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Journal of Toxicology and Environmental Health, Part A
Current Issues

ISSN: 0098-4108 (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/uteh19

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To cite this article: T. G. Ma , Y. H. Ling , G. D. McClure & M. T. Tseng (1990) Effects of trifluoroacetic acid, a halothane metabolite, on C6 glioma cells, Journal of Toxicology and Environmental Health, Part A Current Issues, 31:2, 147-158, DOI: 10.1080/15287399009531444

To link to this article: https://doi.org/10.1080/15287399009531444

Published online: 20 Oct 2009.

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EFFECTS OF TRIFLUOROACETIC ACID, A HALOTHANE METABOLITE, ON C6 GLIOMA CELLS

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Effects of trifluoroacetic acid (TFA) on cell growth, DNA, glycoprotein, and dolichol-linked oligosaccharides synthesis and ribonucleotide triphosphate concentrations were examined in exponentially growing C6 murine glioma cells. One day of treatment with TFA caused a slight concentration-dependent enhancement of cell growth and [^{3}H]thymidine incorporation. Exposure for 1 or 5 d to TFA (0.5–7.0 mM) elevated the [^{3}H]leucine incorporation in a dose- and time-dependent manner. The results suggested that TFA stimulated cell growth and enhanced protein synthesis. TFA also affected [^{3}H]mannose incorporation into glycoproteins and dolichol-linked oligosaccharides in a dose-dependent fashion. In addition, it was found that TFA accelerated lectine-induced cell agglutination. These data suggest that TFA, the principle halothane metabolite, alters plasmalemmal glycoprotein synthesis. These findings should form a basis for further understanding on the mechanism underlying halothane-associated neurotoxicity.

INTRODUCTION

Halothane, 2-bromo-2-chloro-1,1,1-trifluoroethane, is an inhalant anesthetic used frequently for pediatric surgery. Disadvantages of its use include the occurrence of hepatitis or malignant hyperthermia in some individuals (Britt and Gordon, 1969; Farrell et al., 1985). Young rats exposed chronically to a low dose of halothane developed CNS structural abnormalities and functional deficits (Chang et al., 1974, 1976; Quimby et al., 1974). Subsequently, Wiggins et al. (1979) and Patsalos et al. (1980) reported altered myelin synthesis in postnatal rats exposed to halothane. A recent study on 2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) suggested a link between this enzyme and hypomyelination (Nagelhout et al., 1986). The recurrent concern about neurotoxicity in individuals exposed chronically to measurable concentrations of halothane gave the impetus for the current study.

The oxidative and reductive metabolism of halothane is accomplished primarily in the liver (Baden and Rice, 1986). Trifluoroacetic acid (TFA) is the principle hepatic oxidative metabolite. Because of the halogen attachment to the two-carbon chain, TFA is very stable. TFA concen-

Supported in part by NIOSH grant 5RO3-OH02578-02.

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Journal of Toxicology and Environmental Health, 31:147–158, 1990

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trations in serum, liver, and kidney reached their peak between 5 and 16 h (Eckes and Buch, 1985). Acute toxicity of this compound in experimental animals is low; LD_{50} in mice is in excess of 2000 mg/kg (Airaksinen and Tammisto, 1968). Nevertheless, lesions in fetal liver and kidney as well as skeletal malformations in rodents were observed in TFA-treated rodents (Wharton et al., 1979). In human, TFA affects binding of drugs to albumin and induces changes in fat and carbohydrate metabolism (Dale, 1985). The peak plasma bromide concentration, another key metabolite, occurred between 48 and 72 h following halothane anesthesia (Tinker et al., 1976). Thus far, a possible link between TFA and neurotoxicity of the halogenated alkanes has not been reported. The present study was undertaken to determine the impact of TFA on the growth, protein synthesis, and glycosylation of a murine glioma cell, C6, in vitro.

