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In Vivo and In Vitro Evidence Supporting a Role for the Inflammatory Cytokine Interleukin-1 as a Driving Force in Alzheimer Pathogenesis

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Abstract

Interleukin-1 (IL-1), an inflammatory cytokine overexpressed in the neuritic plaques of Alzheimer's disease, activates astrocytes and enhances production and processing of β -amyloid precursor protein (β -APP). Activated astrocytes, overexpressing S100 β , are a prominent feature of these neuritic plaques, and the neurite growth-promoting properties of S100 β have been implicated in the formation of dystrophic neurites overexpressing β -APP in neuritic plaques. These facts collectively suggest that elevated levels of the inflammatory cytokine IL-1 drive S100 β and β -APP overexpression and dystrophic neurite formation in Alzheimer's disease. To more directly assess this driver potential for IL-1, we analyzed IL-1 induction of S100 β expression in vivo and in vitro, and of β -APP expression in vivo. Synthetic IL-1 β was injected into the right cerebral hemispheres of 13 rats. Nine additional rats were injected with phosphate-buffered saline, and seven rats served as uninjected controls. The number of astrocytes expressing detectable levels of S100 β in tissue sections from IL-1-injected brains was 1.5 fold that of either control group ($p < 0.01$), while tissue S100 β levels were approximately threefold that of controls ($p < 0.05$). The tissue levels of two β -APP isoforms (approximately 130 and 135 kDa) were also significantly elevated in IL-1-injected brains ($p < 0.05$). C6 glioma cells, treated in vitro for 24 h with either IL-1 β or IL-1 α , showed significant increases in both S100 β and S100 β mRNA levels. These results provide evidence that IL-1 upregulates both S100 β and β -APP expression, in vivo and in vitro, and support the idea that overexpression of IL-1 in Alzheimer's disease drives astrocytic overexpression of S100 β , favoring the growth of dystrophic neurites necessary for evolution of diffuse amyloid deposits into neuritic β -amyloid plaques.

Keywords

Interleukin-1; S100 β ; β -Amyloid precursor protein; Alzheimer's disease; Down's syndrome; Rats; C6 glioma cells

The global intellectual decline of Alzheimer's disease is accompanied by a profusion of microscopic brain lesions that contain extracellular fibrillar material in a β -pleated sheet

conformation (β -amyloid), abnormal neuronal processes (“dystrophic” neurites) overexpressing β -amyloid precursor protein (β -APP), and activated glia (astrocytes and microglia). The possible interactions of these plaque-associated astrocytes and microglia, their armamentarium of overexpressed cytokines and other molecules, and the known actions of these cytokines and molecules bear sufficient parallels to chronic, focal inflammatory processes found outside the nervous system to suggest that the basic driving pathophysiological mechanism in Alzheimer’s disease is fundamentally an inflammatory one (21).

The plaques of Alzheimer’s disease are thought to originate as diffuse deposits of nonpleated amyloid protein (‘diffuse nonneuritic’ plaques) that progress to more classic ‘diffuse neuritic’ plaques (commonly referred to as immature plaques) composed of β -pleated amyloid intermixed with diffuse amyloid and dystrophic neurites (24). Further amyloid condensation results in formation of a central compact core of amyloid within the larger deposit, yielding a ‘dense core neuritic’ (or mature) plaque. Brains of Alzheimer patients also contain compact deposits of β -amyloid without associated diffuse amyloid and without neuritic elements. These ‘dense core, nonneuritic’ (burned out) plaques are thought to represent an end stage of plaque evolution.

The pathophysiological mechanisms driving plaque evolution, and presumably driving the clinical progression of the disease as well, have only recently begun to be understood. Each stage in the plaque evolution cited above has a distinct and characteristic profile of associated activated microglia overexpressing the inflammatory response cytokine interleukin-1 (IL-1), as detected using anti-IL-1 α antibodies, which recognize the intracellular isoform of IL-1 α (15). These microglia are present in diffuse amyloid deposits (which are devoid of the β -APP⁺ neurites characteristic of neuritic plaques), are abundant in neuritic plaques, and are absent in burned out plaques. Moreover, the pattern of microglial IL-1 α immunoreactivity across brain regions in Alzheimer’s disease mirrors the regional distribution of these β -APP⁺ neuritic plaques (29). Microglia-derived IL-1 may thus be a driving force contributing to astrocytic actions and reactions occurring within the evolving amyloid plaques of Alzheimer’s disease.

