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N-Acetyl-L-cysteine is a pluripotent protector against cell death and enhancer of trophic factor-mediated cell survival *in vitro*

(tumor necrosis factor α/oligodendrocytes/neurons/apoptosis)

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ABSTRACT **We have discovered that *N*-acetyl-L-cysteine (NAC) protects cells against death induced by exposure to noxious stimuli and against programed cell death (apoptosis) associated with exposure to inadequate amounts of trophic factors. NAC prevented glutamate-induced death of oligodendrocytes and tumor necrosis factor α (TNF-α)-induced death of oligodendrocytes and L929 fibroblasts. Moreover, suboptimal doses of NAC plus ciliary neurotrophic factor (which also protects oligodendrocytes against TNF-α-mediated killing) acted synergistically to protect oligodendrocytes against TNF-α-induced death. Protection against death by growth factor deprivation was provided by the combination of (*i*) NAC, vitamin C, or Trolox (a water-soluble analogue of vitamin E) with suboptimal concentrations of protein trophic factors, (*ii*) NAC, vitamin C, or Trolox with progesterone, and (*iii*) NAC with either vitamin C or Trolox; these latter experiments suggest that the addition of tyrosine kinase stimulators is not required to promote cell survival. In all paradigms, NAC was either equally or more effective than the other compounds examined. In light of the long history of therapeutic application of NAC, we suggest that use of this compound may be of interest in conditions where certain toxin-mediated forms of cell death and/or apoptosis contribute significantly to disease.**

The identification of compounds that promote cell survival may provide useful tools for deciphering fundamental mechanisms by which cells live or die and also may yield compounds of potential use in clinical applications. In attempts to identify such reagents, we distinguish between death caused by exposure to toxic stimuli (i.e., "death by murder") and that caused by exposure to suboptimal amounts of necessary trophic factors ("death by neglect"). As well as defining distinct experimental paradigms, these categories may also have relevance for intervention in particular physiological processes. For example, apoptotic cell death during development appears to be due to insufficient quantities of necessary trophic factors in the immediate environment, and supplementation of trophic factors during early development can prevent such cell death in a variety of tissues (1–4). In contrast, cell death following injury more generally appears to be due to cytotoxic stimuli. The extent to which trophic factor supplementation might protect against such death *in vivo* is not yet known.

The model system we primarily utilize to study cell death is the *in vitro* survival of oligodendrocytes, the myelin-forming cells of the central nervous system. Oligodendrocyte death is of interest because of the severe clinical consequences of destruction of these cells, as occurs with demyelination in multiple sclerosis (MS) and in the periventricular white matter injury thought to underlie spastic motor and cognitive deficits frequently seen in premature infants (5–9). In both of these instances, it is thought that cytotoxic stimuli contribute to oligodendrocyte death. In contrast, oligodendrocyte apoptosis during development of the rat optic nerve appears to occur because trophic factor levels are too low to support all of the oligodendrocytes present in this tissue (1, 4).

The death of oligodendrocytes in premature infants recently has been suggested to be due to glutamate exposure, which is toxic for these cells *in vitro* (9). Glutamate toxicity for oligodendrocytes appears to be mediated by uptake of glutamate via a low-affinity uptake system that can operate as an exchange mechanism with intracellular cystine (9). Glutamate uptake by oligodendrocytes is associated with a reduction in intracellular cystine levels (9), followed by a fall in levels of intracellular glutathione, the metabolic product of cystine important in the control of cellular redox state and in protecting cells against damage associated with oxidative stress (10, 11). Death induced by oxidative stress may also be relevant in MS, as it appears that cell death induced by tumor necrosis factor α (TNF-α), which is present in MS lesions and kills oligodendrocytes *in vitro* (12–14), is mediated through reactive oxidative intermediates (15, 16).