MATERIALS AND METHODS

Chemicals

Lectins [concanavalin A (Con A), *Phaseolus vulgaris* agglutinin, *Ricinus communnis* agglutinin (RCA_{120}), *Triticum vulgaris* agglutinin (WGA)], RPMI 1640 tissue culture medium, fetal bovine serum, and trifluoroacetic acid were purchased from Sigma Chemical Co. (St. Louis, Mo.). [6-^{3}H]Mannose (30 Ci/mmol), [4,5-^{3}H]-L-leucine (40 Ci/mmol), [2-^{3}]H-glucosamine (25 Ci/mmol), and [methyl-^{3}H]thymidine were purchased from ICN Radiochemical Inc. (Irving, Calif.). All other chemicals were reagent grade, obtained from commercial suppliers.

Cell Maintenance and Treatment

C6 glioma cells obtained from ATCC (Bethesda, Md.) were maintained as a monolayer with RPMI 1640 supplemented with 5% fetal bovine serum and antibiotics in a humidified CO_2 incubator at 37°C. After 1 d in a 24-well culture dish at a density of 1×10^5 cell/ml, cells were exposed to TFA (0–10 m*M*) for durations up to 5 d. At set intervals, cells were rinsed twice with phosphate-buffered saline (PBS : 50 m*M* Na_2HPO_4, 50 m*M* KH_2PO_4, 145 m*M* NaCl, pH 7.4) before removal by a brief trypsin treatment. Cell number was determined in a Coulter counter (model Zf, Coulter Electronics, Hialeah, Fla.).

Analysis of Ribonucleotide Triphosphate Concentration

After a 24-h incubation, subconfluent C6 cells were TFA exposed for 1 d, washed twice with PBS, and scraped with a rubber policeman. The harvested cells were extracted with cold 0.8 *N* perchloric acid (PCA) for 30 min at 4°C, and the supernant was neutralized with 10 *N* KOH. The extract was fractionated in a Partisil-10 SAX anion-exchange column at

ambient temperature. A linear gradient from 5 m*M* $(NH_4)H_2PO_4$ to 750 m*M* $(NH_4)H_2PO_4$ (pH 3.7) was used. The flow rate was 2 ml/min, and the eluted materials were monitored by a UV detector at an absorbance of 254 nm. The amount of each ribonucleotide triphosphate was determined and calculated with a Laboratory Data Control MP300E system.

Precursor Incorporation into DNA and Protein

After 1 or 5 d of continuous TFA exposure, C6 cells were pulse labeled with 1.0 μCi/ml of $[^3H]$thymidine or $[^3H]$leucine for 1 h, washed 3 times with PBS, scraped from the dishes, and precipitated in cold 10% trichloride acetate (TCA) for 10 min. The TCA-insoluble materials, representing the incorporated thymidine or leucine, were twice rinsed with 5% TCA and trapped on GF/C Whatman filters. The radioactivity in the filter was determined in a liquid scintillation counter.

Synthesis of Glycoproteins and Dolichol-Linked Oligosaccharide

Mannose and glucosamine incorporation into glycoproteins and dolichol-linked oligosaccharide were determined as previous described (Ling et al., 1989). Briefly, cells were TFA treated for 22 h, and incubated with 2.5 μCi/ml of $[^3H]$glucosamine in glucose-free RPMI-1640 medium for 2 h before being washed with PBS. The dolichol-linked oligosaccharides were extracted with chloroform-methanol (2 : 1, v/v) followed by a second extraction with chloroform-methanol-water (1 : 1 : 0.3, v/v/v). Glycoproteins in the cell pellets were hydrolyzed with 1.0 *N* HCl. The radioactivity in both entities was measured as described above.

Cell Agglutination Assay

Exponentially growing C6 cells were exposed to TFA (0–15 m*M*) for 24 h. Cells were scraped from the flask, twice washed with Ca^{2+}, Mg^{2+}-free PBS, and suspended in the same buffer as a concentration of 5×10^6 cells /ml at 4°C. Cell agglutination was measured as described by Hwang et al. (1974). Briefly, a 1.2 ml aliquot of the suspended cells was added to each cuvette, followed by the addition of the lectins (0, 25, or 50 μg). The contents were mixed rapidly by four consecutive inversions. Cuvettes were placed immediately in the chamber of a Gilford Response II spectrophotometer to measure the rate of change of absorbance at 546 nm.

