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Induction of Neuronal Differentiation in PC12 Cells by B-Cell Stimulatory Factor 2/Interleukin 6

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B-cell stimulatory factor 2 (BSF-2) is a lymphokine which induces the final maturation of B cells. BSF-2 acts on a variety of cells other than B cells, and moreover, expression of BSF-2 mRNA is detected in interleukin-1 β-stimulated glioblastoma and astrocytoma cell lines. Here, we studied the function of BSF-2 on pheochromocytoma PC12 cells, a model system for induction of neuronal differentiation. PC12 cells possess specific receptors for BSF-2. The BSF-2-stimulated PC12 cells expressed the c-*fos* proto-oncogene transiently, and they began to change morphologically to neurite-extending cells after several days. The number of voltage-dependent Na^+ channels was also increased.

B-cell stimulatory factor 2 (BSF-2) is a well-identified differentiation factor which acts on B cells and induces final maturation to immunoglobulin-secreting cells (15). Recently, the cDNA for human BSF-2 was molecularly cloned (13), and many of its variety of functions are being explored.

The previously reported 26-kilodalton (kDa) protein, $beta_2$ interferon, hybridoma plasmacytoma growth factor, and hepatocyte-stimulating factor have turned out to be identical with BSF-2 (1, 10, 18, 23, 26). It has been proposed to designate this molecule interleukin 6 (IL-6) (20, 25). Furthermore, BSF-2 mRNA was shown to be expressed not only in T cells, but also in glioblastoma and astrocytoma cell lines when stimulated with IL-1β, suggesting the important role of BSF-2 in the nervous system (25). The rat pheochromocytoma PC12 cell line (8) is a valuable model system for neuronal differentiation. In the presence of nerve growth factor (NGF), PC12 cells change their shape from round cells to neurite-extending cells and acquire properties of sympathetic neurons. Here we show that BSF-2 can induce neuronal differentiation of PC12 cells through specific receptors.

PC12 cells proliferate with a round-shape phenotype without neurites in standard serum-containing culture medium (Fig. 1a). In the presence of NGF, almost all the cells develop neurites within 12 h, and after 6 to 7 days a network of long and profusely branched neurons were observed. Under serum-free conditions, PC12 cells cannot proliferate and most die within a few days. However, in the presence of NGF, the cells remained viable and showed morphological change just as in serum-containing conditions (Fig. 1b).

We first studied the morphological change of PC12 cells induced by BSF-2. The cDNA for human BSF-2 was expressed in *Escherichia coli*, and the recombinant BSF-2 protein was purified to more than 95% homogeneity as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described elsewhere (11). The recombinant BSF-2 possessed immunoglobulin-inducing activity with an estimated specific activity of 5×10^6 U/mg as described elsewhere (11). In the serum-free medium supplemented with recombinant BSF-2, the PC12 cells remained alive and, following a lag of 2 to 3 days, started to extend neurites. In the first 2 to 3 days after the addition of BSF-2, the number of cells increased (data not shown) as in the case of NGF (6). A network of long neurites, as in the NGF-treated cells, was observed 6 days after the addition of BSF-2 (Fig. 1c). Withdrawal of BSF-2 from the culture medium resulted in loss of cell viability even after the cells had morphologically differentiated. Natural BSF-2, partially purified from the culture supernatants of bladder cell carcinoma T24 (12, 13), also induced neurite outgrowth in the cells (Fig. 1d). Affinity-purified polyclonal antibody raised against recombinant BSF-2 but not normal rabbit immunoglobulin G completely inhibited the effect of both recombinant and natural BSF-2, while the anti-BSF-2 antibody had no effect on NGF-induced change (data not shown). These results indicate that the observed morphological change was actually due to the added BSF-2. In serum-containing conditions, neurite formation caused by BSF-2 was not as clear as in serum-deprived conditions.

The presence of BSF-2-specific receptors on PC12 cells was investigated by using ^{125}I-labeled ligand, and binding was performed as described previously (22). The Scatchard analysis (Fig. 2a) demonstrated receptors for BSF-2 with a dissociation constant of 1.8×10^{-9} M, and approximately 1,200 receptors per cell were expressed. The binding of BSF-2 to its receptors was specific, and no significant competition was observed with an excess amount of NGF (Fig. 2b). The numbers of receptors for NGF and epidermal growth factor of PC12 cells were reported as 6×10^4 (17) and 8×10^4 (14) per cell, respectively.