Glutamate toxicity, and reductions in oligodendrocyte glutathione levels associated with glutamate exposure, can be blocked *in vitro* by cystine supplementation (9). Although previous investigators have not reported on the effects of cystine or related compounds on TNF-α-induced cell death, it has been reported for lymphoid cells that some of the consequences of TNF-α exposure on gene transcription are prevented by compounds known to raise intracellular glutathione levels (17–19). Together, these observations raise the theoretical possibility of identifying single compounds that prevent oligodendrocyte death induced by either glutamate or TNF-α. One compound known to effectively raise intracellular glutathione levels, and for which extensive clinical experience also exists, is *N*-acetyl-L-cysteine (NAC) (17, 20–22), a compound of interest as a potential pharmaceutical for AIDS patients (17, 19, 23) as well as being used to replenish liver glutathione following acetaminophen overdose and in treatment of pulmonary disorders (21, 22, 24–27).

We now report that NAC protects oligodendrocytes against death induced by cytotoxic stimuli and also enhances the extent of cell survival obtained with suboptimal concentrations of trophic factors. NAC also blocks TNF-α-induced killing of L929 cells and markedly enhances the number of surviving neurons found in spinal ganglion neuronal cultures exposed to suboptimal quantities of NGF. Our data suggest that several distinct physiological pathways can play a role in preventing programed cell death and also indicate that pre-

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Abbreviations: CNTF, ciliary neurotrophic factor; IGF-I, insulin-like growth factor I; MS, multiple sclerosis; NAC, *N*-acetyl-L-cysteine; NGF, nerve growth factor; TNF-α, tumor necrosis factor α; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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vention of such death with physiologically relevant compounds does not require the addition of activators of tyrosine kinase activity.

MATERIALS AND METHODS

Preparation of Cultures. *Purified oligodendrocytes.* Purified oligodendrocytes were generated from cultures of oligodendrocyte type 2 astrocyte (O-2A; ref. 28) progenitors derived from 7-day-old rats and purified using a specific antibody-capture assay as described (29, 30), except that cells were never exposed to trypsin. Purified cells were grown on poly(L-lysine)-coated glass coverslips (Sigma; M_r 175,000; 20 μg/ml) in chemically defined medium (DMEM/BS; ref. 31) made up in Dulbecco's modified Eagle's medium (DMEM) as described (30). To generate pure cultures of oligodendrocytes the purified O-2A progenitors were grown for 3 days in DMEM/BS to induce differentiation of all cells into oligodendrocytes (28). Parallel coverslips stained with oligodendrocyte-specific antibodies (see below) consisted of 100% oligodendrocytes.

Cultures of embryonic sensory neurons. Cultures were generated from spinal ganglia of 16-day rat embryos by treating single cell suspensions with O4 antibody plus complement to kill Schwann cells (32–34). The remaining cells (the majority of which appeared to be fibroblast-like cells) were plated onto fibronectin-coated coverslips in DMEM/BS at 1000 total cells per coverslip.

L929 fibroblasts. L929 fibroblasts (35) were maintained in flasks in DMEM containing 10% fetal calf serum (FCS). Prior to assays, cells were plated in 96-well plates at a density of 30,000 cells per well in DMEM/FCS.

Death by Murder. The response of L929 fibroblasts to TNF-α was examined by adding TNF-α (2 ng/ml) with or without NAC after cells had settled in their 96-well plates. Cells were analyzed 24 hr later using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) system (Promega) and counted on a plate reader.

The response of oligodendrocytes to TNF-α was examined by plating purified O-2A progenitors at a density of 3000 cells per coverslip. After 3 days, cells were exposed to 10 ng of TNF-α per ml and various concentrations of the compounds indicated for a further 3 days (as indicated in Figs. 1 and 2) before cultures were stained with MTT (see below) for 1 hr and examined under the light microscope (30, 36). The percentage of living (i.e., MTT^+) cells was compared with cultures not exposed to TNF-α.

Glutamate toxicity to oligodendrocytes was examined by plating purified O-2A progenitors at a density of 3500 cells per coverslip in DMEM/BS. After 3 days, oligodendrocytes were exposed to glutamate with or without NAC (single application for both compounds) as indicated in Figs. 1 and 2 for 1 day before cultures were stained with MTT and analyzed as above.