RESULTS

Effects of TFA on Cell Growth

One day exposure to TFA caused a slight concentration-dependent enhancement of cell growth. At 8.38 m*M*, a 28% increase was observed

(Fig. 1). Continual exposure to d 5 resulted in further increase in cell number. However, the relative increase in cell growth for this chronic exposure group, in fact, declined (data not shown).

Effects of TFA on DNA and Protein Synthesis

Exposure to TFA for 1 or 5 d increased the leucine incorporation in C6 cells. The increase in leucine incorporation was dose- and time-dependent. The highest level (216%) was recorded in those treated for 5 d (Fig. 2). A concomitant dose-dependent increase in thymidine incorporation was also found (Fig. 3).

Effects of TFA on Ribonucleotide Triphosphate Concentration

Cellular contents of four ribonucleotide triphosphates were determined and results are presented in Table 1. The high dose of TFA (10 m*M*) suppressed the intracellular ATP, GTP, and CTP ($p < .05$) pool. At lower concentrations TFA elevated ATP levels 11–15%, while it exerted no influence on UTP or CTP. In contrast, intracellular GTP concentration declined in a dose-dependent fashion.

Effects of TFA on Protein Glycosylation

The influence of TFA on mannose and glycosamine incorporation is illustrated in Figs. 4 and 5. Both the dolichol-liked oligosaccharides and glycoprotein synthesis were affected. After 1 d of TFA exposure at 5 m*M* concentration, the rate of [^{3}H]mannose incorporation into glycoproteins and dolichol-linked oligosaccharide was reduced to 71 and 39% of the control, respectively. Lengthening the TFA exposure to 5 d caused further decline in mannose incorporation (53% of control, $p < .05$), but imparted an additional effect on the dolichol-linked oligosaccharides (Table 2). The TFA effect on the glucosamine incorportaion was different. No significant decline in [^{3}H]glucosamine incorporation in glycoprotein and dolichol-linked oligosaccharide was observed, although 13–23% reduction in their incorporation into dolichol-linked oligosaccharide was found in 1-d TFA treatment samples (Fig. 5).

Effects of TFA on Lectin-Induced Cell Agglutination

The treatment of C6 cells with 5 and 15 m*M* TFA for 24 h resulted in accelerated lectin-induced agglutination. Among the lectins tested, only RCA_{120} consistently enhanced cell agglutination. The effect was not found in Con A, RCA_{120}, WGA, or *Phaseolus vulgaris* agglutinin (data not shown). RCA_{120}-induced surface change was dose-dependent between

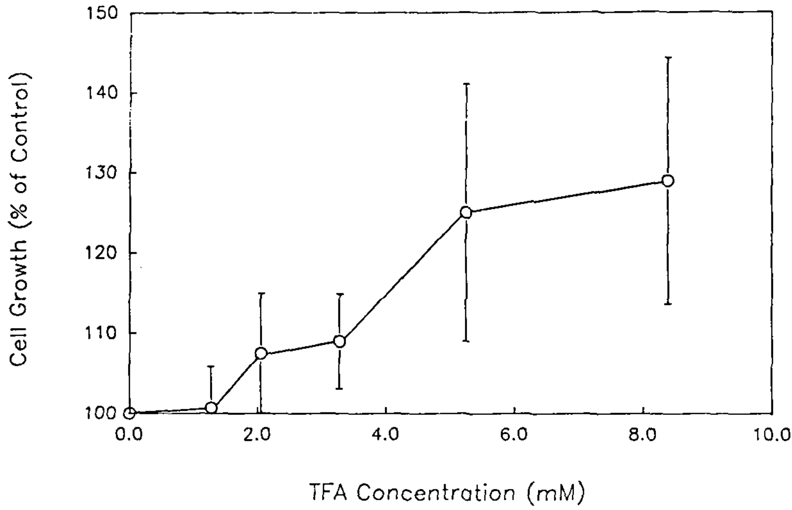

FIGURE 1. Effect of TFA on C6 cell growth. Cells were seeded at a density of 1×10^5 cells/well for 24 h, and exposed to 1.28 m*M* TFA for 22 h. Each value represents the mean of results obtained from triplicate samples of four separate experiments.