IL-1 exists as two isoforms, α and β , that are similar in their cellular origins, regulatory patterns, and effects (22). IL-1 has been shown *in vitro*, but not *in vivo*, to upregulate expression (11,13) and processing (7) of β -APP. IL-1 also activates astrocytes (12), another integral cellular element in β -amyloid plaques. The activated astrocytes associated with the neuritic plaques of Alzheimer’s disease overexpress a neurotrophic cytokine, S100 β (16). The neurite growth-promoting properties of S100 β (18), the proximity of activated astrocytes overexpressing biologically active S100 β to the dystrophic neurites in neuritic plaques (19,28), and the correlation between the density of S100 β -immunoreactive (S100 β ⁺) astrocytes and neuritic plaques in cerebral regions in Alzheimer’s disease (32) implicates this cytokine in the growth of dystrophic neurites in β -amyloid plaques. However, the *in vivo* stimulus that effects this increased expression of S100 β and β -APP in Alzheimer’s disease remains to be identified. The close association of IL-1 α ⁺ microglia and S100 β ⁺ astrocytes in neuritic plaques, together with known IL-1 functions, suggest that IL-1 may be the *in vivo* stimulus that induces astrocytic overexpression of S100 β .

In this study, we directly tested this possibility both *in vivo* and *in vitro*. We also show, for the first time, IL-1-induced overexpression of β -APP *in vivo*. *In vivo*, IL-1 upregulation of tissue S100 β and β -APP levels was examined in rat brain following intracerebral injection of IL-1 β . *In vitro*, IL-1 β upregulation of S100 β and S100 β mRNA was assessed in C6 glioma cells.

METHOD

Animal Preparation and IL-1 Treatment In Vivo

Twenty-nine Sprague-Dawley rats, weighing 300–400 g each, were randomly divided into three groups. Thirteen rats (five for immunohistochemical analysis and eight for Western immunoblot analysis) received intracerebral injections of synthetic IL-1 β (the cleaved active isoform; Cistron, Pine Brook, NH). As controls, nine rats (three for immunohistochemical analysis and six for Western immunoblot analysis) received intracerebral injections of phosphate-buffered saline (PBS, the IL-1 vehicle; “PBS-injected controls”) and seven rats (three for immunohistochemical analysis and four for Western immunoblot analysis) received no injections (“uninjected controls”).

Rats were anesthetized with ketamine and xylazine and placed in a stereotaxic frame. A hole was drilled in the skull at a point 3.6 mm caudal to the bregma, 1.6 mm lateral to the midline. A 5- μ l Hamilton syringe equipped with a 15 μ m-diameter glass micropipette was used to make injections 1.5 mm deep to the dorsal cerebral surface. Injected rats received either 2 μ l of synthetic IL-1 β (2 U) in PBS or 2 μ l of PBS alone. After removal of the needle, Gelfoam (Upjohn, Kalamazoo, MI) was placed over the hole, and the scalp was closed. Three days later, an overdose of sodium pentobarbital was administered and brains were processed either for immunohistochemistry or for Western immunoblot analysis as described below.

Brain Tissue Processing

Brains for immunohistochemistry were perfused with 200 ml PBS followed by 200 ml of 4% paraformaldehyde. Tissue blocks were then embedded in paraffin and sectioned at a thickness of 10 μ m. Tissue sections were permeabilized with 0.2 N HCl, blocked for nonspecific antibody binding and for endogenous peroxidase, and then incubated with S100 β antibody diluted 1:2000 in 2% normal goat serum and processed according to our previously reported peroxidase/diaminobenzidine methods (17). Sections from IL-1 β -injected, PBS-injected, and uninjected rats were processed together to ensure uniformity of immunoreaction. S100 β ⁺ cells were counted at a magnification of 250 diameters in five microscopic fields (each field = 0.4 mm²) in dorsal cortex immediately adjacent to the injection site in each animal.

