

---

**CELL BIOLOGY AND METABOLISM:**  
**A Predominant Apoptotic Death Pathway**  
**of Neuronal PC12 Cells Induced by**  
**Activated Microglia Is Displaced by A**  
**Non-apoptotic Death Pathway Following**  
**Blockage of Caspase-3-dependent Cascade**

Kazunari Tanabe, Hiroshi Nakanishi,  
Hidefumi Maeda, Tsuyoshi Nishioku, Koichi  
Hashimoto, Shyh-Yuh Liou, Akifumi  
Akamine and Kenji Yamamoto  
*J. Biol. Chem.* 1999, 274:15725-15731.

---

Access the most updated version of this article at <http://www.jbc.org/content/274/22/15725>

Find articles, minireviews, Reflections and Classics on similar topics on the [JBC Affinity Sites](#).

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 59 references, 26 of which can be accessed free at  
<http://www.jbc.org/content/274/22/15725.full.html#ref-list-1>

# A Predominant Apoptotic Death Pathway of Neuronal PC12 Cells Induced by Activated Microglia Is Displaced by A Non-apoptotic Death Pathway Following Blockage of Caspase-3-dependent Cascade\*

(Received for publication, January 12, 1999, and in revised form, March 12, 1999)

Kazunari Tanabe<sup>‡§</sup>, Hiroshi Nakanishi<sup>‡</sup>, Hidefumi Maeda<sup>§</sup>, Tsuyoshi Nishioku<sup>‡</sup>, Koichi Hashimoto<sup>¶</sup>, Shyh-Yuh Liou<sup>¶</sup>, Akifumi Akamine<sup>§</sup>, and Kenji Yamamoto<sup>‡¶</sup>

From the Departments of <sup>‡</sup>Pharmacology and <sup>§</sup>Conservative Dentistry II, Faculty of Dentistry, Kyushu University, Fukuoka 812-8582, Japan and <sup>¶</sup>Tsukuba Research Laboratories, Nippon Glaxo Ltd., Tsukuba 300-4247, Japan

Activated microglia have been implicated in the regulation of neuronal cell death. However, the biochemical mechanism for neuronal death triggered by activated microglia is still unclear. When treated with activated microglia, neuronal PC12 cells undergo apoptosis accompanied by caspase-3-like protease activation and DNA fragmentation. Apoptotic bodies formed were subsequently phagocytosed by neighboring activated microglia. Pretreatment of the cells with the caspase-3-like protease inhibitor *N*-acetyl-Asp-Glu-Val-Asp-aldehyde did not reverse this cell death. Although Bcl-2 overexpression in the cells caused the inhibition of caspase-3-like protease activity and DNA fragmentation and the effective interference of apoptosis induced by deprivation of trophic factors, it could not suppress the activated microglia-induced neuronal death. At the electron microscopic level, degenerating cells with high levels of Bcl-2 were characterized by slightly condensed chromatin forming irregular-shaped masses, severely disintegrated perikarya, and marked vacuolation. Various protease inhibitors tested did not inhibit this cell death, whereas the radical oxygen species scavenger *N*-acetyl-L-cysteine significantly suppressed this death. Altogether, our study provides an alternative death pathway for the activated microglia-induced neuronal death by blockage of the caspase-3 protease cascade.

Apoptosis or programmed cell death is distinguished from lytic or necrotic cell death by specific biochemical and structural events. The most characteristic features of apoptosis are the activation of caspase cascades, nuclear and cytoplasmic condensation, blebbing of cytoplasmic membranes, and apoptotic body formation. It is generally accepted that apoptosis is a fundamental cell death pathway contributing to the regulation of tissue development and homeostasis and that dysregulated apoptosis may cause a variety of pathologic states such as autoimmune disease and malignancy. Therefore, apoptosis appears to be highly controlled by a complex interplay between regulatory proteins such as Bcl-2 and its family members. However, the biochemical mechanism involved in the function of these proteins has not been fully elucidated.

\* This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom all correspondence should be addressed. Tel.: 81-92-642-6339; Fax: 81-92-642-6342; E-mail: kyama@dent.kyushu-u.ac.jp.

Bcl-2 is a 26-kDa integral membrane protein that is found in the nuclear envelope, the part of the endoplasmic reticulum, and the outer mitochondrial membrane. Several reports have reported that overexpression of Bcl-2 can delay or prevent apoptosis by a diverse number of death-promoting signals in some cell systems (1–8). Moreover, the study of Bcl-2-knockout mice has revealed that this protein is involved in regulation of cellular redox in response to oxidative stress (9). It has also been shown that Bcl-2 inhibits the activation of caspases by blocking cytochrome *c* release from mitochondria (10–13) and prevents the loss of mitochondrial membrane potential (14, 15). In addition to the anti-apoptosis activity, recent studies have revealed that Bcl-2 also promotes regeneration of severed neuronal axons (16), regulates neuronal differentiation (17), and controls cell proliferation (18–22).

So far, it has been shown that microglia are activated by cytokines to produce neurotoxic agents such as nitric oxide and reactive oxygen intermediates (23–26). Therefore, it is considered that activation of microglia contributes to expansion of neuronal injury and has important pathogenetic implications in neurodegenerative diseases such as Alzheimer's disease, multiple sclerosis, and human immunodeficiency virus-associated dementia (27–30). Despite the clinical importance, the precise intracellular mechanism for neuronal death triggered by activated microglia is currently unknown.

To elucidate the biochemical mechanism for activated microglia-induced neuronal death, control and Bcl-2-transfected neuronal PC12 cells were treated with activated microglia that were challenged with a combination of interferon- $\gamma$ /lipopolysaccharide (LPS)<sup>1</sup> in the presence and absence of the caspase-3-like protease inhibitors.

## EXPERIMENTAL PROCEDURES

**Materials**—Ac-DEVD-CHO, pepstatin A, leupeptin, Ac-YVAD-MCA, and Ac-DEVD-MCA were purchased from Peptide Institute Inc. (Osaka, Japan). Calpain inhibitor I was obtained from Bachem (Torrance, CA). *N*-Acetyl-L-cysteine (NAC) was obtained from Nacalai Tesque Inc. (Tokyo, Japan). Recombinant rat IFN- $\gamma$  and LPS (*Escherichia coli* serotype 055:B5) were purchased from Genzyme Corporation (Cambridge, MA) and Sigma, respectively. Nerve growth factor (NGF) was obtained from Chemicon International Inc. (Temecula, CA). OX42 and OX6 were purchased from Serotec Ltd (Bicester, UK). Anti-human Bcl-2 antibody was purchased from Genosys Biotechnologies Inc. (Cambridge, UK). Papain and DNase were from Worthington Biochemical (Freehold, NJ).

