# U.S. ARMY MEDICAL RESEARCH INSTITUTE OF CHEMICAL DEFENSE

## USAMRICD-TR-03-07

# Optimization of Glial Fibrillary Acidic Protein Immunoreactivity in Formalin-fixed, Paraffin-embedded Guinea Pig Brain Sections

Christina P. Tompkins
Tracey A. Hamilton
John P. Petrali
Robert K. Kan

September 2003

Approved for public release; distribution unlimited

U.S. Army Medical Research
Institute of Chemical Defense
Aberdeen Proving Ground, MD 21010-5400

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

| 1. REPORT DATE *(DD-MM-YYYY)* | 2. REPORT TYPE | 3. DATES COVERED *(From - To)* |
|---|---|---|
| September 2003 | Technical Report | January 2003 to June 2003 |

**4. TITLE AND SUBTITLE**
Optimization of Glial Fibrillary Acidic Protein Immunoreactivity in Formalin-fixed, Paraffin-embedded Guinea Pig Brain Sections

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**

**5c. PROGRAM ELEMENT NUMBER**
61384

**6. AUTHOR(S)**
Tompkins, CP, Hamilton, TA, Petrali, JP, and Kan, RK

**5d. PROJECT NUMBER**
TC1

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-UV-CC
3100 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

**8. PERFORMING ORGANIZATION REPORT NUMBER**
USAMRICD-TR-03-07

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

US Army Medical Research Institute of Institute of Chemical Defense
ATTN: MCMR-UV-RC
3100 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for public release; distribution unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**
Glial fibrillary acidic protein (GFAP) is an astrocyte-specific intermediate filament whose expression has been shown to be a sensitive marker of toxin-induced brain injury. The present study was designed to employ microwave-assisted antigen retrieval to optimize GFAP immunostaining in formalin-fixed, paraffin-embedded guinea pig brain sections using a variety of commercially available GFAP antibody clones. Of the 7 clones tested for cross-reactivity, following microwave antigen retrieval, mouse clone GA-5 from NeoMarkers was found to be the most responsive, yielding specific GFAP staining in both astrocytic cell bodies and processes. Our observations indicate that different antibody clones must be examined to obtain superior immunolocalization of epitopes uncovered by microwave antigen retrieval. This finding undoubtedly will have important applications in our efforts aimed at determining neuropathological consequences in the guinea pig following exposure to chemical warfare nerve agent.

**15. SUBJECT TERMS**
GFAP, astrocytes, immunohistochemistry, formalin fixation, and paraffin embedding

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | UNLIMITED | 13 | John P. Petrali |
| UNCLASSIFIED | UNCLASSIFIED | UNCLASSIFIED | | | 19b. TELEPHONE NUMBER *(include area code)* 410-436-2334 |

## ACKNOWLEDGEMENTS

The authors acknowledge branch chief MAJ J. Scot Estep and former division chief LTC Roxanne Baumgartner. The first author would also like to acknowledge the U.S. Army Medical Research Institute of Chemical Defense for providing additional support of this research through Battelle.

## 1. INTRODUCTION

Astrocytes are supporting cells in the brain that maintain the functional capacity of neurons. Following injury to the brain, as a result of trauma, disease, genetic disorders, or chemical insult, astrocytes become reactive (Eng et al., 2000). Reactive astrocytosis, also known as astrogliosis, is morphologically characterized by extensive proliferation and hypertrophy of astrocytes and their cytoplasmic processes in and beyond the site of injury (Eng and Ghirnikar, 1994). Biochemically, the hallmark of astrogliosis is the rapid synthesis and accumulation of glial fibrillary acidic protein (GFAP), a major component of glial intermediate filaments (Reier, 1986; Eng, 1985).

GFAP was found to be a sensitive marker of neurotoxicity associated with exposure to high (symptomatic) doses of chemical warfare nerve agents (Baille-Le Crom et al., 1995; Zimmer et al., 1997, 1998). In this regard, immunodetection of GFAP is ideally suited for studying the pathological consequences of repeated exposures to low-dose chemical warfare nerve agent. The present study was designed to develop optimal GFAP immunohistochemical staining on formalin-fixed, paraffin-embedded guinea pig brain sections using microwave-assisted antigen retrieval (MAR) technique. Once established the procedure will be incorporated into studies confirming neuropathology observed with microtubule-associated protein 2 (MAP-2) immunostaining, which was consistently found to be elevated in the CA-2 sub-region of the hippocampus following repeated, daily VX injection.