Death by Neglect. Purified O-2A progenitors were plated at 1000–2500 cells per coverslip, as indicated in figure legends. After 3 days of growth in DMEM/BS, the resulting cultures of pure oligodendrocytes were switched to DMEM alone, a condition that induces apoptosis unless cells are exposed to appropriate trophic factors (4, 30, 37). Cultures of oligodendrocytes received various concentrations of NAC, ciliary neurotrophic factor (CNTF), insulin-like growth factor I (IGF-I), vitamin C, Trolox (glydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble vitamin E analogue; ref. 38), or progesterone alone or in combinations as indicated in *Results*. After 3 further days, cultures were analyzed as for experiments examining oligodendrocyte death induced by exposure to toxic stimuli.

To examine protection of embryonic sensory neurons against apoptosis associated with exposure to suboptimal amounts of trophic factors, cultures were fed with various doses of nerve growth factor (NGF) on days 1 and 2, in the presence or absence of 0.5 mM or 1 mM NAC. Cultures were analyzed on day 3 and neurons were defined by morphology and by labeling with the TuJ1 antibody against β_3-tubulin (39).

MTT Assay. The assay was performed as described (4, 36) and additionally combined with immunofluorescence. Following completion of MTT staining, cells were fixed with 4% paraformaldehyde and stained with anti-GalC antibodies as described. Live cells appeared with dark blue cytoplasm in bright-field phase microscopy.

Glutathione Measurements. Measurements were carried out by the method of Tietze (40).

Compounds Utilized. NGF, glutamate, vitamin C, progesterone, and NAC were from Sigma, IGF-I was from Precision Research Biochemicals (London), TNF-α and CNTF were from Promega, and Trolox was from Fluka. All trophic factors used were recombinant proteins.

Immunocytochemistry. Antibodies used, as described (30, 32), for cell-type identification and in panning protocols were as follows: mouse IgG3 monoclonal antibody against galactocerebroside (GalC; ref. 41); A2B5 mouse IgM monoclonal antibody (42); anti-Ran-2 mouse IgG2 monoclonal antibody (43); the neuron-specific TuJ1 antibody against β_3-tubulin (ref. 39, a kind gift of A. Frankfurter).

RESULTS

NAC Protects Against Death Induced by Glutamate and by TNF-α. As expected from the protective effects of cystine and cysteine (9), NAC protected purified oligodendrocytes against glutamate-induced cell death. Glutamate killed up to 85% of oligodendrocytes within 24 hr at concentrations of 10 μM to 2 mM (Fig. 1*A*). In contrast, in cultures exposed to ≤100 μM glutamate plus 1 mM NAC, the numbers of live oligodendrocytes were not reduced and cultures exposed to 2 mM glutamate plus 1 mM NAC exhibited only a slight fall in cell number. Also, as expected from previous studies (11, 20–22), growth of oligodendrocytes in the presence of NAC was associated with increases in levels of intracellular glutathione. Cells grown in control medium contained 27 ng of glutathione per μg of protein, as compared with 75 and 181 ng/μg of protein for cells grown in the presence of 1 mM NAC for 7 or 15 hr, respectively.

NAC also protected oligodendrocytes against cell death induced by exposure to 10 ng of TNF-α per ml, a dose that yielded a plateau killing of 50–60% of unprotected oligodendrocytes over 3 days (Fig. 1*B*, dose–response for TNF-α not shown). Significant protection against TNF-α-mediated killing was conferred by 0.1 mM NAC and complete protection by 1 mM NAC. One millimolar NAC afforded similar protection against 50 ng of TNF-α per ml (data not shown). As seen for glutamate toxicity (9), cystine and cysteine also conferred some protection against TNF-α-mediated killing of oligodendrocytes, although less than NAC (data not shown). The antioxidants vitamin C and Trolox were never as effective as even 10 μM NAC and rescued only 25–35% as many oligodendrocytes as did 1 mM NAC when applied at their maximal nontoxic doses (10 μM vitamin C and 100 μM Trolox; data not shown).

NAC also prevented TNF-α-mediated killing of L929 fibroblasts, a cell line highly sensitive to TNF-α-induced apoptosis (38). Cultures exposed to TNF-α for 24 hr contained virtually no living cells; in contrast, L929 cultures exposed to TNF-α in the presence of 1 mM NAC could not be distinguished from untreated control cultures (Fig. 1*C*).