The c-*fos* proto-oncogene is rapidly and transiently induced in PC12 cells following exposure to NGF (3, 5, 16, 19). As shown in Fig. 3, BSF-2 also induced c-*fos* mRNA within 30 min, although the level of induction was less than that observed with NGF stimulation. The peak induction was observed 45 min after exposure to BSF-2, and very little induction was observed after 60 min.

We studied the change of the number of the voltage-dependent Na^+ channels to see whether BSF-2 could cause functional differentiation as well as morphological conversion. Saxitoxin is a neurotoxin which binds specifically to the Na^+ channels at the same site as another neurotoxin, tetrodotoxin. The difference in binding of [^{3}H]saxitoxin in the presence and absence of excess amounts of tetrodotoxin represents specific binding to the Na^+ channel. As shown in

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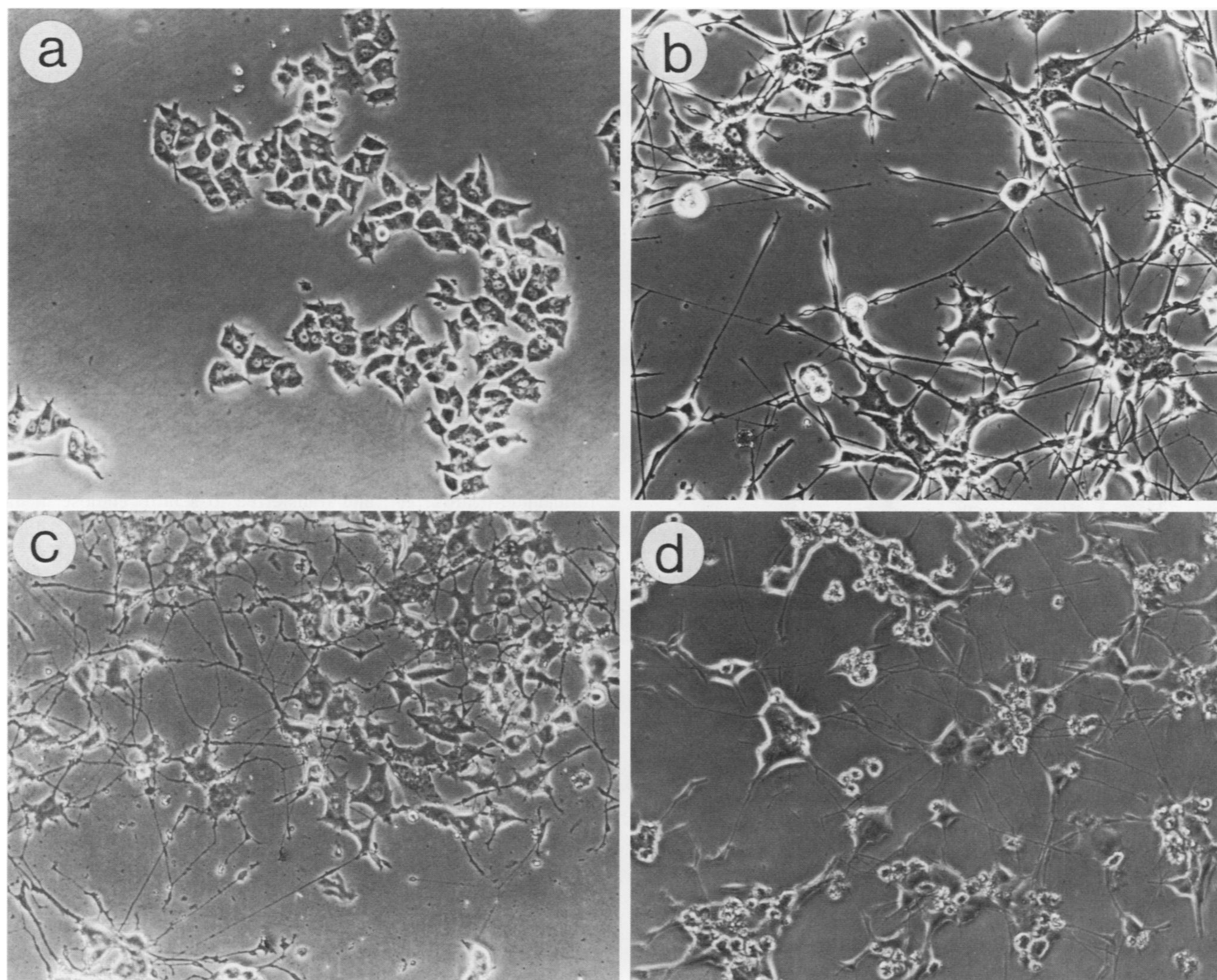