<sup>1</sup> The abbreviations used are: LPS, lipopolysaccharide; ANOVA, analysis of variance; LDH, lactate dehydrogenase; MCA, aminomethylcoumarin; NAC, *N*-acetyl-L-cysteine; NGF, nerve growth factor; ROS, reactive oxygen species; TUNEL, terminal dUTP nick-end labeling; Ac-DEVD-CHO, *N*-Acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-YVAD-MCA, Ac-Try-Val-Ala-Asp-aminomethylcoumarin.

RPMI 1640 medium and Dulbecco's modified Eagle's medium were from Life Technologies, Inc. (Grand Island, NY). Calceinacetoxymethyl ester was from Molecular Probes (Eugene, OR). ApopTag kit was from Oncor (Gaithersburg, MD).

**Cell Transfection**—The pheochromocytoma cell line PC12 was transfected with the plasmid pSTneoB and human Bcl-2/pcDNA/Amp by the electroporation method (7). The transfected cells were selected by their resistance to G418 (800  $\mu\text{g/ml}$ ) for 2 months in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal calf serum (FCS). The resulting stable neomycin resistant clonal PC12 cell line and Bcl-2 overexpressing clonal cell line were termed Vector N2 and Bcl-2 N2, respectively.

**PC12 Cell Culture**—Stock PC12 cells were cultured in RPMI 1640 medium containing 10% heat-inactivated horse serum, 5% FCS, 50 units/ml penicillin, and 100  $\mu\text{g/ml}$  streptomycin. Neuronally differentiated PC12 cells were obtained by plating  $3\text{--}4 \times 10^4$  cells onto 24-well collagen-coated dishes in the presence of 50 ng/ml NGF for a period of 8–10 days in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% FCS.

**Microglial Primary Culture**—Microglia were isolated from a primary culture of the rat brain as described previously (7, 31). Briefly, the cerebral cortex from 3-day-old male Wistar rats was minced and treated with papain (90 units/ml) and DNase (2000 units/ml) at 37 °C for 15 min. The mechanically dissociated cells were seeded into plastic flasks at a density of  $10^7$  per  $300\text{ cm}^2$  in Dulbecco's modified Eagle's medium, 0.3%  $\text{NaHCO}_3$ , 50 units/ml penicillin, 100  $\mu\text{g/ml}$  streptomycin, and 10% FCS, and maintained at 37 °C in a 10%  $\text{CO}_2$ , 90% air atmosphere. Subsequent medium replacement was carried out every 3 days. After 10–14 days in culture, floating cells and weakly attached cells on the mixed primary cultured cell layer were isolated by gentle shaking of the flask for 3–5 min. The resulting cell suspension was transferred to plastic dishes (Falcon 1001, Lincoln Park, NJ) and allowed to adhere at 37 °C. Unattached cells were removed after 30 min, microglia were isolated as strongly adhering cells. About 90% of these attached cells were positive for OX42 or OX6, makers for macrophage/microglial cell types.

**Assay for Cell Survival**—Isolated microglia were directly plated into 24-well dishes containing neuronal PC12 cells, and then IFN- $\gamma$  (100 units/ml) and LPS (1 ng/ml) were added in the medium. In some experiments, isolated microglia were plated into cell culture inserts (0.4- $\mu\text{m}$  pore size, Falcon 3095) and placed in 24-well dishes containing neuronal PC12 cells. Cell death was quantified by the measurement of the cytosolic enzyme lactate dehydrogenase (LDH) released into the culture medium from degenerating cells. For determination of LDH activity, the culture medium was collected from each culture well, and then the well was washed with Dulbecco's modified Eagle's medium containing HEPES. The medium was centrifuged at  $200 \times g$  for 5 min to sediment nonadherent cells. The supernatant was transferred to a fresh tube, and pelleted cells were lysed in 1 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 0.5% Triton X-100 (lysing buffer). Cells remaining on the well were lysed in 1 ml of lysis buffer. LDH activities in the culture medium (supernatant) and in the cell lysates were measured spectrophotometrically. The percentage of cell death was calculated by the following formula: cell death = (LDH activity in the culture medium/total LDH activity)  $\times$  100. LDH levels in the culture medium and the cell lysate from microglia (generally less than 10% of the total LDH) were determined in the sister cultures and subtracted from the levels in co-culture experimental systems.

**In Situ DNA Nick-end Labeling**—Apoptosis of neuronal PC12 cells after co-culture with activated microglia were detected by terminal dUTP nick-end labeling (TUNEL) using the ApopTag kit. Neuronal Vector N2 and Bcl-2 N2 cells plated on the chamber slides at various days after co-culture with activated microglia were fixed by 4% paraformaldehyde for 30 min at room temperature. After washes in phosphate-buffered saline, cells on the slides were stained for TUNEL following the protocol provided by the manufacturer.

**Electrophoresis and Immunoblotting**—Detailed electrophoresis and immunoblotting procedures were described previously (31, 32). Briefly, cells in the dishes were washed with phosphate-buffered saline and mechanically removed. Cells were pelleted by centrifugation and resuspended in phosphate-buffered saline containing 0.1% Triton X-100. The soluble cell extracts were prepared by ultrasonication followed by centrifugation at  $105,000 \times g$  for 30 min. The extracts were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions in 7–12% gradient gels after the heat treatment in the solubilizing buffer at 100 °C for 5 min. For immunoblotting, the proteins on gels were electrophoretically transferred to nitrocellulose membranes and then immunostained with anti-human Bcl-2 antibody (10  $\mu\text{g/ml}$ ), essentially

according to the procedure described previously (32). As a control, the primary antibody was replaced by preimmune mouse IgG.

**Assays for Caspase Activity**—Assays for caspase-3-like proteases (caspase-2, -3, and -7) and caspase-1-like proteases (caspase-1 and -4) were performed at pH 7.4 by using fluorogenic synthetic tetrapeptide substrates (100  $\mu\text{M}$ ), Ac-DEVD-MCA and Ac-YVAD-MCA, respectively. After the removal of the culture medium at the appropriate time, the cells were lysed in 500  $\mu\text{l}$  of lysing buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100). After centrifugation, 100  $\mu\text{l}$  of cleared lysates were subjected to assays for caspase-3-like and caspase-1-like proteases with the respective substrates at pH 7.4. Fluorescence was measured with excitation at 360 nm and emission at 460 nm using CytoFluor II fluorometer (PerSeptive, Eugene, OR). The measurements were performed in triplicate, and the activity was expressed as change in fluorescence units per hour per 10,000 cells.