For Western immunoblot analysis, injected hemispheres (or analogous hemispheres from uninjected controls) were divided into three coronal sections. The section containing the injection site was homogenized and processed to obtain supernatant samples for determination of S100 β and β -APP, as previously described (17,27).

IL-1 Treatment In Vitro and Cell Culture Preparation and Processing

C6 rat glioma cells (American Type Culture Collection, Rockville, MD) were seeded at 3×10^6 cells in 75 ml filter-cap flasks (CoStar, Cambridge, MA), each with 25 ml of media containing Minimal Essential Medium (MEM, Gibco, Grand Island, NY), pH 7.4; 15% equine serum, and 2.5% fetal bovine serum (FBS, Hyclone Laboratories, Inc., Logan, UT). Cells were allowed to reach subconfluency (3 days), and were then treated in triplicate with 25 ml fresh media with or without IL-1 β (1 U/ml) for 1, 6, and 24 h. Triplicate sets of subconfluent sister cultures were treated for 24 h with IL-1 β or IL-1 α (1 U/ml). After treatment, the cells were trypsinized, pelleted at $1,000 \times g$ for 10 min, at 4°C, and counted. Cellular extracts for S100 β protein analysis were prepared from triplicate preparations of rinsed cell pellets (10^7 cells/100 μ l PBS), which were then frozen, thawed on ice, and sonicated (six pulses). Supernatants from $16,000 \times g$ (5 min, at 4°C) centrifugation were

analyzed for total protein as previously described (17). Relative levels of S100 β were determined by Western immunoblot analysis as previously described (17).

Rinsed cells (10^7) from parallel triplicate preparations were pelleted twice at $1,000 \times g$ (10 min, at 4°C). TriReagent™ RNA extraction reagent (Molecular Research Center, Inc., Cincinnati, OH) was added to each preparation for assay of S100 β mRNA by Northern blot hybridization as described below.

Western Immunoblot Procedures and Analysis

A specific S100 β antibody (32) that recognizes monomeric and dimeric S100 β was used. This antibody is now commercially available from East Acres Biologicals (Southbridge, MA) and SWant (Bellinzona, Switzerland). Monoclonal anti- β -APP antibody (clone 22C11), which recognizes 120–135 kDa β -APP isoforms in human brain (27), was obtained from Boehringer–Mannheim Biochemica (Indianapolis, IN). Polyclonal antiglial fibrillary acidic protein antibody (GFAP; Dako, Carpinteria, CA) was used for Western immunoblot analysis.

Western immunoblot analyses of S100 β , GFAP (28), and of β -APP were performed as previously described (17). Briefly, nitrocellulose immunoblots were incubated overnight in 10 ml of 10% nonfat dry milk, with 0.01% sodium azide, containing either β -APP antibody at a 1:100 dilution or S100 β antibody at a 1:500 dilution, and for 1 h with either 5 μ Ci of 125 I-labeled goat anti-rabbit (for S100 β and GFAP) or goat antimouse (for β -APP) IgG. Washing was as previously described (17). Immunoblots were exposed overnight to Kodak XAR film. The optical density (OD) of immunoreactive bands was measured as previously described (28) to estimate the levels of immunoreactive S100 β , β -APP, and GFAP in tissue samples.

Micro In Situ and Northern Hybridization Analyses of S100 β mRNA

Micro in situ hybridization analysis of the relative levels of S100 β mRNA in C6 glioma cells was performed as previously described (34). Briefly, cells were seeded at 20,000 cells/200 μ l media (MEM, containing 15% equine serum and 2.5% FBS) in wells of microtiter well strips (Corning, NY) and treated with media alone, or media containing 1 U/ml of IL-1 β for 1, 4, 6, and 24 h. After rinsing twice with PBS, cells were fixed and permeabilized, dehydrated in ethanol as previously described (34), and incubated overnight at 44°C with 2×10^5 cpm/50 μ l of denatured rat 32 P-labeled S100 β cDNA [pSB-1, generously donated by Dr. Alexander Marks, Toronto; (10)], and then washed with $0.2 \times$ SSC (1.8 g NaCl, 0.9 g sodium citrate/l, pH 7) containing 0.25% sodium dodecyl sulfate (SDS), at 47°C for 10 min. To estimate total polyadenylated mRNA in each well, cells were incubated with 2×10^5 cpm/50 μ l of 3 H polyuridylylate (NEN Research Products, Boston, MA) in $4 \times$ SSC and processed as previously described (34). For assessment of radioactive labeling and estimation of relative S100 β mRNA levels, individual wells were placed in vials with 3 ml scintillation cocktail (Biosafe II, Research Products International, Mount Prospect, IL) and counted in a Beckman LS 7000 scintillation counter.