## 2. MATERIALS AND METHODS

### *2.1 Tissue Preparation*

Routinely formalin-fixed (10% neutral phosphate buffered formalin for 18 hr) guinea pig brains were sectioned coronally in a guinea pig brain matrix (ASI Instruments, Warren, MI). Brain samples were processed in paraffin, sectioned serially at 5μm, and mounted on positively charged slides (Fisher Scientific, Pittsburgh, PA). Brain sections were allowed to dry at room temperature for 24 hr and then processed for MAR immunohistochemistry.

### *2.2 Microwave Pretreatment*

Following dewaxing in xylene and hydration to distilled water, sections were incubated in 5% hydrogen peroxide for 15 min at room temperature to suppress endogenous peroxidase activity. Sections were then rinsed in running tap water and washed thoroughly with distilled water. Ten mM citric acid monohydrate (Sigma-Aldrich; St Louis, MO; Lot 30H-0627) was used as an antigen retrieval solution. Citric acid solution was prepared according to formula (.21g/100ml) and adjusted to pH 6.0 by adding 2N NaOH, while monitoring with a pH meter (Beckman Instruments, Fullerton, CA). MAR procedure was performed as described in USAMRICD-TR-02-06 (Pleva et al., 2002). Sections were boiled in the microwave oven (Pelco 3440 Max, Ted Pella, Inc., Redding, CA) in plastic Coplin jars for two 5-min cycles, with the power of the

microwave was set at 100%. Each cycle was broken into two equal time periods of 2.5 min so that more AR solution could be added to compensate for loss due to boiling over and to avoid drying out the tissue sections. Following two cycles of boiling in the microwave for a total time of 10 min, sections were allowed to cool at room temperature for a minimum of 20 min prior to processing for GFAP immunohistochemistry.

### *2.3 GFAP Immunohistochemistry*

Indirect GFAP immunohistochemistry was performed using the avidin-biotin-peroxidase complex (ABC) method of Hsu et al. (1981). Brain sections, rinsed twice in phosphate buffered saline (PBS), pH 7.4 (Sigma-Aldrich, St. Louis, MO; Lot 12K8203), were incubated in 5% normal serum derived from the host for the secondary antibody for 30 min at 4°C to block non-specific binding sites of tissue immunoglobulins to secondary antibody. Sections were then incubated with GFAP antibody for 18 hr at 4°C. The specific clones of GFAP antibodies and their dilution are summarized in Table 1. Following two washes with PBS, sections were incubated with biotinylated secondary antibody (1:200 dilution) (Vector, Burlingame, CA) for 1 hr at room temperature, washed twice with PBS, and allowed to react with ABC reagent (Vector, Burlingame, CA) for 30 min at room temperature. The presence of GFAP immunoreactivity was visualized as a brown precipitate after incubating sections in DAB-$H_2O_2$ solution (Sigma-Fast DAB tablet sets, Sigma-Aldrich, St. Louis, MO) for 5 min. The tablets were dissolved in 6ml of distilled water instead of 5ml as recommended by the manufacturer, a modification made to attenuate the rate of peroxidase-DAB-$H_2O_2$ reaction. Finally, sections were counterstained with 0.8% cresyl violet acetate for morphology and topography and mounted with Permount® (Fisher Scientific, Fair Lawn, NJ) for light microscopic examination. Negative control sections were treated in an identical manner except that incubation in primary antibody or microwave pretreatment was omitted. Human sections were used as positive controls, since all antibodies were generated against human GFAP.