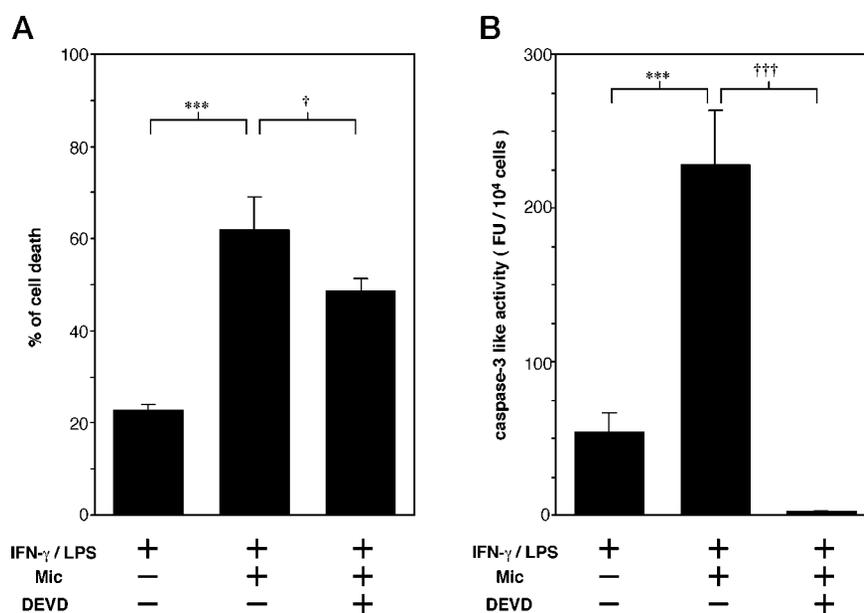
**Electron Microscopic Analysis**—At 52 h after the treatment with activated microglia, neuronal Vector N2 or Bcl-2 N2 cells on a glass slide chamber (Falcon) were fixed with 4% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) containing 1% glutaraldehyde. After washing with 0.1 M sodium phosphate buffer, the cells were dehydrated in a graded series of ethanol and then embedded in Epon 812. Serial ultrathin sections were cut and mounted on nickel grids and stained with 4% uranyl acetate and lead citrate and then examined with a Hitachi H-7000 electron microscope.

**Statistical Analysis**—Data are expressed as means  $\pm$  S.D. The significance of differences between groups was determined with two-way analysis of variance (ANOVA), followed by Scheffe's post hoc test for multiple comparison when *F* ratios reached significance.

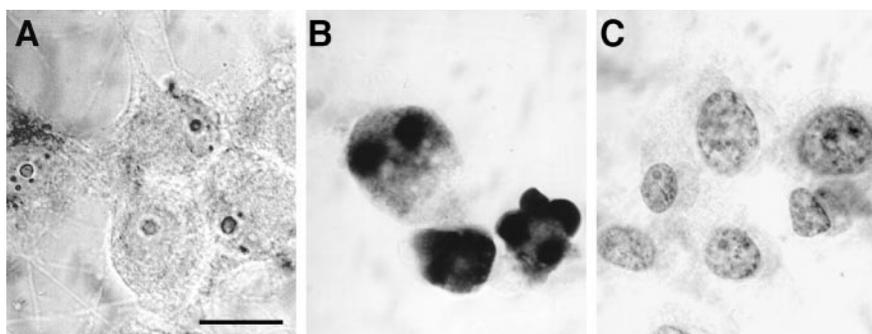
## RESULTS

**Effects of Ac-DEVD-CHO on Activated Microglia-induced Death of Neuronal PC12 Cells**—The cell viability of neuronal PC12 cells following co-culture with rat microglia immunostimulated with IFN- $\gamma$  (100 units/ml)/LPS (1 ng/ml) was examined in the serum-free and NGF-containing medium. When the neuronal cells were cultured for 72 h in the absence of activated microglia, approximately 20% of the total cells were dead (Fig. 1A). The neuronal cells, when treated with IFN- $\gamma$ /LPS in the absence of microglia, exhibited no significant increase of their death. However, when the neuronal PC12 cells were treated with activated microglia, approximately 60% of the neuronal cells were dead at 72 h after co-culture. Importantly, no significant increase of the neuronal death was induced by the treatment with either unstimulated microglia or activated microglia plated on culture inserts to avoid direct contact with the neuronal cells (data not shown). The activated microglia-induced neuronal death was accompanied by the significant increase of the caspase-3-like protease activity determined with Ac-DEVD-MCA as the substrate (Fig. 1B). When the neuronal cells were pretreated with the caspase-3-like protease inhibitor Ac-DEVD-CHO (100  $\mu\text{M}$ ) for 24 h, the caspase-3-like protease activity in the cells was completely inhibited, but the microglia-induced neuronal death was only partially inhibited, as compared with that of the untreated neuronal cells. The result indicates that inhibition of the caspase-3-like protease activity is not sufficient to inhibit activated microglia-induced neuronal death.

**Morphological Characteristics of Activated Microglia-induced Death of Neuronal PC12 Cells**—At 72 h after treatment with activated microglia, the majority of neuronal cells became TUNEL-positive and contained various sizes of multiple apoptotic bodies (Fig. 2B), indicating the occurrence of apoptotic cell death. When Ac-DEVD-CHO (100  $\mu\text{M}$ ) was added to the culture medium at 24 h before and during treatment with activated microglia, the majority of neuronal cells were not stained with TUNEL (Fig. 2C). However, damage of neuronal cells was clearly evident by morphological alterations such as their retracted neurites. Neither TUNEL-positive staining nor neuronal damage was observed in the neuronal cells treated with IFN- $\gamma$ /LPS in the absence of activated microglia (Fig. 2A).



**FIG. 1. Effects of Ac-DEVD-CHO on the action of activated microglia on neuronal PC12 cells.** *A*, induction of neuronal cell death during co-culture with activated microglia. Both Ac-DEVD-CHO-treated and control neuronal PC12 cells were co-cultured with the activated microglia for 72 h. Then cell viability was measured by the LDH assay. Each column and bar represents the mean  $\pm$  S.D. of three to five individual experiments. \*\*\*,  $p < 0.001$  as compared with the IFN- $\gamma$ /LPS-treated neuronal PC12 cells in the absence of microglia. †,  $p < 0.05$  as compared with the activated microglia-treated neuronal cells in the absence of Ac-DEVD-CHO (two-way ANOVA). *B*, activation of caspase-3-like proteases in the activated microglia-treated neuronal PC12 cells. After treatment with the activated microglia for 72 h, both control and Ac-DEVD-CHO-treated neuronal PC12 cells were lysed, and the caspase-3-like protease activity in their cell extracts were measured using the fluorogenic substrate. Each column and bar represents the mean  $\pm$  S.D. of three to five individual experiments. \*\*\*,  $p < 0.001$  as compared with the IFN- $\gamma$ /LPS-treated neuronal cells in the absence of microglia. †††,  $p < 0.001$  as compared with the activated microglia-treated neuronal cells in the absence of Ac-DEVD-CHO (two-way ANOVA).