For Northern blot analysis, total RNA was extracted from approximately 10^7 cells, using 3 ml TriReagent™ RNA isolation reagent according to the manufacturer's instructions, and RNA samples were processed using modifications of published protocols (25). Briefly, equal amounts of total RNA (5 μ g) from each sample were subjected to electrophoresis on a 1.2% agarose, 5% formaldehyde gel, and then transferred to GeneScreen+™ membrane (NEN Research Products, Doraville, GA) by capillary action with $10 \times$ SSC for 12 h. The membrane was baked at 80°C for 2 h, prehybridized with 50% formamide containing 1% SDS, 1 M NaCl, 10% dextran sulfate, 0.2 mg/ml salmon sperm DNA, and 50 mM Tris-HCl,

pH 7.5, for 4 h at 42°C, and then hybridized for 24 h, at 42°C, with 100 ng ³²P-labeled rat S100β cDNA (1 × 10⁹ cpm/μg DNA, labeled by using a random-primed DNA labeling kit, Boehringer–Mannheim, Indianapolis, IN). Membranes were then washed twice with 2× SSC, containing 1% SDS, at 65°C for 20 min and exposed to Kodak XAR film. The density of bands was measured at 560 nm using a computer-assisted analysis package on a Beckman DU-62 spectrophotometer.

Statistical Analysis

Differences between data from the three rat groups, and between treated and untreated cell cultures, were assessed for statistical significance using Student's *t*-test.

RESULTS

IL-1 Upregulation of S100β In Vivo

Immunohistochemical analysis showed prominent S100β⁺ astrocytes in IL-1β-injected rat brains (Fig. 1). Relative to their smaller counterparts in controls, many S100β⁺ astrocytes were identified as activated (9) with enlarged somas, prominent processes, and euchromatic nuclei. These S100β⁺ cells were more intensely immunoreactive (Fig. 1) and were more numerous in IL-1β-injected brains: 50% more numerous than in PBS-injected and 70% more numerous than in uninjected brains (Fig. 2; *p* < 0.05 in each case). Western immunoblot analyses (examples shown in Fig. 3) showed a clear increase in S100β levels in IL-1β-injected brain compared with PBS-injected controls (uninjected not shown). Densitometric analysis showed the S100β⁺ bands from IL-1β-injected brains to be approximately threefold as dense than those from PBS-injected or uninjected controls (Table 1; *p* < 0.001 for each comparison).

IL-1 Upregulation of S100β and S100β mRNA In Vitro

Examples of Northern and Western blots of S100β mRNA and S100β in C6 glioma cells, treated for 24 h in vitro with IL-1β or IL-1α, are shown in Fig. 4A and B. S100β mRNA was significantly elevated following 24 h of IL-1β treatment (*p* < 0.002) or IL-1α treatment (*p* < 0.02). As shown in Fig. 4B and C, cellular S100β levels were also elevated following 24 h of treatment with either IL-1β or IL-1α (*p* < 0.002 in each case). In contrast, neither IL-1β nor IL-1α treatment altered the levels of the astrocyte intermediate filament protein, GFAP (Fig. 4B). The levels of ribosomal (28S + 18S) RNA were similar in control and treated cells (0.6 ± 0.05 and 0.7 ± 0.04 OD/μg total RNA). Micro in situ hybridization analysis confirmed a doubling of S100β mRNA levels after 24 h of IL-1β treatment (13,000 ± 1,900 cpm vs. 6,500 ± 800 in PBS-treated cultures, for three microwells at each time point; *p* < 0.05; Fig. 5). The levels of polyadenylated RNA were similar in these IL-1β-treated and PBS-treated C6 cells.