NAC Is More Effective than CNTF at Protecting Oligodendrocytes from Death Induced by Toxic Stimuli. Oligodendrocytes can also be protected from the lethal effects of TNF-α by CNTF (14), but even 50 ng of CNTF per ml did not protect cells as well as 0.1 mM NAC (compare Fig. 1 with Fig. 2*A*).

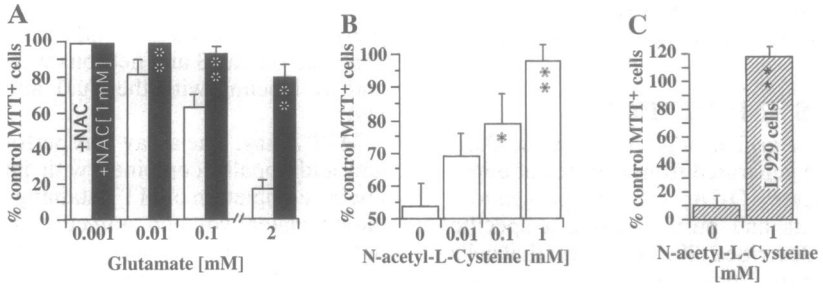

FIG. 1. (*A*) Glutamate. Pure oligodendrocyte cultures were prepared, treated with glutamate, and analyzed as in the text (initial plating density = 3500 cells per coverslip). The percentage of living (i.e., MTT^+) cells was compared with cultures not exposed to glutamate (ordinate). The results shown are means ± SEM of two experiments containing eight replicates for each condition. Control values (100%) = 403 ± 42 cells. The significance values compared to controls are as indicated: *, $P < 0.01$; **, $P < 0.005$ (Student's *t* test) for these and all following experiments. (*B*) TNF-α. Oligodendrocytes were prepared as above (plating density = 3000 cells per coverslip), exposed to 10 ng of TNF-α per ml with or without NAC for 3 days, and analyzed as in *A*. Values shown are means ± SEM for quadruplicate coverslips from one experiment; experiments with NAC were repeated 10 times with similar results. Control values (100%) = 778 ± 10 cells. (*C*) L929 fibroblast cultures exposed to TNF-α without NAC contained almost no living cells, whereas L929 cultures exposed to TNF-α plus 1 mM NAC showed no signs of extensive cell death. The ordinate represents the percentage of cells that were alive (calculated from absorption values) as compared with cultures not treated with TNF-α. Values shown are means ± SEM for quadruplicate wells from one of two experiments, both of which yielded similar results. Control values (100%) = MTT absorption of 1.17.

We did find a striking synergy between these compounds, however. Cultures exposed to 0.5 ng of CNTF per ml plus 0.01 or 0.1 mM NAC contained significantly more live oligodendrocytes than those exposed to NAC alone (see Fig. 1) or to CNTF alone at 0.5 ng/ml (which had no significant effect on survival). At higher doses of CNTF, the combined effect of the two compounds appeared additive (Fig. 2A).

As CNTF and NAC both rescued oligodendrocytes from TNF-α-induced cell death, these compounds might have exerted their effects through similar mechanisms. This seems unlikely, however, as CNTF did not protect oligodendrocytes against glutamate-induced death (Fig. 2*B*).

NAC Enhances Neuron and Oligodendrocyte Survival in Paradigms of Apoptosis Associated with Exposure to Suboptimal Amounts of Trophic Factors. Cells exposed to inadequate amounts of appropriate trophic factors die from apoptosis (1–3, 44). The protective effect of NAC against this form of cell death was examined for sensory neurons and oligodendrocytes.

Dramatic effects of NAC on cell survival were observed in cultures of spinal ganglion neurons, derived from day 16 rat embryos and exposed to suboptimal doses of NGF (the appropriate trophic factor for 70–85% of these neurons; refs. 45 and 46). Cultures exposed to NAC and either 1 or 10 ng of NGF per ml contained 300–1000% more neurons than those exposed to NGF alone (Fig. 3*A*). Analysis of DNA synthesis by BrdUrd incorporation (47) confirmed that this increase in neuronal number was not due to cell division, strongly implicating differential cell survival as the reason for the difference.