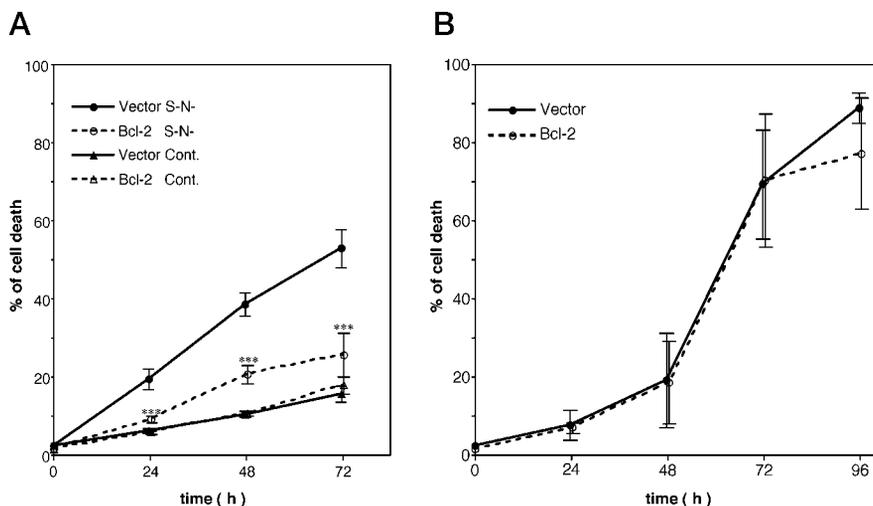
**FIG. 2. TUNEL staining in neuronal PC12 cells following co-culture with activated microglia in the presence and absence of Ac-DEVD-CHO.** *A*, neuronal PC12 cells at 48 h after the treatment of IFN- $\gamma$ /LPS in the absence of microglia. Scale bar, 20  $\mu$ m. *B*, neuronal PC12 cells at 48 h after treatment with the activated microglia. TUNEL-positive neuronal cells contained multiple apoptotic bodies. *C*, neuronal PC12 cells pretreated with Ac-DEVD-CHO at 48 h after the treatment with activated microglia. Most of the neuronal cells were not stained with TUNEL but showed morphological changes such as retracted neurites, indicating their damage.

**Effects of Overexpression of Human Bcl-2 on Activated Microglia-induced Cell Death of Neuronal PC12 Cells**—We then examined the effect of high levels of Bcl-2 protein in the neuronal cells on the activated microglia-induced neuronal death. The stable neomycin-resistant clonal PC12 cells without (Vector N2) or with human Bcl-2 protein (Bcl-2 N2) were differentiated to neurons by NGF treatment. Then both serum and NGF were deprived from cultures of the respective cell types. As shown in Fig. 3A, neuronal Vector N2 cells were markedly induced to neuronal death by the trophic factor deprivation in a time-dependent manner, whereas neuronal Bcl-2 N2 cells were more resistant to death induced by deprivation of trophic factors (Fig. 3A). These data indicate that Bcl-2 is expressed highly enough in neuronal Bcl-2 N2 cells to suppress cell death induced by deprivation of trophic factors. In contrast, no significant difference with regard to the extent of the activated microglia-induced neuronal death was observed between Vector N2 and Bcl-2 N2 cells, indicating that high levels of Bcl-2

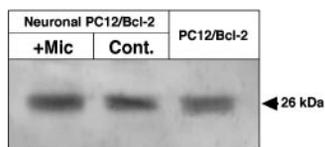
cannot protect the neuronal cells from the activated microglia-induced cell death (Fig. 3B).

To examine the possibility that the lack of protective effect of Bcl-2 expression on the activated microglia-mediated neurotoxicity may be because of the changes of its cellular levels or molecular forms during incubation, the cellular levels of Bcl-2 protein were analyzed by immunoblotting (Fig. 4). When the same amounts of proteins from the cell extracts of neuronal Bcl-2 N2 before and after treatment with activated microglia for 72 h were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblot analysis, single immunoreactive bands with an apparent molecular mass of 26 kDa were observed with both samples at the same intensity, and their electrophoretic mobilities were also indistinguishable. Therefore, it is unlikely that the activated microglia-induced neuronal death of Bcl-2 N2 cells is caused by either the reduction of cellular levels of Bcl-2 or its molecular change.

In the neuronal Bcl-2 N2 cells, the proteolytic activities of



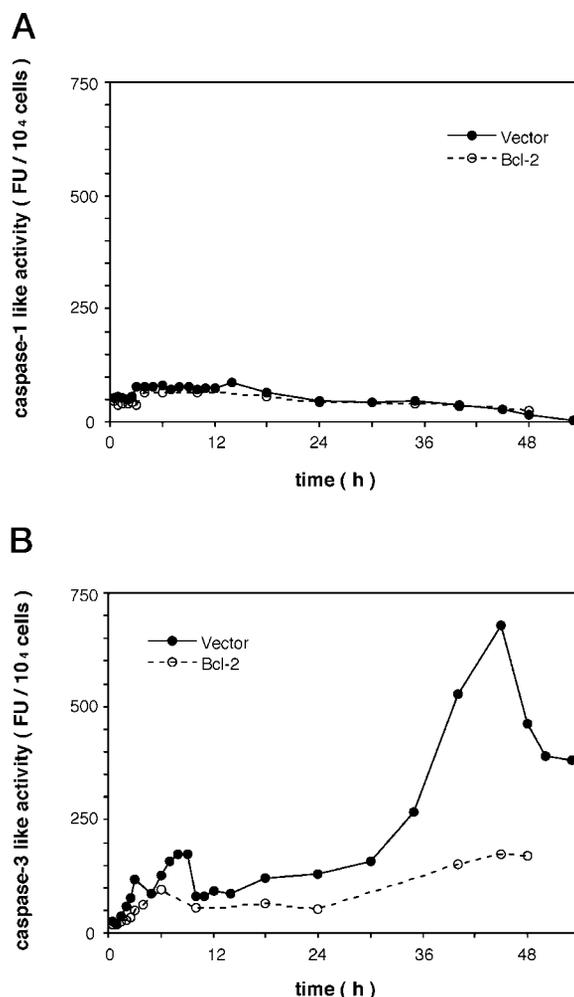
**FIG. 3. Effects of Bcl-2 overexpression in neuronal PC12 cells on their death induced by deprivation of trophic factors (A) and treatment with activated microglia (B).** A, time-course of changes in cell viability after deprivation of both NGF and serum in the neuronal Vector N2 (filled circle) and Bcl-2 N2 (open circle) cells. Cell viability was measured by the LDH assay. More than 50% of the control Vector N2 cells died at 72-h postdepletion, whereas about 75% of the Bcl-2 N2 cells were apparently viable at the same time point. Each point and bar represents the mean  $\pm$  S.D. of five experiments, respectively. \*\*\*,  $p < 0.001$  as compared with Vector N2 cells (two-way ANOVA). B, time course of changes in cell viability after treatment with activated microglia in neuronal Vector N2 (filled circle) and Bcl-2 N2 (open circle) cells. No significant difference in the extent and profile of cell death was observed between Vector N2 and Bcl-2 N2 neuronal cells. Each point and bar represents the mean  $\pm$  S.D. of five to ten individual experiments, respectively.