FIG. 1. Morphological conversion of PC12 cells treated with NGF or BSF-2. PC12 cells were seeded onto plastic culture dishes, grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and 5% horse serum, and incubated at 37°C in 5% CO_2. The culture medium was changed every 2 days. (a) PC12 cells grown in growth medium. (b) PC12 cells cultured in serum-free medium containing 2.5S NGF (50 ng/ml) (Takara Shuzo) for 7 days. (c) PC12 cells cultured in serum-free medium containing 24 ng of recombinant BSF-2 per ml for 6 days. (d) PC12 cells cultured for 9 days in serum-free medium containing 11 ng of natural BSF-2 partially purified as described elsewhere (12) per ml.

Table 1, the specific binding of saxitoxin per milligram of protein in the BSF-2-treated cells increased six- to sevenfold as in the NGF-treated cells after an 11-day treatment. The results indicate that BSF-2, like NGF, induces the formation of voltage-dependent Na^+ channels. In the previous report (21), binding was measured more than 2 weeks after the addition of NGF, and the increase was about ninefold. However, the BSF-2-treated cells lost viability after about 10 days (see below). Therefore, we measured the binding of the toxin at an earlier stage. The values of binding with NGF-treated cells in our results were lower than those reported by previous workers (21).

As described above, PC12 cells differentiated by BSF-2 acquire a number of neuronal properties similar to those induced by NGF. Some differences, however, were noted. First, there was a longer lag period (2 to 3 days) until clear morphological change was observed than in the case of NGF. Second, in contrast to NGF, BSF-2 cannot maintain the viability of the differentiated PC12 cells beyond 10 days

TABLE 1. Specific binding of [^{3}H]saxitoxin to PC12 cells cultured in various conditions for 11 days[a]

Addition to culture (ng/ml)	Mean specific [^{3}H]saxitoxin binding (fmol/mg of protein) ± SE
None	3.81 ± 1.19 (n = 5)
NGF (50)	22.2 ± 1.88[b] (n = 4)
Recombinant BSF-2[c] (20)	26.3 ± 5.59[b] (n = 4)

[a] Binding of saxitonin was measured by using [^{3}H]saxitoxin (Amersham; TRK.877; 68 Ci/mmol) at 20 nM, essentially as described elsewhere (21). Each experimental value represents the average for four independent cultures. Data are the mean of four to five independent experiments, as indicated.

[b] $P < 0.01$ compared with no-addition value.

[c] In serum-free medium.