Table I. Monoclonal mouse and polyclonal rabbit GFAP antibodies used. Antibodies were diluted as recommended by the manufacturer.

| Clone | Manufacturer | Host | Dilution |
|---|---|---|---|
| GA-5+6F2 | NeoMarkers, Fremont, CA | Mouse | 1:100 |
| GA-5 | NeoMarkers, Fremont, CA | Mouse | 1:200 |
| G-A-5 | Sigma-Aldrich, St. Louis, MO | Mouse | 1:200 |
| GFP/6F2 | Novocastra, Newcastle upon Tyne, UK | Mouse | 1:100 |
| 4A11,1B4,2E1 | BD PharMingen, San Diego, CA | Mouse | 1:100 |
| polyclonal | Dako, Carpinteria, CA | Rabbit | 1:200 |
| polyclonal | NeoMarkers, Fremont, CA | Rabbit | 1:100 |

### *2.4 Evaluation of Immunohistochemical Staining*

Slides were examined using an Olympus BX61 microscope. Each slide was graded based on three criteria: stain intensity, stain specificity, and stain uniformity. If all three criteria were met, a grade of +++ was assigned. Likewise, a grade of ++ was assigned if two of the three criteria were met, and + if only one of the criteria was met. A -/+ was assigned if stain was weak, and a - was assigned if stain was absent. The differences in GFAP staining in the dentate gyrus of the hippocampus were photographically documented using a Spot RT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

## 3. RESULTS

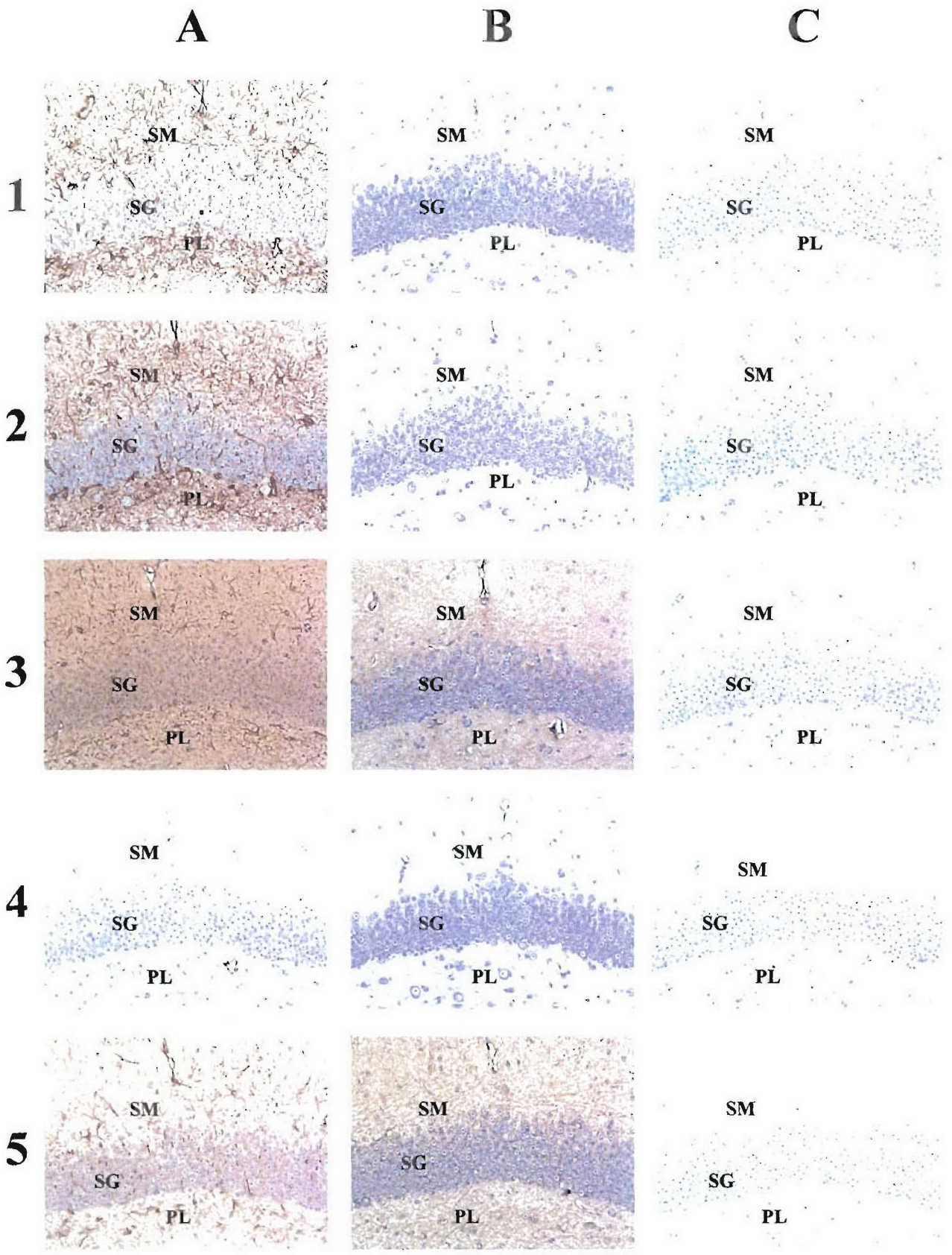
Table II summarizes the results of GFAP immunostaining obtained using five different monoclonal mouse antibodies and two different polyclonal rabbit antibodies. Generally, clones of GFAP antibody were ineffective when sections were processed without microwave pretreatment (Figure 1, Column B). However, weak GFAP immunoreactivity with high background staining was observed with clone G-A-5 from Sigma-Aldrich (Figure 1, Column B3) and clone 4A11+1B4+2E1 from BD PharMingen (Figure 1, Column B5). Sections processed with microwave pretreatment, but without primary antibody, showed no specific GFAP immunoreactivity (Figure 1, Column C).