IL-1 Upregulation of β-APP In Vivo

Western immunoblot analysis showed clear increases in two distinct β-APP⁺ bands (molecular weights of 130 and 135 kDa) in IL-1β-injected brains (examples shown in Fig. 6). The densities in supernatants from IL-1β-injected rats were significantly elevated, relative to the two control groups (*p* < 0.05 in each case), which did not differ from each other (Table 2).

DISCUSSION

We find IL-1β upregulation of S100β in vivo and in vitro, and IL-1α upregulation of S100β in vitro. In addition, we show IL-1β upregulation of β-APP in vivo. This suggests that the

overexpression of IL-1 in Alzheimer's disease is causally related to the concomitant astrocyte overexpression of S100 β (16, 19, 28) as well as to the neuritic overexpression of β -APP early in Alzheimer's disease (23). IL-1-induced excessive expression of S100 β may be the result of a direct effect of IL-1, as IL-1 receptors are present on astrocytes (4), or an indirect effect, as IL-1 is known to stimulate astrocyte activation (12). In either case, the increase in the levels of S100 β above the increase in the number of S100 β^+ astrocytes, indicates that individual S100 β^+ astrocytes contain more S100 β as a result of exposure to increased levels of IL-1 β .

Our finding of IL-1 β upregulation of β -APP expression in vivo supports the idea that the overexpression of IL-1 α (15) and IL-1 β (8) in Alzheimer's disease induces excessive synthesis and processing of β -APP. Such IL-1-induced excessive synthesis (11, 13) and processing (7) would generate β -APP fragments, some of which are neurotrophic (2,20,26). These neurotrophic β -APP fragments could stimulate further neurite growth with consequent further demand for synthesis of β -APP. In addition, IL-1-induced upregulation of S100 β could indirectly promote increased β -APP expression through the known neurotrophic and neurite extension-promoting properties of S100 β (5, 18). The resultant neurite growth would similarly demand production of more β -APP as a component of new neurite membrane formation. Thus, IL-1, via direct or indirect effects, may influence the growth of neurites and the concomitant increase in β -APP synthesis through either the actions of S100 β or neurotrophic fragments of β -APP. Therefore, IL-1 provides a plausible molecular introduction to a pathogenic sequence (15) involved both in the formation of dystrophic neurites and in further amyloid deposition, and, thus, in the evolution of amyloid deposits into neuritic β -amyloid-containing plaques.

The finding of upregulation of S100 β and β -APP by IL-1 may also explain the greater-than-1.5-fold elevation in the expression of these two chromosome 21 gene products (1, 14,30) in Down's syndrome (3,30), as IL-1, which is not a chromosome 21 gene product (6), is known to be overexpressed early in Down's syndrome (16). In view of the interactions between these proteins, shown here, and their potential importance in the pathogenesis of neuritic plaques in Alzheimer's disease (15, 19, 28), we suggest that IL-1 induction of S100 β and β -APP may explain, in part, the precocious appearance (33) of neuritic plaques at middle age in patients with Down's syndrome.

In summary, this first demonstration of an effect of IL-1 on S100 β and β -APP expression in vivo provides experimental confirmation of an important link in a proposed pathogenic sequence underlying the progression of lesions in Alzheimer's disease. It is also key evidence for the idea (21,31) that elevated levels of IL-1 in Alzheimer's disease and in Down's syndrome are of seminal pathogenic import in the development of the neuropathological changes that characterize these two disorders. In addition, this demonstration supports our original suggestion (16) that IL-1 up-regulation of S100 β expression is a concept that is generalizable to other neurodegenerative conditions in which there is microglial activation with IL-1 overproduction.

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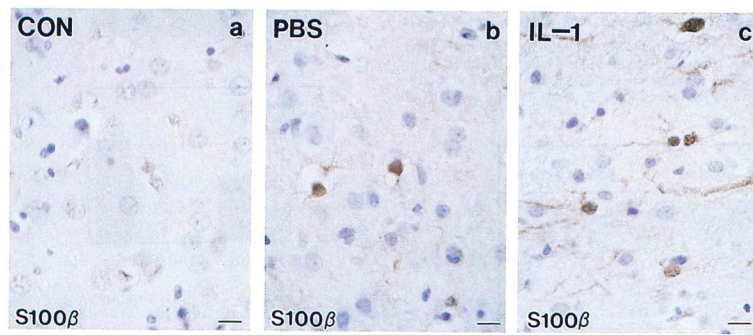


FIG. 1. S100 β -immunoreactivity (S100 β ⁺) in tissue sections of rat cerebrum showing increases in number, size, and immunoreactive intensity of S100 β ⁺ astrocytes (brown) in IL-1 β -injected animals. As illustrated here, some S100 β ⁺ astrocytes could also be found in PBS-injected animals, but the number of these cells was not significantly different from controls (see Fig. 2). (A) Uninjected control rat; (B) PBS-injected rat; (C) IL-1 β -injected rat. Bars = 15 μ m.