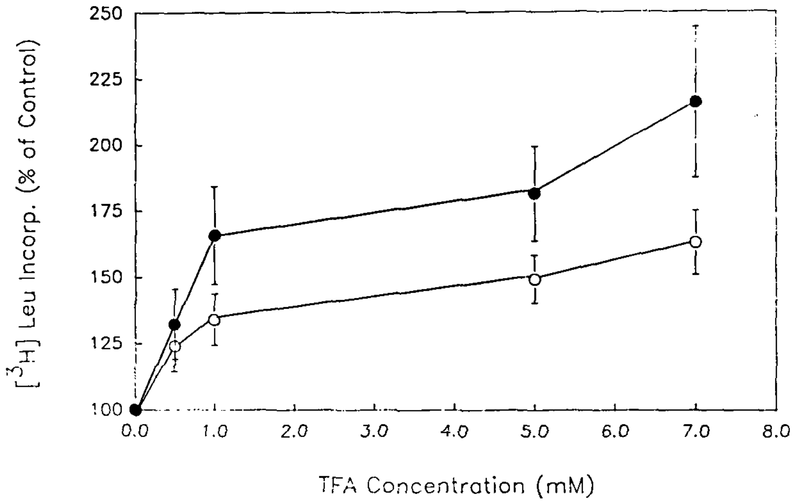

FIGURE 2. Effect of TFA on [^{3}H]leucine incorporation. C6 cells were exposed to various concentrations of TFA for 1 d (O) or 5 d (●) and pulse labeled with 1.0 μCi/ml of [^{3}H]leucine for 1 h. Cells were PBS washed, collected by filtration, and the radioactivity therein was measured. Each point represents the mean of triplicate samples from four separate experiments.

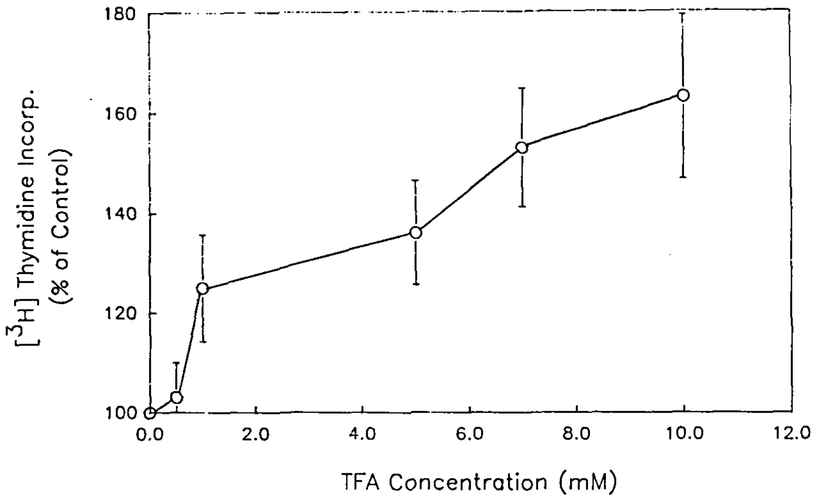

FIGURE 3. Effect of TFA on [^{3}H]thymidine incorporation. C6 cells were exposed to various concentrations of TFA for 1 d and pulse labeled with 1.0 μCi/ml of [^{3}H]thymidine for 1 h. The radioactivity in cells was measured as described in Fig. 2. Each point represents the mean of triplicate samples from four separate experiments.

25 and 50 μg/1.2 ml. Within a span of 12 min, more than 50% of the cell became agglutinated with a combined treatment of 15 m*M* TFA and 50 μg/1.2 ml RCA_{120} (Fig. 6). The effect of TFA on RCA_{120}-induced C6 cell agglutination was dose dependent at 25 and 50 μg/1.2 ml of RCA (Fig. 6).