Following MAR, the immunoreactivity of GFAP was highly influenced by the clone of the antibody used. Mouse clones GA-5+6F2 (Figure 1, Column A1) and GA-5 (Figure 1, Column A2) from NeoMarkers produced specific and intense GFAP immunoreactivity in astrocytic cell bodies and processes. Staining was less intense with rabbit polyclonal antibody from NeoMarkers (Figure 1, Column A7). Increased background staining was noted with clone 4A11+1B4+2E1 from BD PharMingen (Figure 1, Column A5) and clone G-A-5 from Sigma-Aldrich (Figure 1 Column A3). Very weak immunoreactivity was produced with clone GFP/6F2 from Novocastra (Figure 1, Column A4). No GFAP immunoreactivity was observed with polyclonal antibody from Dako (Figure 1, Column A6).

Human positive control sections were used to ensure that microwave pretreatment did not attenuate the responsiveness of the antigen to the antibody. Specific GFAP immunoreactivity without background staining was established in human positive control sections using clones GA-5+6F2 (Figure 2A) and GA-5 (Figure 2B) from NeoMarkers, clone 4A11+1B4+2E1 (Figure 2E) from BD PharMingen, clone GFP/6F2 (Figure 2D) from Novocastra, and polyclonal antibody from NeoMarkers (Figure 2G). In contrast, clone G-A-5 (Figure 2C) from Sigma-Aldrich yielded GFAP immunoreactivity with high background staining. No GFAP immunoreactivity was detected with polyclonal antibody from Dako (Figure 2F).

Table II. GFAP immunoreactivity of seven commercially available antibodies.

| Clone | Manufacturer | With AR | Without AR | Negative Control | Positive Control |
|---|---|---|---|---|---|
| GA-5+6F2 | NeoMarkers, Fremont, CA | +++ | - | - | +++ |
| GA-5 | NeoMarkers, Fremont, CA | +++ | - | - | +++ |
| G-A-5 | Sigma-Aldrich, St. Louis, MO | + | -/+ | - | + |
| GFP/6F2 | Novocastra, Newcastle upon Tyne, UK | -/+ | - | - | +++ |
| 4A11+1B4+2E1 | BD PharMingen, San Diego, CA | ++ | -/+ | - | +++ |
| polyclonal | Dako, Carpinteria, CA | - | - | - | - |
| polyclonal | NeoMarkers, Fremont, CA | ++ | - | - | +++ |