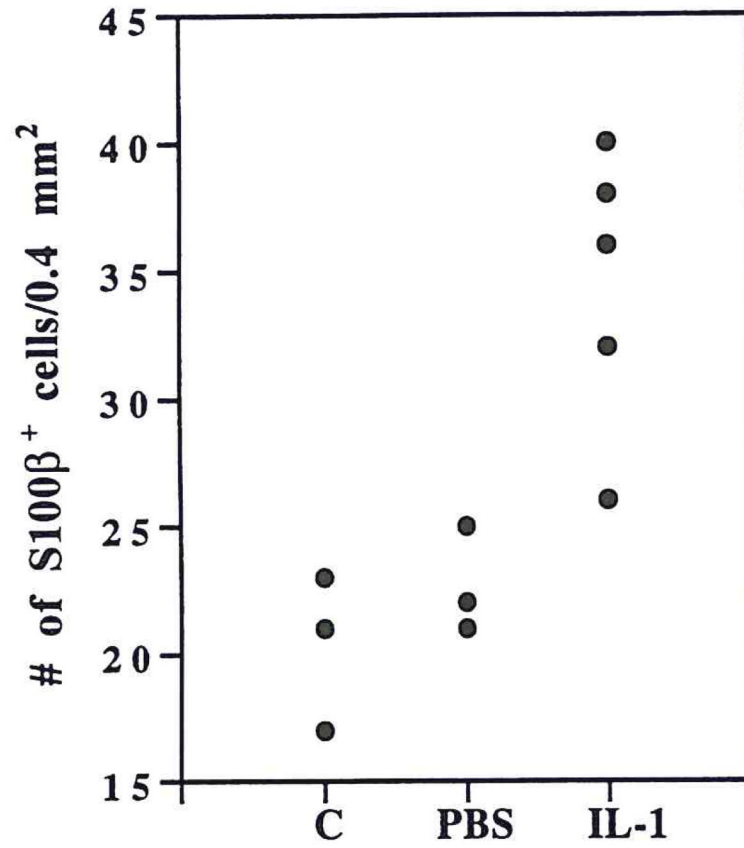


FIG. 2. Numbers of S100β-immunoreactive cells in five separate 250-diameter fields in brains from five IL-1β-injected; three PBS-injected, and three uninjected control rats.

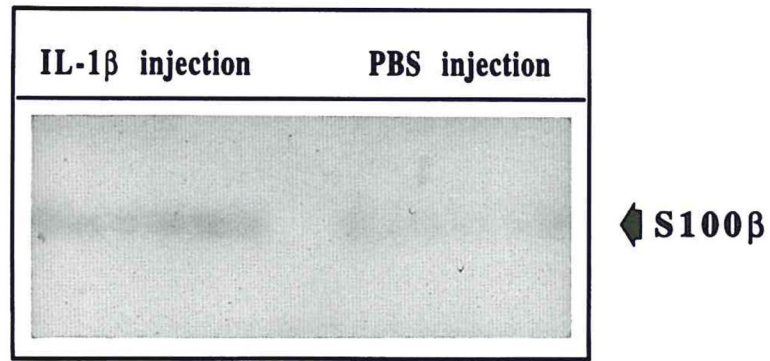


FIG. 3. Examples of Western immunoblot showing increased expression of S100 β in tissue extracts from IL-1 β -injected (IL-1 β -injection) brain compared to PBS-injected (PBS injection) brain. Results from three IL-1 β -injected and three PBS-injected animals are shown.