**FIG. 4. Bcl-2 protein expression in neuronal Bcl-2 N2 cells during co-culture with activated microglia.** Western blot analysis was performed on the neuronal Bcl-2 N2 cells before and after treatment with activated microglia for 72 h to ensure the unchanged expression of Bcl-2 protein during the process of the activated microglia-induced neuronal death. The same amount of protein (30  $\mu$ g) of each cell extract was subjected to SDS-polyacrylamide gel electrophoresis and analyzed with anti-human Bcl-2 antibody. Right lane, undifferentiated Bcl-2 N2 cells; middle lane, neuronal Bcl-2 N2 cells; left lane, neuronal PC12 N2 cells after treatment with activated microglia for 72 h.

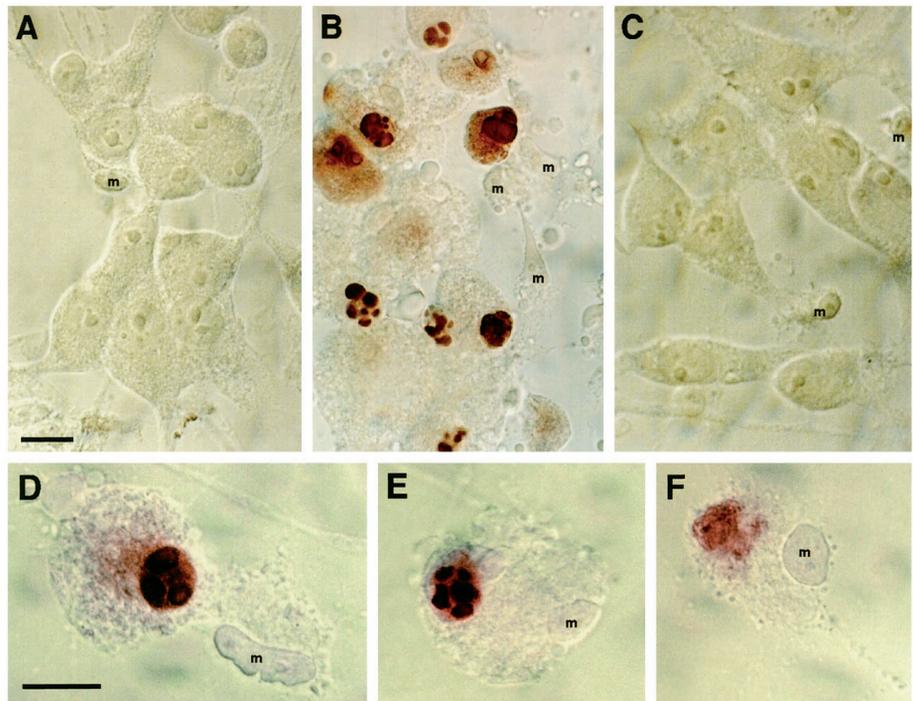
caspase-1-like and caspase-3-like proteases were determined after treatment with activated microglia by use of the fluorogenic substrates Ac-YVAD-MCA and Ac-DEVD-MCA, respectively (Fig. 5). The hydrolytic activity of Ac-YVAD-MCA was barely detectable in the extracts of both neuronal Vector N2 and Bcl-2 N2 cells during the whole period of co-culture with activated microglia. No significant activity of Ac-DEVD-MCA was detected in the extract of neuronal Bcl-2 N2 cells during the co-culture, although only a slight increase in the activity was observed around 42 h after the co-culture. In contrast, the extract of neuronal Vector N2 cells showed a gradual increase in the hydrolytic activity of Ac-DEVD-MCA to attain the peak value at around 42 h after the treatment with activated microglia. This activity profile appeared to be correlated with their death as measured by LDH activity in the culture medium per total LDH activity. No hydrolytic activity of either Ac-YVAD-MCA or Ac-DEVD-MCA was detected in cytosolic extracts of microglia. Moreover, various protease inhibitors, other than Ac-DEVD-CHO, such as the aspartic proteinase inhibitor pepstatin A (30  $\mu$ M for 48 h), the cysteine proteinase inhibitor leupeptin (100  $\mu$ M for 24 h), and the calpain inhibitor I (30  $\mu$ M, for 24 h) had no significant inhibitory effect on either the Ac-DEVD-MCA hydrolytic activity or the death of neuronal Bcl-2 N2 cells following co-culture with activated microglia (data not shown).

#### Inhibition of DNA Fragmentation in Neuronal PC12 Cells



**FIG. 5. Time-course of changes in the caspase-1-like (A) and caspase-3-like proteolytic activities (B) after treatment with activated microglia in the cell extracts of control and Bcl-2-overexpressing neuronal PC12 cells.** A, time course of caspase-1-like protease activity. The activity was determined with the fluorogenic substrate Ac-YVAD-MCA. B, time course of caspase-3-like protease activity that was determined with Ac-DEVD-MCA.

**FIG. 6. TUNEL stainings on control and Bcl-2-overexpressing neuronal PC12 cells after treatment with activated microglia for 48 h.** *A*, the neuronal Vector N2 (control) cells that were treated with unstimulated microglia. *B* and *D–F*, the control cells after treatment with activated microglia. TUNEL-positive apoptotic bodies were frequently attached to microglia (*D*) and engulfed by microglia (*E* and *F*) and degraded (*F*). *C*, the neuronal PC12 cells overexpressing Bcl-2 (Bcl-2 N2) after treatment with activated microglia. The cells were scarcely stained by TUNEL but had retracted neurites and accumulated vacuoles in the perikarya. The nuclei of microglia were indicated by *m* in panels *A–F*. Scale bars in panels *A* and *D* = 10  $\mu$ m.