A
B
C
1
2
3
4
5
SM
SG
PL

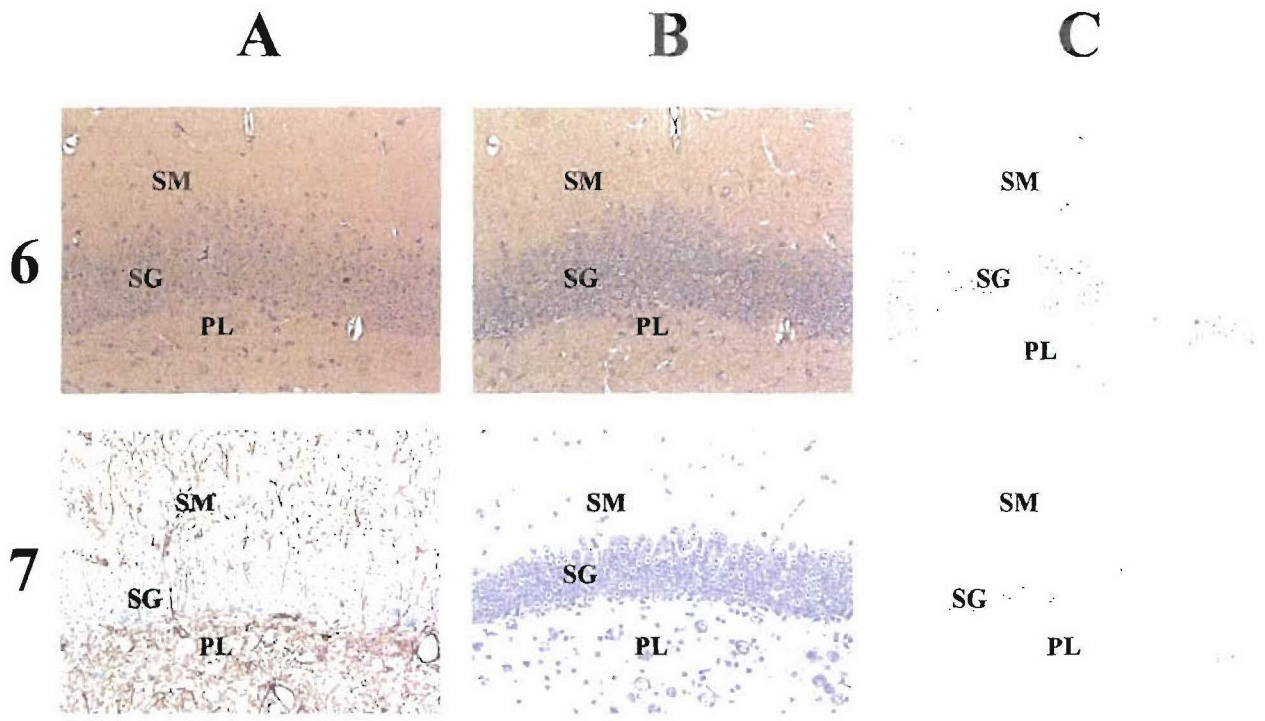



**Figure 1.** GFAP immunoreactivity in the dentate gyrus processed with microwave pretreatment (Column A), without microwave pretreatment (Column B), and without primary antibody (Column C) using clones GA-5+6F2 (1), GA-5 (2), G-A-5 (3), GFP/6F2 (4), 4A11+1B4+2E1 (5), polyclonal from Dako (6), and polyclonal from NeoMarkers (7). SM, Stratum moleculare; SG, Stratum granulosum; PL, Polymorphic layer.