DISCUSSION

The toxic effects of TFA are poorly understood. Early studies that suggested a low degree of TFA toxicity in experimental animals in-

TABLE 1. Effects of TFA on Ribonucleotide Triphosphates[a]

TFA (m*M*)	Ribonucleotide triphosphate (% of control)			
	ATP	GTP	UTP	CTP
1	111 ± 29	93 ± 9	96 ± 17	99 ± 33
5	115 ± 23	89 ± 11	102 ± 18	106 ± 21
10	80 ± 16	73 ± 16[b]	99 ± 41	62 ± 21[b]

[a]C6 cells were exposed to various concentrations of TFA for 1 d. The cells were extracted with PCA and centrifuged. The supernatant was fractionated in a Partisil-10 SAX anion exchange column and eluted with $NH_4H_2PO_4$ linear gradient. The eluted materials were read by a UV detector at an absorbance of 254 nm.

[b]Significant at $p < .05$.

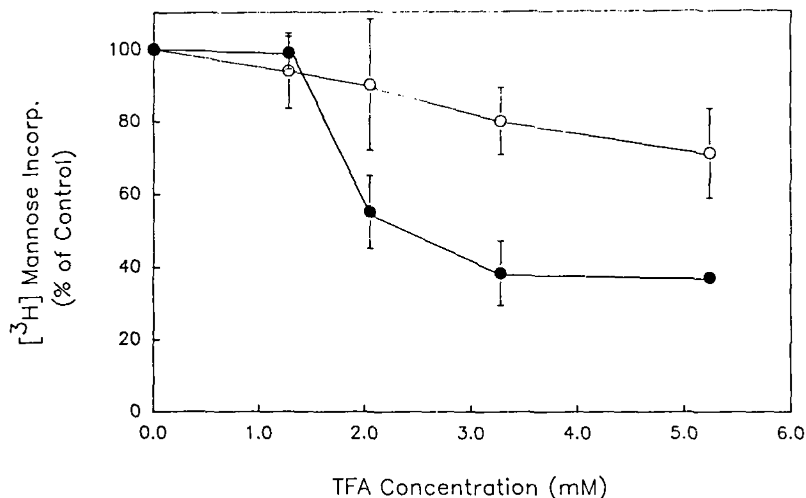

FIGURE 4. Effect of TFA on [^{3}H]mannose incorporation into glycoproteins and dolichol-linked oligosaccharides. C6 cells were treated with varying concentrations of TFA for 22 h and labeled with 2.5 μCi [^{3}H]mannose for 2 h. Cells were washed and extracted. The radioactivity in glycoproteins (O) and dolichol-linked oligosaccharides (●) was measured as described in Materials and Methods. Each point represents the mean of triplicate samples from four separate experiments.

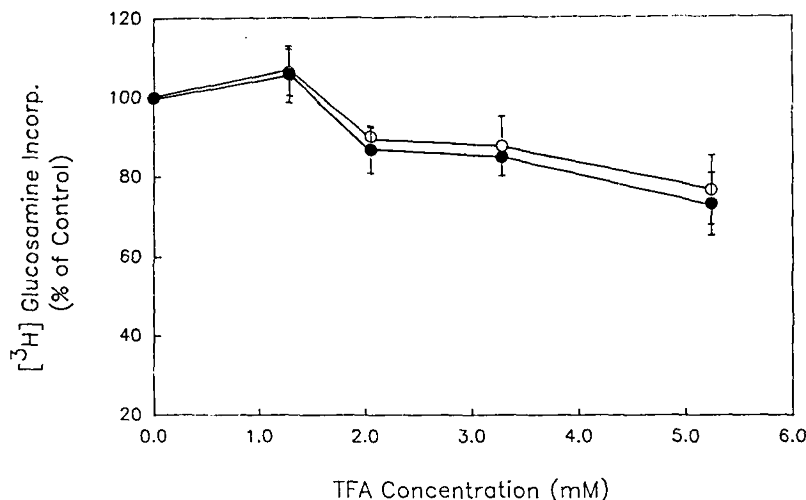

FIGURE 5. Effect of TFA on [^{3}H]glucosamine incorporation into glycoproteins and dolichol-linked oligosaccharides. C6 cells were treated with TFA as described in Fig. 4 and labeled with 5.0 μCi [^{3}H]glucosamine. Radioactivity in glycoproteins (O) and dolichol-linked oligosaccharides (●) was measured as described in Fig. 4. Each point represents the mean of quadruplicate samples from two separate experiments.