Exposure to NAC also markedly enhanced the extent of oligodendrocyte survival obtained with suboptimal quantities of trophic factors. Cultures of pure oligodendrocytes treated with any dose of CNTF examined, and most doses of IGF-I, contained significantly more live oligodendrocytes if cultures were also exposed to 1 mM NAC (Fig. 3 *B* and *C*). Of particular interest, the presence of NAC was associated with the presence of significant numbers of live oligodendrocytes in cultures exposed to doses of CNTF or IGF-I that by themselves had little or no effect on cell survival. In contrast, NAC was not by itself sufficient to rescue cells from death associated with growth factor deprivation.

Vitamin C and Trolox, two other compounds with antioxidant activity could also cooperate with trophic factors to promote oligodendrocyte survival. Although neither compound had any effect when applied alone, significant numbers of oligodendrocytes survived when either compound was added with 0.5 ng of CNTF per ml (Fig. 3 *D* and *E*).

Promotion of oligodendrocyte survival by antioxidants did not require simultaneous exposure of cells to trophic factors capable of stimulating receptor tyrosine kinases (for NGF and IGF-I; ref. 48) or receptors associated with tyrosine kinases (for CNTF; ref. 49). For example, progesterone, applied alone at concentrations equal to or less than that used in DMEM/BS (i.e., 63 ng/ml), had little or no effect on oligodendrocyte survival but, when applied together with NAC, promoted highly significant levels of cell survival (Fig. 4*A*). Combinations of progesterone plus vitamin C or Trolox also promoted survival, but to only 50% of the values obtained with NAC (data not shown). In addition, application of NAC together with either vitamin C or Trolox (Fig. 4*B*)

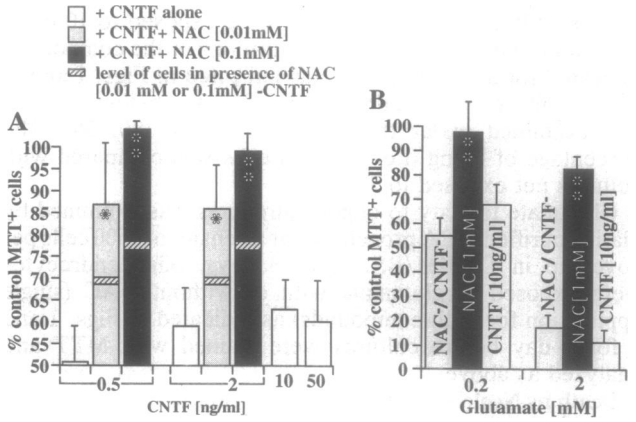

FIG. 2. (*A*) Cultures were prepared as in Fig. 1*B*, and oligodendrocytes were exposed for 3 days to TNF-α (10 ng/ml) with or without the indicated doses of CNTF with or without NAC. For low doses of CNTF (0.5 ng/ml), the presence of CNTF plus NAC led to a greater rescue than expected from a simple additive interaction between these two compounds. No doses of CNTF tested (up to 50 ng/ml) rescued >26% of the population that was killed by TNF-α. Values shown are means ± SEM for quadruplicate coverslips from one of four experiments, all of which yielded similar results. Control values (100%) = 275 ± 30 cells. (*B*) Cultures of purified oligodendrocytes were prepared as in Fig. 1*A* and were exposed to glutamate with or without NAC or CNTF for 1 day before MTT labeling. CNTF did not confer significant protection against glutamate-mediated killing at the dose shown, nor at lower doses (data not shown). Values shown are means ± SEM for quadruplicate coverslips from one of three experiments, all of which yielded similar results. Control values (100%) = 640 ± 84 cells.