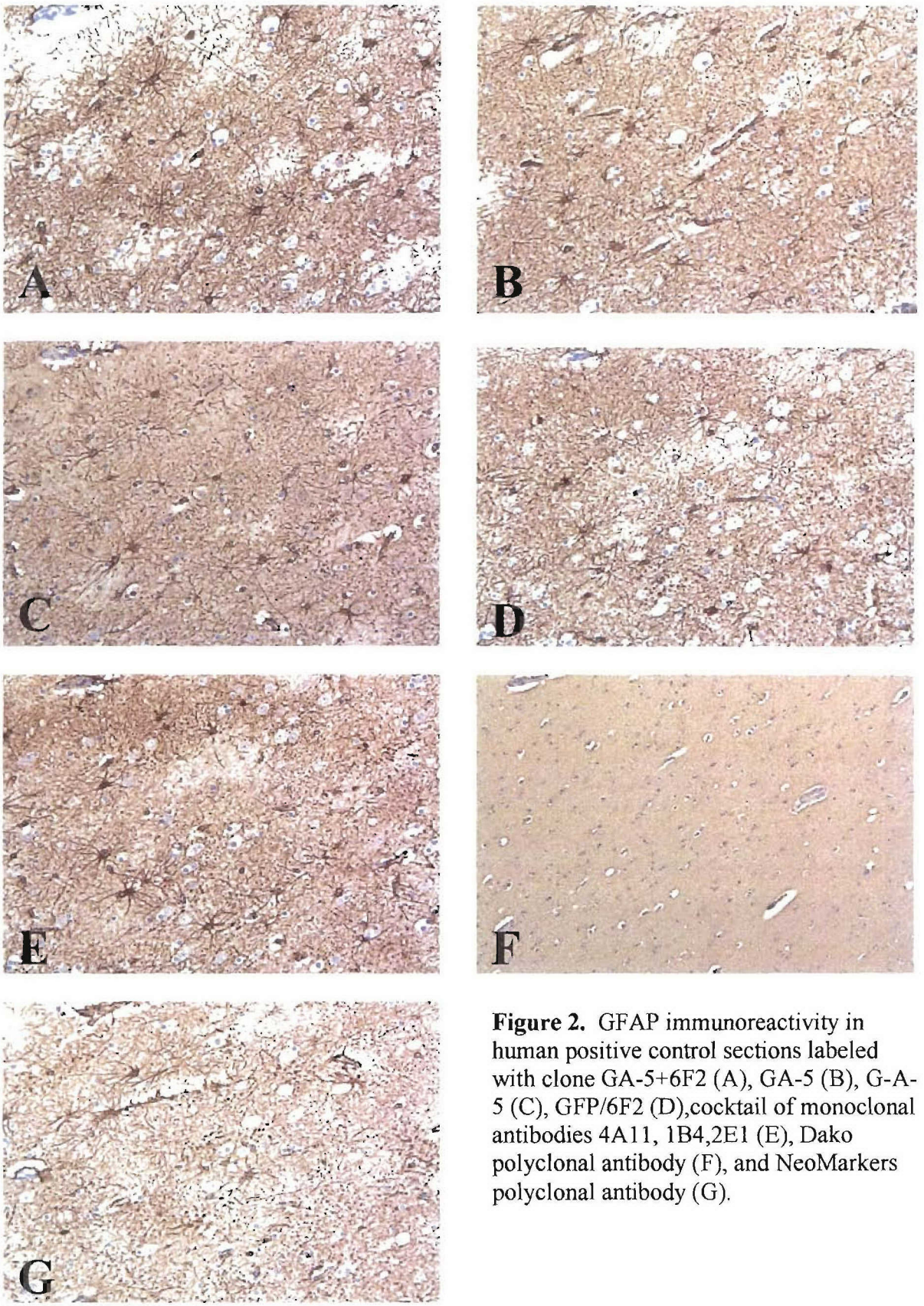



**Figure 2.** GFAP immunoreactivity in human positive control sections labeled with clone GA-5+6F2 (A), GA-5 (B), G-A-5 (C), GFP/6F2 (D),cocktail of monoclonal antibodies 4A11, 1B4,2E1 (E), Dako polyclonal antibody (F), and NeoMarkers polyclonal antibody (G).

## 4. DISCUSSION

Alterations of GFAP immunoreactivity have been used extensively as an investigative diagnostic tool to study astrocytic response following brain injury (Eng and Shiurba, 1988). An early biomarker of toxicity of the brain is an increase in GFAP staining (O'Callaghan, 1991). In this study, we utilized an established antigen retrieval method for the immunodetection of GFAP in formalin-fixed, paraffin-embedded guinea pig brain sections using seven different commercially available GFAP antibodies.