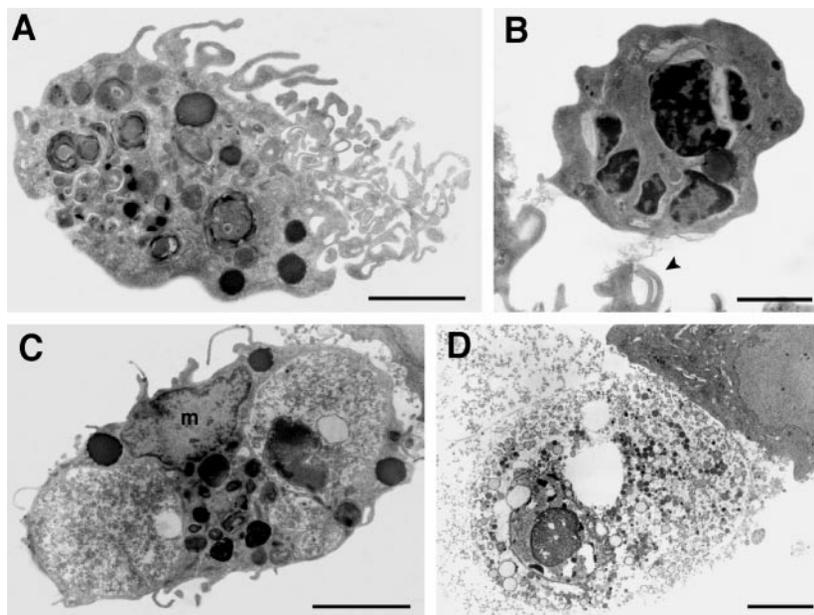
**Overexpressing Bcl-2 during the Process of Degeneration Induced by Activated Microglia**—At 48 h after treatment with activated microglia, the majority of neuronal Vector N2 cells became TUNEL-positive and contained various sizes of multiple apoptotic bodies (Fig. 6*B*). A number of TUNEL-positive apoptotic bodies were attached to neighboring microglia and were engulfed by the microglia (Fig. 6, *D* and *E*). Some of the TUNEL-positive apoptotic bodies appeared to be extensively degraded in microglia (Fig. 6*F*). Approximately 40% of the total TUNEL-positive apoptotic bodies were engulfed by microglia. On the other hand, the majority of neuronal Bcl-2 N2 cells were not stained by TUNEL even at 48 h after treatment with activated microglia but were nevertheless clearly damaged by the treatment as evidenced by their retracted neurites and accumulated vacuoles in their perikarya (Fig. 6*C*). No staining was observed in neuronal Vector N2 cells co-cultured with unstimulated microglia (Fig. 6*A*).

**Ultrastructural Analyses of Neuronal Vector and Bcl-2 N2 Cells Treated with Activated Microglia**—After treatment with IFN- $\gamma$ /LPS, most of the microglia showed characteristic ruffled membrane structures on their cell surface and had a large number of phagosome-like dense bodies containing phagocytosed materials (Fig. 7*A*). Fig. 7*B* shows an apoptotic body of neuronal Vector N2 cells containing distinct chromatin condensation in the vicinity of activated microglia (an arrowhead indicates the ruffled cell membrane of microglia). Apoptotic bodies of neuronal Vector N2 cells were found to be frequently phagocytosed by activated microglia. Nuclei of Vector N2 cells were also characterized by chromatin uniformly aggregated into large discrete masses abutting on the nuclear membrane (Fig. 7*C*). In contrast, in the activated microglia-treated neuronal Bcl-2 N2 cells, their chromatin was slightly condensed to form irregular shaped masses (Fig. 7*D*), but their nuclear membranes were still maintained and formed some boundaries between nuclear and cytoplasmic matrixes. Importantly, these degenerating Bcl-2 N2 cells frequently showed severely disintegrated perikarya, dilated rough endoplasmic reticulum, and swollen mitochondria, suggesting that the degenerating process is similar to the necrotic death pathway rather than the apoptotic death pathway.

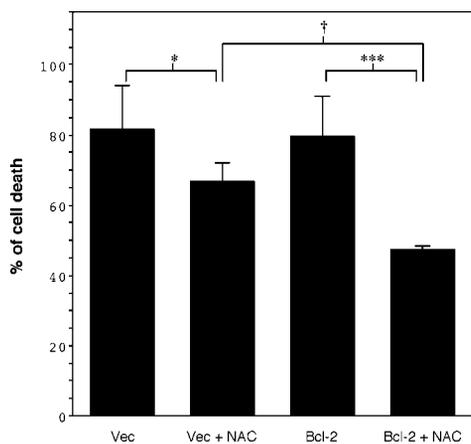
**Effects of N-Acetyl-L-cysteine on Activated Microglia-induced Death**—Several lines of evidence have revealed a role of reactive oxygen species (ROS) as a mediator of cell death. Indeed, generation of ROS has been commonly observed after treatment with agents such as tumor necrosis factor- $\alpha$  (33), LPS (34), and ceramide (35). Trophic factor deprivation can also stimulate the ROS production (36). In addition, ROS scavengers, such as NAC, are known to inhibit cell death induced by various death stimuli (9, 37–39). Therefore, to elucidate the involvement of ROS in the activated microglia-induced death of neuronal PC12 cells, we examined the effect of NAC (60 mM). Both neuronal Vector N2 and Bcl-2 N2 cells, when treated with NAC (60 mM) for 1 h before and during the treatment with activated microglia, exhibited significant resistance to the microglia-induced neuronal death (Fig. 8). It was also noted that NAC-treated neuronal Bcl-2 N2 cells were more resistant to killing by activated microglia than NAC-treated neuronal Vector N2 cells.

## DISCUSSION

The present study has clearly demonstrated that activated microglia can effectively induce the apoptotic cell death of neuronal PC12 cells. This apoptosis was accompanied by caspase-3-like protease activation and morphological changes characteristic of apoptosis, such as nuclear and cytoplasmic condensation, DNA fragmentation, and the formation of apoptotic bodies that were finally phagocytosed by neighboring microglia. This demonstrates the predominance of the caspase-3-executed apoptotic cell death of neuronal cells by activated microglia. When the neuronal cells were pretreated with AC-DEVD-CHO, no activation of caspase-3-like proteases was observed. Nevertheless, activated microglia-induced neuronal death occurred, suggesting that the activation of caspase-3-like proteases is not essential for the execution of activated microglia-induced neuronal death. However, since the morphological hallmarks of apoptosis, such as chromatin condensation, DNA fragmentation, and apoptotic body formation, were not observed in this process, the microglia-induced neuronal death executed in the presence of inhibitor seems to be non-apoptotic. There is increasing evidence that cells can die without chroma-



**FIG. 7. Electron microscopic graphs of neuronal Vector N2 and Bcl-2 N2 cells after treatment with activated microglia.** A, typical activated microglia having ruffled membrane structures on the cell surface. B, an apoptotic body with uniformly condensed chromatin of Vector N2 cells close to the ruffled cell membrane of microglia (an arrowhead). C, apoptotic bodies of neuronal Vector N2 cells engulfed by microglia (its nuclei was indicated by *m*). D, a degenerating neuronal Bcl-2 N2 cell after treatment with activated microglia, where its nuclear membrane was still maintained to form boundaries between the nuclear and the cytoplasmic matrix. Scale bars, 2  $\mu\text{m}$  (A and B) and 4  $\mu\text{m}$  (C and D).



**FIG. 8. Inhibition of activated microglia-induced death by NAC in neuronal Vector N2 and Bcl-2 N2 cells.** Both control (Vector N2) and Bcl-2-overexpressing neuronal PC12 cells (Bcl-2 N2) were pretreated with 60 mM for 1 h, and then the cells were treated with the activated microglia for 72 h in the presence of NAC (60 mM). Cell viability was measured by the LDH assay. Each column and bar represents the mean  $\pm$  S.D. of four to eight individual experiments. \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$  as compared with the respective nontreated neuronal cells; †,  $p < 0.05$  as compared with the NAC-treated Vector N2 cells (two-way ANOVA).

tin condensation and DNA fragmentation after apoptotic stimuli in the presence of the inhibitor for caspase-3-like proteases (40–42). Recent evidence also indicates that caspase-3 is not required for apoptosis in the PC12 cells (43) and that thymocytes isolated from caspase-3 knockout mice exhibit normal Fas-induced apoptosis (44). Taken together, the present data indicate that although caspase-3-like proteases are essential for chromatin condensation and DNA fragmentation, they are not required for other apoptotic events in the cytoplasm or the cell death itself. It should also be noted that this inhibitor rescued the same cells from death induced by trophic factor deprivation.

