested by the method of Chomczynski and Sacchi (15), with minor modifications. The RNA samples were quantitated by absorbance at 260 nm. Total RNA from pure neuronal cultures (20 μ g) and cocultures (10 μ g) was separated on a 1% agarose/0.66 M formaldehyde gel, transferred to GeneScreenPlus (NEN) by capillary blotting, and baked under vacuum. The blot was prehybridized at 65°C in a solution containing 50% (vol/vol) formamide, 1% SDS, 1 M NaCl, 2 $\times$ Denhardt's reagent, and denatured salmon sperm DNA at 100 μ g/ml.

The *Bam*HI-*Hind*III fragment of a full-length cDNA to murine IL-1 β (Genentech) was subcloned into pBluescribe KS(-) (Stratagene). A ³²P-labeled RNA probe was synthesized with T7 polymerase by using standard techniques and hybridized (5 $\times$ 10⁵ cpm/ml) to the blot at 65°C for 24 hr. The blot was washed sequentially in 2 $\times$ SSC/1% SDS, 0.2 $\times$ SSC/1% SDS, and 0.1 $\times$ SSC/1% SDS at 65°C, exposed to Kodak XAR film, and developed after 48 hr.

RESULTS

In vivo, the SCG contains a number of cell types including neurons, Schwann cells, fibroblasts, satellite cells, and macrophages (10). Sympathetic ganglia grown as dissociated cultures contain the same cell types. By contrast, as described in *Methods*, cultures treated with cytosine arabinoside are >99% pure neuronal, as defined by neurofilament staining and morphological criteria (see ref. 10). Further, macrophages were not detected after immunostaining with the mouse monoclonal antibody AD1, which recognizes a cytoplasmic antigen on rat macrophages (data not shown).

To test the hypothesis that endogenous IL-1 β regulates substance P expression in sympathetic ganglia, we first asked whether the SCG synthesizes the cytokine. Dissociated SCG were grown as pure neuronal cultures and as cocultures of neurons and nonneuronal cells, and the culture media were examined for IL-1 β immunoreactivity by immunoblot analysis. Because serum may contain IL-1 β (although none was detected by our immunoanalysis), after the first 36 hr in culture the cells were maintained for 7 days in a defined serum-free medium (N2, ref. 14). Immunoblot analysis with an antibody directed against murine IL-1 β (1 μ g/ml; R & D Systems) demonstrated that medium from both neurons cultured alone and neurons cocultured with ganglion nonneuronal cells contained an immunoreactive species that comigrated with murine IL-1 β at $\approx$ 17 kDa (Fig. 1). Additional immunoreactive bands at $\approx$ 30 and 14 kDa are presumably other forms of the cytokine (see *Discussion*). Interestingly

FIG. 1. Pure neuronal cultures and cocultures with nonneuronal cells release IL-1 β . Dissociated pure neuronal cultures and cocultures (Cocul) were grown as described. After 36 hr in culture cells were switched to defined N2 media. On day 8, the culture medium was collected and processed for immunoblot analysis, by using the ECL detection system for visualization (Amersham). Media collected from both pure neuronal cultures (left two lanes) and cocultures (third and fourth lanes) contained an immunoreactive band that comigrated with recombinant murine IL-1 β (mIL-1 β). Recombinant murine IL-1 β migrated at $\approx$ 17 kDa (fifth lane). The higher- and lower-molecular-mass bands in the experimental lanes corresponded to $\approx$ 30 kDa and 14 kDa, respectively, and may represent other forms of the cytokine.

the amount of cytokine in the medium was about the same for both types of culture.

To further characterize expression of IL-1 β by cultured sympathetic neurons, total RNA was collected both from pure neuronal cultures and from neurons cocultured with ganglion nonneuronal cells and subjected to RNA blot analysis (Fig. 2). IL-1 β mRNA was detected from both types of culture. However, levels of IL-1 β mRNA were markedly higher in cocultures, suggesting that the nonneuronal cells up-regulate neuronal IL-1 β mRNA levels or synthesize it themselves. Nevertheless, based on evidence from both protein and RNA blots, we conclude that IL-1 β is synthesized and released from cultured SCG neurons.

To test the hypothesis that endogenous IL-1 β regulates substance P expression, intact SCG were cultured in the presence of an antagonist at the IL-1 β receptor (IL-1ra, 500 ng/ml; see ref. 11) and examined for substance P as described (7). Treatment with IL-1ra suppressed (but did not completely block) the increase in substance P that normally occurs in these cultures (Fig. 3). As reported (7), treatment with IL-1 β (1 ng/ml) increased substance P expression in explants, to 2.5 times control levels. IL-1ra almost completely blocked the effect of exogenous IL-1 β , indicating the

FIG. 2. Pure neuronal cultures and cocultures (Cocul) with nonneuronal cells contain IL-1 β mRNA. On day 10, cultures were harvested for total RNA, which was fractionated and probed for IL-1 β mRNA as described. IL-1 β mRNA was detected in lanes for both pure neuronal cultures (left two lanes) and cocultures (right lane), with markedly higher signals in the cocultures.