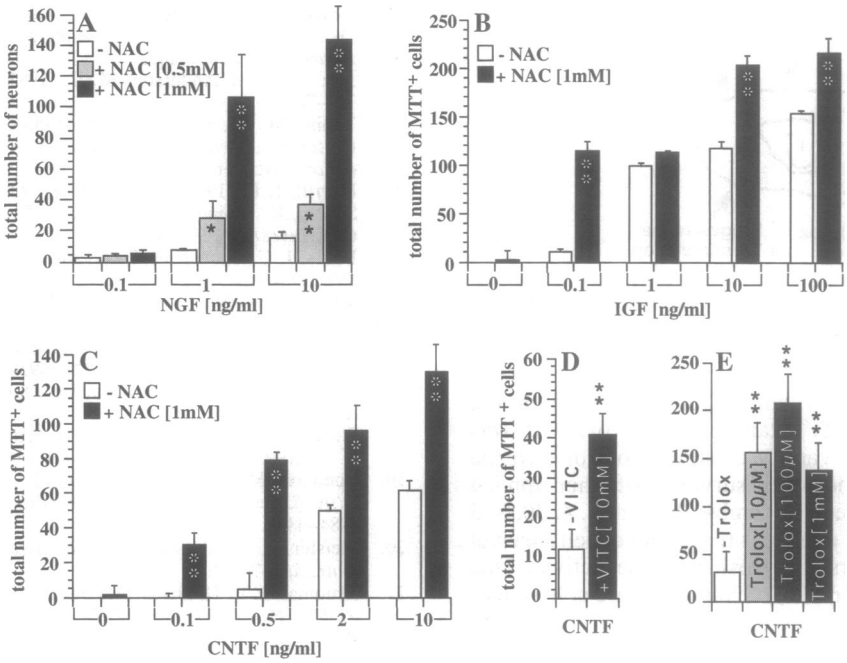

FIG. 3. (*A*) Cultures of spinal ganglion neurons were prepared and analyzed as in the text. There was no BrdUrd labeling of neurons, nor was there any correlation between the extent of BrdUrd labeling in nonneuronal populations and the number of neurons present (data not shown). Values shown are means ± SEM for quadruplicate coverslips from one of three experiments, all of which yielded similar results. (*B* and *C*) Cells were prepared as in Fig. 1*B* (initial plating density = 2500 or 2000 cells per coverslip, respectively). After 3 days in DMEM/BS, oligodendrocytes were switched to DMEM alone to which was added IGF-I (*B*) or CNTF (*C*) with or without 1 mM NAC. NAC alone did not promote cell survival, but the presence of 1 mM NAC was associated with highly significant (**) increases in the extent of survival observed with either CNTF or IGF-I applied by itself. Values shown are means ± SEM for quadruplicate coverslips from one of three experiments, all of which yielded similar results. (*D*) Cells were prepared as above, except that the initial plating density was 1000 cells per coverslip, and cultures of pure oligodendrocytes were switched to DMEM alone containing 0.5 ng of CNTF per ml (a concentration that did not significantly protect cells on its own; see also *C*) with or without vitamin C. The application of CNTF and vitamin C together was associated with highly significant (**) increases in the extent of survival observed (see also Fig. 4). Values shown are means ± SEM for quadruplicate coverslips from one of two experiments, both of which yielded similar results. (*E*) Experiments were conducted as above (initial plating density = 2000 cells per coverslip), but oligodendrocytes were switched to DMEM containing 0.5 ng of CNTF per ml with or without Trolox. The application of CNTF and Trolox together was associated with highly significant (**) increases in oligodendrocyte survival. Values shown are means ± SEM for five coverslips from one experiment.

also promoted highly significant levels of oligodendrocyte survival. In contrast, the combination of vitamin C plus Trolox had no such effects, indicating that NAC was acting through mechanisms at least partially distinct from those relevant to the action of these other two antioxidants.

DISCUSSION

We have found that NAC is able to promote cell survival in the two distinct experimental paradigms of death by "murder" and by "neglect." In the former case, NAC prevented the death of oligodendrocytes induced by glutamate or TNF-α and also prevented TNF-α-induced death of L929 cells. NAC also acted in synergy with CNTF to prevent killing of oligodendrocytes by TNF-α. In the analysis of death by neglect, exposure to NAC was associated with dramatic increases in the numbers of live oligodendrocytes, or spinal ganglion neurons, found in cultures exposed to suboptimal amounts of trophic factors. To our knowledge, there has been no previous demonstration of a compound that can act synergistically with established trophic factors to prevent both death induced by cytotoxic stimuli and death induced by exposure to suboptimal amounts of trophic factors. Moreover, NAC promoted significant levels of oligodendrocyte survival when applied with vitamin C, Trolox, or progesterone in the absence of any added stimulators of tyrosine kinase activity.