Then we examined whether high levels of Bcl-2 protein in neuronal PC12 cells inhibited the activated microglia-induced

neuronal apoptosis. The results indicated that Bcl-2 overexpression effectively protected the neuronal cells from apoptosis induced by deprivation of both serum and NGF, whereas it did not abrogate the activated microglia-induced neuronal death. In either case, the caspase-3-like protease activity was effectively inhibited in neuronal PC12 cells overexpressing Bcl-2 protein, but the trophic factor deprivation-induced cell death only was prevented. Importantly, the Bcl-2-overexpressing cells after treatment with activated microglia showed morphological changes different from apoptosis. These results suggest that apoptotic signaling pathways dependent on the activation of caspase-3-like proteases would be changed to non-apoptotic signaling pathways independent of the activation of caspase-3-like proteases.

It has recently been shown that, in addition to the caspase-3-like proteases, some other intracellular proteolytic systems are identified as executioners to activate cell death. Cathepsin D, a typical lysosomal aspartic proteinase, has been shown to mediate cytokine-induced apoptosis of some cell types (45, 46). Calpain, a calcium-dependent cysteine proteinase, has also been shown to mediate cell death in some cell systems (47–51). However, it is unlikely that the intracellular proteolytic machinery mediated by either cathepsin D or calpain is responsible for the activated microglia-induced neuronal death of Bcl-2 overexpressing PC12 cells in the present study because pepstatin A, leupeptin, or calpain inhibitor I failed to protect the cells from death.

Recently, several lines of evidence support a role for ROS as a mediator of both apoptosis and necrosis. Hydrogen peroxide and superoxide caused apoptosis in a variety of cell lines (9, 52, 53). In addition, antioxidants such as NAC can inhibit apoptosis of neuronal PC12 cells and sympathetic neurons induced by trophic factor deprivation (54, 55). ROS and resulting cellular redox changes are known to be part of the signal transduction pathway during apoptosis. On the other hand, ROS toxicity has been also shown to result in necrosis of some cell types (56, 57). More recently, Vercaemmen *et al.* (58, 59) have reported that enhanced production of ROS is responsible for TNF-induced necrosis of L929 cells in the presence of caspase inhibitors.

Accordingly, we attempted to determine whether ROS was important in the activated microglia-induced death of neuronal Vector N2 and Bcl-2 N2 cells. The results indicated that NAC significantly but partially decreased the activated microglia-induced death of both cell types, suggesting that ROS production is partially involved in death pathways of both neuronal PC12 cells. It should also be noted that NAC showed more effective protection in the neuronal Bcl-2 N2 cells than the control Vector N2 cells against the activated microglia-induced cell death. This suggests that the activated microglia-induced death of neuronal Bcl-2 cells is accompanied by the enhanced production of ROS. Although the enhanced production of ROS may be one of the mediators for non-apoptotic death of neuronal Bcl-2 N2 cells, the precise mechanism is to be elucidated in future studies.

In conclusion, it must be pointed out that activated microglia predominantly induce caspase-3-like protease-executed apoptosis of neuronal PC12 cells but alternatively trigger non-apoptotic cell death when the caspase-3-like protease cascade is inhibited by protease inhibitors and Bcl-2 overexpression. Thus, it is more likely that whether activated microglia induce apoptosis or non-apoptosis, the neuronal cells depend on the prominence of caspase-3-like protease activation in the death process as well as other factors such as the ROS production. These findings also suggest that adaptation to one type of death pathway may render cells more susceptible to alternative death pathways and that the hypersensitive response of the neuronal cells may be caused by the process of adaptation to the microglia-induced apoptosis. This could be an effective backup mechanism to execute the activated microglia-induced neuronal death. To our knowledge, the data presented here provide the first evidence for a possible mechanism for the regulation of neuronal death by activated microglia.

**Acknowledgments**—We thank Drs. N. Takai and D. F. Sastradipura for assistance with the preparation of primary cultured microglia. We also thank Jeremy Hengst for critical reading of the manuscript.