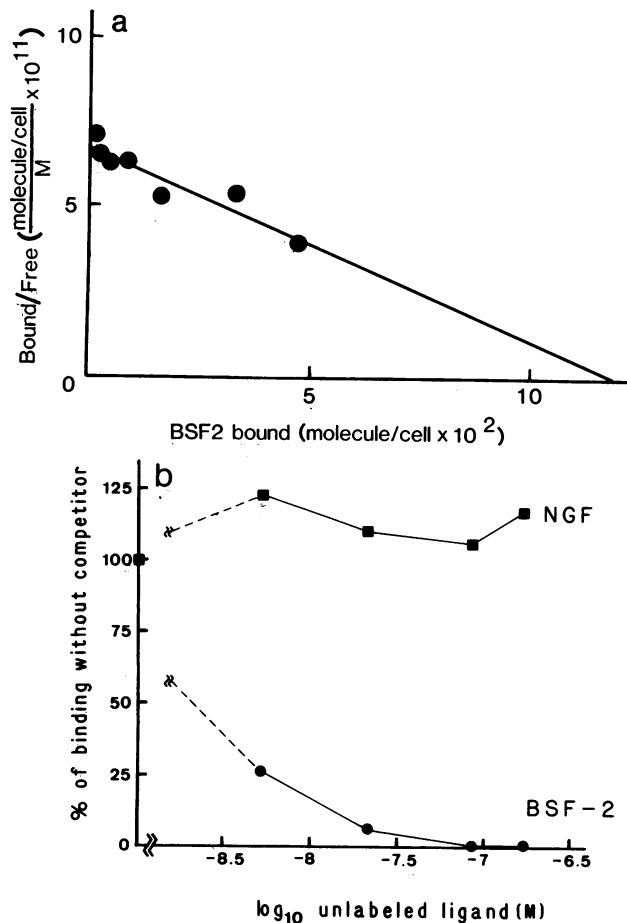

FIG. 2. Characterization of receptors for BSF-2 on PC12 cells. (a) Scatchard plot of the specific binding of [^{125}I]BSF-2 to PC12 cells. PC12 cells (10^6) in 70 μl of binding medium with various amount of [^{125}I]BSF-2 in the presence or absence of a 200-fold excess of unlabeled BSF-2 were incubated for 1 h at room temperature. Data represent the mean specific binding of duplicate samples; the variation was usually less than 8%. Concentration of the labeled ligand used for this assay was in the range of 7.7 pM to 1.2 nM. (b) Competition for the binding of [^{125}I]BSF-2 to PC12 cells by unlabeled BSF-2 (●) or NGF (■). PC12 (2.5×10^6) cells in 70 μl of 1.4 nM [^{125}I]BSF-2 were incubated in the presence of the indicated concentrations of BSF-2 or NGF. Data represent the mean percentage of the specific binding without competitor for duplicate samples.

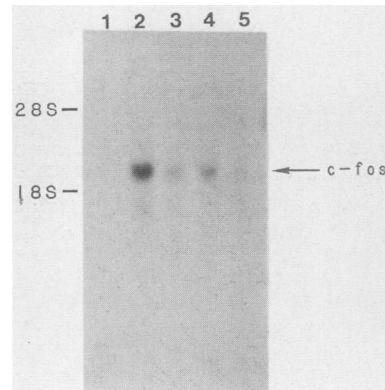

FIG. 3. Induction of *fos* mRNA by NGF or BSF-2 in PC12 cells. PC12 cells were untreated (lane 1) or treated with 2.5S NGF (50 ng/ml) for 30 min (lane 2) or with recombinant BSF-2 (20 ng/ml) for 30 min (lane 3), 45 min (lane 4), or 60 min (lane 5) in growth medium. Total RNA (25 μg per lane) was isolated, separated on a 1.2% formamide-agarose gel, and transferred to a nitrocellulose membrane. The c-*fos* mRNA was detected by hybridization with a ^{32}P-labeled c-*fos*-specific probe [prepared from 3.24-kb *Xho*I-*Nco*I fragment of pc-*fos*(human)-1] (2) in stringent conditions (24).

in serum-free conditions. Thus, BSF-2 can initiate neuronal differentiation but does not support long-term culture of neuronal cells. Third, a significant increase in acetylcholinesterase activity, specific for neuronal differentiation by NGF (7), was not detected in the case of BSF-2 within 6 days (data not shown), although the increase by NGF was already observed after 3 to 4 days of exposure.

In this paper, we report that pheochromocytoma PC12 cells differentiate to neuronal cells in response to BSF-2. We can speculate that BSF-2 may function as a nerve cell differentiation factor. Expression of BSF-2 mRNA in astrocytoma or glioblastoma cells induced by IL-1β (25) strongly supports this, since IL-1 is also expressed in microglia under physiological conditions (4). Recently, it was reported (9) that a 56-kDa neurotropic factor, termed neuroleukin, was produced by lectin-stimulated T cells and induced immunoglobulin synthesis. BSF-2 could be another example of a well-identified differentiation factor that acts in both the immune and nervous systems.

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