TABLE 2. Effects of TFA on [^{3}H]Mannose Incorporation into Glycoproteins and Dolichol-Linked Oligosaccharides[a]

TFA (mM)	[^{3}H]Mannose Incorporation (% of control) Glycoprotein	Dolichol-linked oligosaccharides
1	81 ± 32	93 ± 18
5	53 ± 31[b]	70 ± 8[b]
10	45 ± 17[c]	70 ± 18

[a]C6 cells were exposed to various concentrations of TFA for 5 d, then labeled with [^{3}H]mannose, washed and extracted as described in Fig. 4. The radioactivity in glycoproteins and dolichol-linked oligosaccharides was measured. Each value is the mean of three separate experiments.
[b]Significant at $p < .05$.
[c]Significant at $p < .01$.

cluded a slight elevation of ATP in liver and selected muscles (Airaksinen and Tammisto, 1968), inhibition of glycolysis in rats intraperitoneally injected with TFA (Rosenberg et al., 1970), inhibition of creatine phosphokinase (Airaksinen et al., 1970), and declined levels of carbohydrate metabolism intermediates such as the lactate, pyruvate, glycero-1-phosphate, glucose 6-phosphate, glucose, and glycogen (Stier et al., 1972). Collectively these data suggest that TFA affects metabolism of the liver, but they do not account directly for the specific effects on the CNS. In this study the effect of TFA on glial components of the CNS was examined. Even though the in vitro TFA concentration exceeded that of the in vivo levels reported in the rodent (Eckes and Buch, 1985), the relatively mild impact of TFA on C6 cells is consistent with tissue responses of nonneuronal origin. The results indicated that TFA stimulated cell growth, enhanced protein synthesis, but did not affect significantly levels of ATP. The latter suggests that TFA only minimally affected the cell's energy metabolism.

The enhanced leucine uptake, besides indicating a generalized protein synthesis, could signify an altered energy requirement for these cells. Since leucine can be catabolized to acetoacetate and acetyl-coenzyme A, its uptake may help to ease the possible decrease in acetyl-coenzyme A. Furthermore, an intermediate in the catabolism of leucine is 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA). HMGCoA could give rise to mevalonate, which is necessary for the induction of CNPase in oligodendrocytes (Langan and Volpe, 1987). Others found that conversion of HMGCoA to mevalonate by the enzyme HMGCoA reductase depends upon the integrity of the enzyme's sulfhydroyl group (Roitelman and Schechter, 1986). An altered regula-