#### REFERENCES

- Garcia, I., Martinou, I., Tsujimoto, Y., and Martinou, J.-C. (1992) *Science* **258**, 302–304
- Allsopp, T. E., Wyatt, S., Paterson, H. F., and Davies, A. M. (1993) *Cell* **73**, 295–307
- Batistatou, A., Merry, D. E., Knorsmeyer, S. J., and Gree, n L. A. (1993) *J. Neurosci.* **13**, 4422–4428
- Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., Ord, T., and Bredesen, D. E. (1993) *Science* **262**, 1274–1277
- Zhong, L.-T., Sarafian, T., Kane, D. J., Charles, A. C., Ma, S., Edwards, R. H., and Bredesen, D. E. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 4533–4537
- Offen, D., Berat P. M., Cheung N. S., Pascoe C. J., Hochman A., Gorodin S., Melamed E., Bernard, R., and Bernard, O. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 5789–5794
- Takai, N., Nakanishi, H., Tanabe, K., Nishioku, T., Sugiyama, T., Fujiwara, M., and Yamamoto, K. (1998) *J. Neurosci. Res.* **54**, 214–222
- Tanabe, H., Eguchi, Y., Kamad, S., Martinou, J.-C., and Tsujimoto, Y. (1998) *Eur. J. Neurosci.* **9**, 848–856
- Hockenbery, D., Oltvai, Z. N., Yin, X., Milliman, C. L., and Korsmeyer, S. J. (1993) *Cell* **75**, 241–251
- Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. (1997) *Science* **275**, 1132–1136
- Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) *Cell* **91**, 479–489
- Reed, J. C. (1997) *Nature* **387**, 773–776
- Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T.-I., Jones, D. P., and Wang, X. (1997) *Science* **275**, 1129–1132
- Shimizu, S., Eguchi, Y., Kamiike, W., Wagri, S., Uchiyama, Y., Matsuda, H., and Tsujimoto, Y. (1996) *Oncogene* **13**, 21–29
- Zamzami, N., Susin, S. A., Marchetti, P., Hirsch, T., Gomez-Monterrey, I., Castedo, M., and Kroemer, G. (1996) *J. Exp. Med.* **183**, 1533–1544
- Chen, D. F., Schneider, G. E., Martinou, J.-C., and Tonegawa, S. (1997) *Nature* **385**, 434–439
- Zhang K.-Z., Westberg, J. A., Holtta, E., and Andersson, L. C. (1996) *Proc. Natl. Acad. Natl. U. S. A.* **93**, 4504–4508
- Pietenpol, J. A., Papadopoulos, N., Markowitz, S., Willson, J. K., Kinzler, K. W., and Vogelstein, B. (1994) *Cancer Res.* **54**, 3714–3717
- Borner, C. (1996) *J. Biol. Chem.* **271**, 12695–12698
- Uhlmann, E. J., D'Sa-Eipper, C., Subramanian, T., Wagner, A. J., Hay, N., and Chinnadurai, G. (1996) *Cancer Res.* **56**, 2506–2509
- O'Reilly, L. A., Huang, D. C., and Strasser, A. (1996) *EMBO J.* **15**, 6979–6990
- Vairo, G., Innes, K. M., and Adams, J. M. (1996) *Oncogene* **13**, 1511–1519
- Zielasek, J., Tausch, M., Toyka, K. V., and Hartung, H. P. (1992) *Cell Immunol.* **141**, 111–120
- Betz-Corradin, S., Mauer, J., Donini, S. D., Quattrocchi, E., and Ricciardi-Castagnoli, P. (1993) *Glia* **7**, 255–262
- Colton, C. A., Snell, J., Chernyshev, O., and Gilbert, D. L. (1994) *Ann. N. Y. Acad. Sci.* **738**, 54–63
- Chao, C. C., Hu, S., and Peterson, P. K. (1995) *J. Leukocyte Biol.* **58**, 65–70
- McGeer, P. L., and McGeer, E. G. (1995) *Brain Res. Rev.* **21**, 195–218
- Meda, L., Cassatella, M. A., Szendrei, G. I., Otvos, L., Jr., Baron, P., Villalba, M., Ferrari, D., and Rossi, F. (1995) *Nature* **374**, 647–650
- Navikas, V., and Link, H. (1996) *J. Neurosci. Res.* **45**, 322–333
- Nuovo, G. J., and Alfieri, M. L. (1996) *Mol. Med.* **2**, 358–366
- Sastradipura, D. F., Nakanishi, H., Tsukamoto, T., Nishishita, K., Sakai, H., Kato, Y., Gotow, T., Uchiyama, Y., and Yamamoto, K. (1998) *J. Neurochem.* **70**, 2045–2956
- Nakanishi, H., Amano, T., Sastradipura, D. F., Yoshimine, Y., Tsukuba, T., Tanabe, K., Hirotsu, I., Ohono, T., and Yamamoto, K. (1997) *J. Neurochem.* **68**, 739–749
- Albrecht, H., Tschopp, J., and Jongeneel, C. V. (1994) *FEBS Lett.* **351**, 45–48
- Wang, J. H., Redmond, H. P., Watson, R. W., and Bouchier-Hayes, D. (1997) *Am. J. Physiol.* **272**, C1543–C1551
- Garcia-Ruiz, C., Colell, A., Mari, M., Morales, A., and Fernandez-Checa, J. C. (1997) *J. Biol. Chem.* **272**, 11369–11377
- Atabay, C., Cagnoli, C. M., Kharlamov, E., Ikonovic, M. D., and Nanev, H. (1996) *J. Neurosci. Res.* **43**, 465–475
- Lafon, C., Mathieu, C., Guerrin, M., Pierre, O., Vidal, S., and Valette, A. (1996) *Cell Growth Differ.* **7**, 1095–1104
- Cossarizza, A., Franceschi, C., Monti, D., Salvioli, S., Bellesia, E., Rivabene, R., Biondo, L., Rainaldi, G., Tinari, A., and Malorni, W. (1995) *Exp. Cell Res.* **220**, 232–240
- Islam, K. N., Kayanoki, Y., Kaneto, H., Suzuki, K., Asahi, M., Fujii, J., and Taniguchi, N. (1997) *Free Radical Biol. Med.* **22**, 1007–1017
- Xiang, J., Chao, D. T., and Korsmeyer, S. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 14559–14563
- Toyoshima, F., Moriguchi, T., and Nishida, E. (1997) *J. Cell Biol.* **139**, 1005–1015
- McCarthy, N. J., Whyte, M. K. B., Gilbert, C. G., and Evan, G. I. (1997) *J. Cell Biol.* **136**, 215–227
- Stefanis, L., Park, D. S., Yan, C. Y., Farinelli, S. E., Troy, C. M., Shelanski, M. L., and Greene, L. A. (1996) *J. Biol. Chem.* **271**, 30663–30671
- Kuida, K., Zheng, T. S., Na, S., Kuan, C.-y., Yang, D., Karasuyama, H., Rakic, P., and Flavell, R. (1996) *Nature* **384**, 368–372
- Deiss L. P., Galinka, H., Berissi, H., Cohen, O., and Kimchi, A. (1996) *EMBO J.* **15**, 3861–3870
- Wu, G. S., Saftig, P., Peters, C., and El-Deiry, W. S. (1998) *Oncogene* **16**, 2177–2183
- Seubert, P., Larson, J., Oliver, M., Jung, M. W., Braudry, M., and Lynch, G. (1988) *Brain Res.* **460**, 189–194
- Seubert, P., Lee, K., and Lynch, G. (1989) *Brain Res.* **492**, 366–370
- Siman, R., and Noszek, J. C. (1988) *Neuron* **1**, 279–287
- Sarin, A., Adams, D. H., and Henker, P. A. (1993) *J. Exp. Med.* **178**, 1693–1700
- Squier, M. K., Miller, A. C., Malkinson, A. M., and Cohen, J. J. (1994) *J. Cell. Physiol.* **159**, 229–237
- Bladier, C., Wolvetang, E. J., Hutchinson, P., de Haan, J. B., and Lola, I. (1997) *Cell Growth Differ.* **8**, 589–598
- Gardner, A. M., Xu, F., Fady, F. J., Duffey, D. C., Tu, Y., and Lichtenstein, A. (1997) *Free Radical Biol. Med.* **22**, 73–83
- Slater, A. F., Nobel, C. S., and Orrenius, S. (1995) *Biochim. Biophys. Acta* **1271**, 59–62
- Ferrari, G., Yan C. Y. I., and Greene, L. A. (1995) *J. Neurosci.* **15**, 2857–2866
- Goossens, V., Grooten, J., De Vos, K., and Fiers, W. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 8115–8119
- Higuchi, M., Honda, T., Proske, R. J., and Yeh, E. T. (1998) *Oncogene* **17**, 2753–2760
- Vercammen, D., Beyaert, R., Denecker, G., Goossens, V., Van Loo, G., Declercq, W., Grooten, J., and Vandenabeele, P. (1998) *J. Exp. Med.* **187**, 1477–1485
- Vercammen, D., Brouckaert, G., Denecker, G., Van de Craen, M., Declercq, W., Fiers, W., and Vandenabeele, P. (1998) *J. Exp. Med.* **188**, 919–930