The spectrum of protective activity offered by NAC makes the mechanism(s) of action of this molecule particularly interesting. At present, there are two major known modes of action for NAC, (*i*) as a scavenger of free radicals and (*ii*) to augment intracellular levels of glutathione (11, 18, 20–22). Both of these antioxidative activities may contribute to protection against cell death induced by TNF-α or glutamate.

In contrast to its possible mode of action in protecting against cell death induced by toxic stimuli, the role of NAC in promoting cell survival in conditions of trophic factor deprivation may be more complex. Our experiments revealed three different combinations of factors that can prevent cell death in this situation: (*i*) the combination of known trophic factors with NAC, vitamin C, or Trolox, (*ii*) the combination

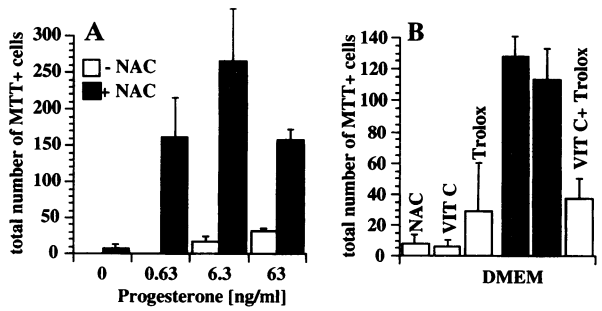

FIG. 4. Oligodendrocytes were prepared as in Fig. 3 *B–E* (initial plating density = 2500 cells per coverslip) and switched to DMEM alone to which was added progesterone with or without 1 mM NAC (*A*) or vitamin C or Trolox with or without 1 mM NAC (*B*). Progesterone promoted oligodendrocyte survival when applied in combination with NAC, vitamin C, or Trolox, and the combination of 1 mM NAC with either vitamin C or Trolox was also associated with highly significant increases (**) in cell survival. Values shown are means ± SEM for quadruplicate coverslips from one of two (*A*) or three (*B*) experiments, all of which yielded similar results.

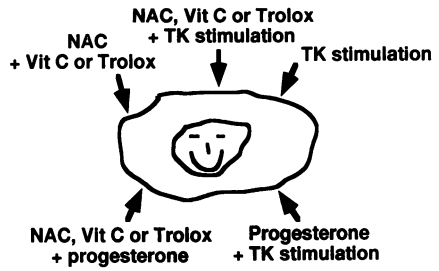

FIG. 5. Summary of pathways that can prevent cell death associated with exposure to suboptimal concentrations of trophic factors. Vit C, vitamin C; TK, tyrosine kinase.

of progesterone with NAC, vitamin C, or Trolox, and (*iii*) the combination of NAC with vitamin C or Trolox. It was previously known that a variety of stimulators of tyrosine kinase activity will promote cell survival (whether applied alone or, with greater effect, in combination; refs. 4, 30, and 40) and that progesterone enhances the extent of cell survival obtained with stimulators of tyrosine kinase activity (see materials and methods of ref. 42). Thus, there are at least five combinations of molecules involved in normal aspects of cellular regulation that can be applied to cells to prevent cell death associated with growth factor deprivation (Fig. 5), suggesting that multiple pathways can contribute to the prevention of apoptosis in this situation; strikingly, it seems that some of these pathways promote survival without requiring addition of agonists for receptor tyrosine kinases or receptors associated with tyrosine kinases. That such a variety of exogenous stimuli can prevent apoptosis raises the question of whether there is a similar multiplicity of intracellular metabolic pathways functioning in causing this process. Further, it seems to us to be of particular interest to determine why NAC is so singularly effective at enhancing cell survival in all of the paradigms we have analyzed.

The identification of pharmaceutical compounds that promote cell survival may be of use in understanding the cellular mechanisms that prevent cell death and may also be of therapeutic utility. Our present data suggest that NAC expresses *in vitro* many of the characteristics of such a potentially useful compound. NAC is also known to be effective *in vivo*—for example, in blockade of acute TNF-α toxicity in rats (50)—and has already been used safely in humans for >30 years (21, 22, 25–27). Thus, we suggest that NAC may be a promising candidate for examination in conditions where cell death by apoptosis or oxidative stress is involved in the manifestation of disease.

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