FIG. 3. IL-1ra, an antagonist at the IL-1 receptor, decreases substance P expression in cultured SCG explants. Intact neonatal rat SCG were placed in culture and treated as described. The explants were harvested after 48 hr, and substance P content was determined for each ganglion. IL-1ra suppressed substance P to 60.75 ± 36.4 pg of substance P per ganglion, as compared with values in untreated explants of 153 ± 43.3 pg of substance P per ganglion. Exogenous IL-1 β increased substance P levels to 401 ± 53.2 pg per ganglion; this effect was largely blocked by IL-1ra (83.2 ± 28.4 ; does not differ from IL-1ra alone). Values are expressed as mean pg of substance P per ganglion $\pm$ SD (for control, IL-1ra, IL-1 β , and IL-1 β + IL-1ra treatments $n = 4$, $n = 4$, $n = 5$, and $n = 5$, respectively). *, Differs from control at $P < 0.05$.

efficacy of the antagonist action (Fig. 3). These observations suggest that endogenous IL-1 β governs, at least in part, the increase in substance P seen with explantation of the SCG.

We sought to determine whether the IL-1 β antagonist had the same action on substance P synthesis in dissociated as in explant culture. The synthesis of substance P induced in neurons cocultured with nonneuronal cells was suppressed by $\approx 50\%$ by IL-1ra (500 ng/ml), indicating that endogenous IL-1 β , at least partly, regulates substance P synthesis under these conditions (Fig. 4, left two bars, $P < 0.05$). As reported (7), dissociated cultures of SCG neurons synthesized substance P only in the presence of ganglion nonneuronal cells (data not shown). Substance P is localized exclusively in the neurons in these cultures (10). In addition, IL-1 β did not increase neuronal substance P levels in pure neuronal cultures (data not shown, ref. 7). These data support the hypothesis that endogenous IL-1 β acts through or in conjunction with the nonneuronal cells in the ganglion to stimulate neuronal substance P expression by an, as yet, unknown mechanism.

DISCUSSION

These observations demonstrate that cultured sympathetic neurons synthesize and release IL-1 β and that endogenous IL-1 β participates in the regulation of neuronal substance P expression. It should be noted, however, that the expression of IL-1 β mRNA is much greater in cocultures than in pure neuronal cultures, yet the levels of IL-1 β present in the culture media of either pure neuronal or cocultures do not appear to differ. This apparent dissociation between levels of mRNA and protein synthesis has been found in other systems with respect to the IL-1 family, suggesting these results may reflect a common translational regulatory mechanism for

FIG. 4. IL-1ra decreases substance P expression in SCG neurons cocultured with ganglion nonneuronal cells. Dissociated neurons were grown as cocultures as described. Experimental cultures were treated with IL-1ra (500 ng/ml; Synergen) and/or human IL-1 β (1 ng/ml; Genentech), and levels of substance P were measured. IL-1ra suppressed substance P expression to half of levels in untreated control cultures. IL-1ra largely blocked the effects of IL-1 β treatment [IL-1ra + IL-1 β does not differ from control but is larger than IL-1ra alone ($P < 0.05$), indicating that blockade was not complete]. No detectable substance P was found in pure neuronal cultures under any of the described treatment conditions (data not shown). Values are expressed as mean fg of substance P per neuron $\pm$ SEM ($n = 15$ cultures for each group). *, Differs from control values by $P < 0.05$; **, differs from control values by $P < 0.001$.

production of the cytokine (16). *In situ* hybridization should demonstrate whether or not IL-1 β mRNA is found exclusively in neurons. IL-1 β can exist in a number of forms, including membrane-associated proteins of 31 kDa and 22 kDa, as well as soluble molecules of 17.5 kDa and 14.3 kDa (1–3). Thus, the discrepancy between release and mRNA content might also indicate that a membrane-associated form is the predominant form in cocultures. However, crude cell homogenates probed for IL-1 β immunoreactivity failed to detect differences between pure neuronal and coculture conditions (data not shown), arguing against this hypothesis. Nevertheless, the release of more than one form of IL-1 β probably accounts for the two additional bands recognized by the antibody (Fig. 1).

These observations suggest the intriguing possibility that in the initial immune response to injury to the SCG, IL-1 β participates in regulation of substance P expression and perhaps other factors as well. IL-1 β alone does not induce substance P expression in neurons (7), although its release by neurons may stimulate the ganglion nonneuronal cells to express any number of molecules, membrane-associated and/or diffusible, which then induce substance P expression in the neurons. Alternatively, factor(s) released by the non-neuronal cells may permit sympathetic neurons to respond to IL-1 β by stimulating substance P expression. However, experiments using conditioned medium from cocultures treated with the cytokine failed to stimulate substance P in pure neuronal cultures or to make the neurons responsive to IL-1 β . Several factors have been shown to stimulate substance P in cultured SCG neurons, including ciliary neurotrophic factor, leukemia inhibitory factor, and membrane-associated neurotransmitter-stimulating factor (9, 17), but it is not known whether any of these agents are regulated by IL-1 β .

Substance P, in turn, may regulate IL-1 β expression in the SCG. Substance P has been shown to stimulate IL-1 β release by the P388D1 murine macrophage cell line (3) and to act synergistically with the cytokine to stimulate production of prostaglandin E2 by BALB 3T3 cells (18). Thus, neuronal substance P may act as an autocrine agent to stimulate IL-1 β synthesis in a positive-feedback loop and/or facilitate its actions, ultimately modulating injury and immune responses by sympathetic neurons.

In summary, this paper presents evidence for the mediation of substance P expression by endogenous IL-1 β in SCG cultures. This action requires and may proceed via the ganglion nonneuronal cells in these cultures. In addition, the specific expression of the cytokine by cultured sympathetic neurons was demonstrated by RNA and immunoblot analyses. IL-1 β and substance P may serve as a link between the nervous and immune systems, particularly in injury responses and repair processes regulated by the autonomic nervous system.

We thank Mrs. Anne Barnecott for her invaluable help in preparing this manuscript. This work was supported by National Institutes of Health Grants NS20013 and NS20778.

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