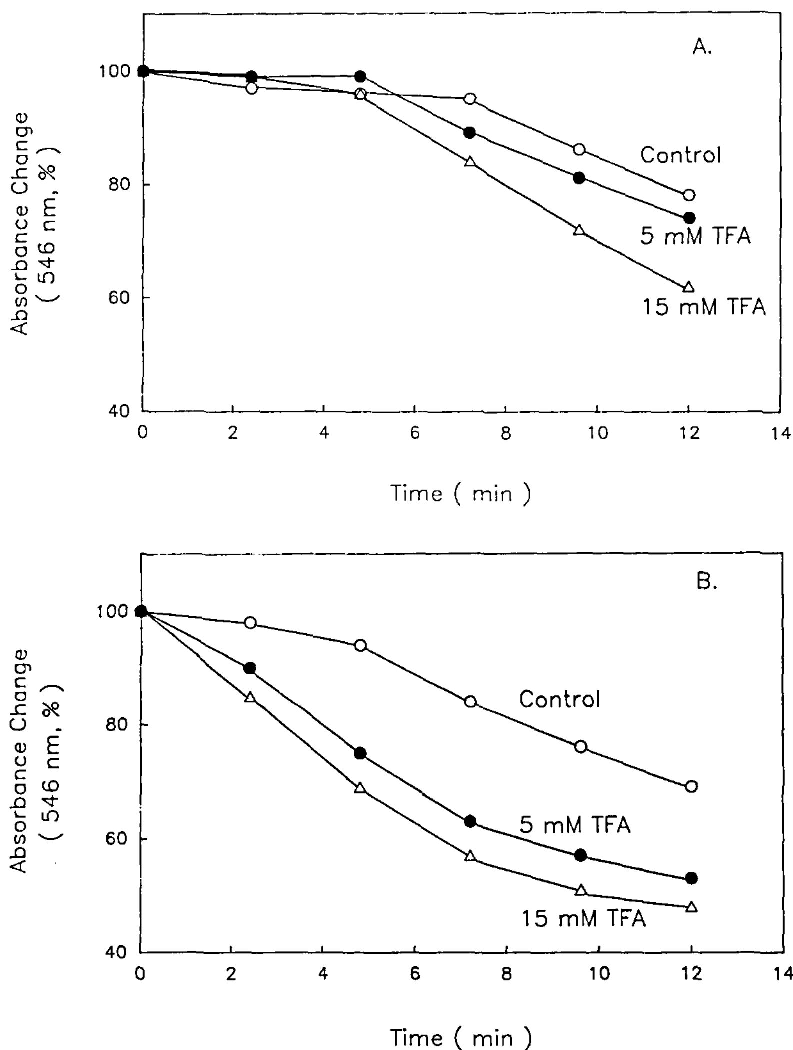

FIGURE 6. Effect of TFA on RCA-induced C6 cells agglutination. C6 cells were respectively exposed to 0 (○), 5 (●) and 15 m*M* (△) of TFA for 1 d. Cells were scraped from the flask and suspended in Ca^{2+}, Mg^{2+}-free PBS. A 1.2-ml aliquot of the suspended cells was added to the cuvette, followed by the addition of RCA_{120} at (*a*) 25 μg or (*b*) 50 μg. The change of absorbance at 546 nm was measured.

tion of HMGCoA reductase would then affect not only cholesterol biosynthesis but also the expression of CNPase, and could account for the in vivo deficit of myelination and the decrease in CNPase specific activity after exposure to halothane.

Protein carbohydrate complexes, a component of the plasmalemma, play a role in regulating cell growth, recognition, and differentiation

(Jain, 1988; Hughes, 1976). Modification of the glycoprotein structure can profoundly affect the functionality of the cell. Examples of such a change have been noted in membranes of transformed cells (Stanley et al., 1980) and in xenobiotic-treated cells (Metcalf et al., 1987; Hernandez et al., 1987). The observed dose- and time-dependent decrease in [^{3}H]mannose incorporation into glycoprotein and dolichol-linked oligosaccharide in TFA-treated C6 cells demonstrated that synthesis of glycoprotein was affected by TFA, since mannose comprises the internal core of many oligosaccharide chains. Early studies indicated that transformation of a mannose-rich precursor, coupled with a cleavage of several terminal mannose units and the insertion of additional mannose units, leads to major changes in the structure and function of the cellular glycoproteins (Robbins et al., 1977; Lazo et al., 1979). The suppression of glycoprotein synthesis may be specific, since TFA treatment induced cellular proliferation with elevated leucine incorporation but not glycoprotein synthesis. Among the ribonucleotide triphosphates assayed, TFA suppressed only GTP in a dose-dependent fashion. GTP is essential for the synthesis of mannosyl-containing glycoproteins. It is possible that guanosine sugar nucleotide activation is acutely sensitive to TFA in C6 cells and that the conversion of inosinic acid to xanthylic acid or some other biosynthesis pathway of guanine nucleotides is affected specifically. Further study is needed to delineate the possible relationship between TFA and depletion of inosine monophosphate (IMP) dehydrogenase activity.

Perhaps the most direct evidence supporting TFA-induced membrane change came from the lectin-induced cell agglutination study. The loss of terminal sialyl residues in TFA-treated cells could lead to an increase in the amount of terminally exposed galactose residues. Such surfaces bind more readily to RCA, and hence the cells became more sensitive to the treatment of this particular lectin. Additional examination of the possible relationship between the altered glycoprotein synthesis in TFA-treated cells and myelin deficiency-related disorders (Hogan and Greenfield, 1984) is necessary to further the understanding of halothane-induced neurotoxicity.

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Received July 19, 1989
Accepted April 5, 1990