The major finding of the present study is that optimization of GFAP immunostaining following antigen retrieval is strongly influenced by the specificity and avidity of GFAP antibody clone. While clone GFP/6F2 from Novocastra produced very weak GFAP immunoreactivity, clone GA-5+6F2 from NeoMarkers yielded optimal GFAP immunostaining. Moreover, purified clone GA-5 from NeoMarkers also generated optimal GFAP immunostaining. These observations indicate that clone GA-5 is more responsive in binding to recovered GFAP epitope than are clones GFP and 6F2.

In addition to clone selection, the results indicate that the quality of antibodies from different manufacturers is also an important parameter. For example, clone G-A-5 from Sigma produced high background staining with increased nonspecific staining in neuronal perikarya, whereas clone GA-5 yielded specific GFAP staining in both astrocytic cell bodies and processes without high background staining. It is strongly recommended that when evaluating different clones of antibody, identical clones from different manufacturers be tested, as well.

## 5. CONCLUSIONS

In summary, optimal immunoreactivity of GFAP in guinea pig sections is obtainable using clones GA-5+6F2 or GA-5 in combination with microwave pretreatment. Because our results indicate that the 6F2 component of the GA-5+6F2 antibody is not cross-reactive with guinea pig, we conclude that NeoMarkers purified clone GA-5 is most efficient for use on paraffin-embedded guinea pig brain sections. This application will no doubt aid our evaluation of changes in GFAP expression in guinea pig brains following exposure to nerve agent. By using regional assessments of GFAP, it should be possible to localize areas of damage within discrete brain regions. This approach would serve as a foundation for guiding studies aimed at determining neuropathology induced by nerve agent toxicity.

## LITERATURE CITED


Baille-Le Crom, V., J.M. Collombet, P. Carpentier, G. Brochier, M.F. Burckhart, A. Foquin, I. Pernot-Marino, G. Rondouin, and G. Lallement. (1995) Early regional changes of GFAP mRNA in rat hippocampus and dentate gyrus during soman-induced seizures. NeuroReport 7:365-369.

Eng, L.F. (1985) Glial fibrillary acidic protein (GFAP): the major protein of glial intermediate filaments in differentiated astrocytes. J. Neuroimmunol. 8:203-14.

Eng, L.F., and R.S. Ghirnikar. (1994) GFAP and astrogliosis. Brain Pathol. 4:229-37.

Eng, L.F., R.S. Ghirnikar, and Y.L. Lee. (2000) Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000). Neurochem. Res. 25(9-10):1439-51.

Eng, L.F., and R.A. Shiurba. (1988) Glial fibrillary acidic protein: a review of structure, function and clinical application. In: Neuronal and Glial Proteins: Structure, Function, and Clinical Application, Vol. 2 (Marangos P.J., Campbell I., Cohen R.M., Eds.), pp. 339-59. Academic Press, New York.

Hsu, S.M., L. Raine, and H. Fanger. (1981) The use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques. A comparison between ABC and unlabeled antibody (PAP) procedure. J. Histochem. and Cytochem. 29(4): 577-80.

O'Callaghan, J.P. (1991) Assessment of neurotoxicity: use of glial fibrillary acidic protein as a biomarker. Biomed. Environ. Sci. 4: 197-206.

Pleva, C.M., T.A. Hamilton, J.P. Petrali, and R.K. Kan. (December, 2002) Determining optimal antigen retrieval conditions for microtubule-associated protein 2 immunohistochemistry in the guinea pig brain. USAMRICD-TR-02-06. US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground.

Reier, P.J. (1986) Gliosis following CNS injury: the anatomy of astrocytic scars and their influences on axonal elongation. In: Astrocytes: Cell Biology and Pathology of Astrocytes (Federoff S. and Vernadakis A., Eds.), pp. 263-324. Academic Press, New York.

Zimmer, L.A., M. Ennis, and M.T. Shipley. (1997) Soman-induced seizures rapidly activate astrocytes and microglia in discrete brain regions. J. Comp. Neurol. 378:482-492.

Zimmer, L.A., M. Ennis, R.G. Wiley, and M.T. Shipley. (1998) Nerve gas-induced seizures: role of acetylcholine in the rapid induction of Fos and glial fibrillary acidic protein in the piriform cortex. J. Neurosci. 18(10):3897-3908.