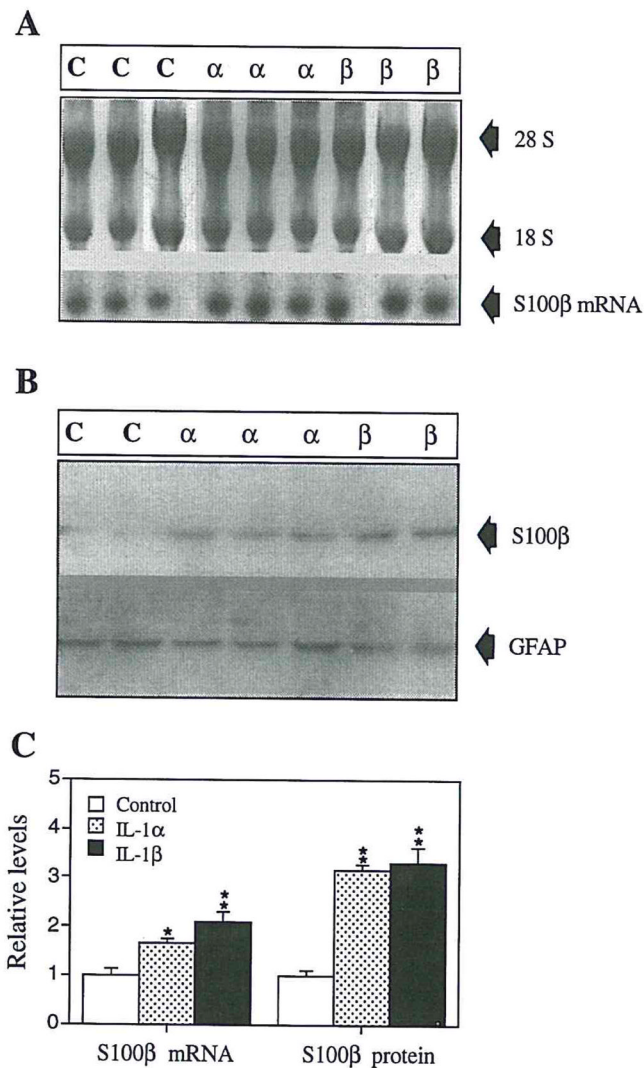


FIG. 4. IL-1 upregulation of S100β and S100β mRNA in C6 glioma cells in vitro. (A) Northern hybridization blots showing S100β mRNA with corresponding 28S and 18S levels from IL-1β-treated (β), IL-1α-treated (α), and untreated (C) cells. (B) Western immunoblot probed with anti-S100β and anti-GFAP antibodies showing results from IL-1β-treated (β), IL-1α-treated (α), and untreated (C) cells. (C) S100β and S100β mRNA levels in IL-1β-treated, IL-1α-treated, and untreated (control) C6 glioma cells. * $p = 0.02$; ** $p < 0.002$ compared to control values.

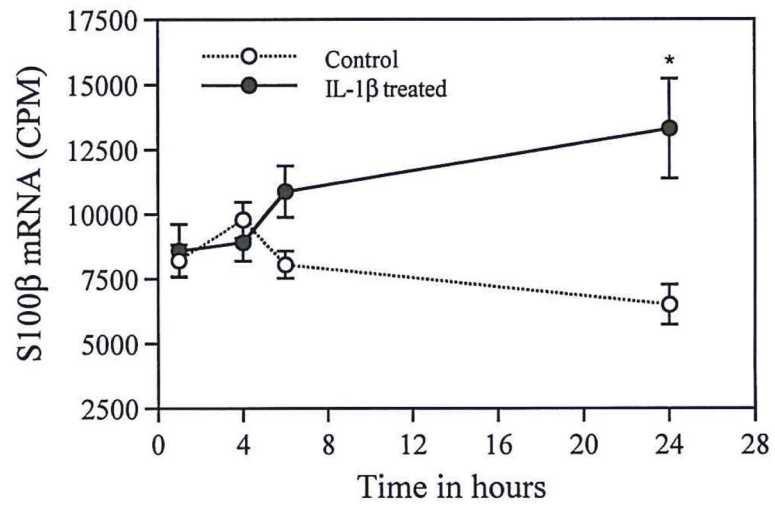


FIG. 5. S100 β mRNA levels (radioactive counts per min, CPM) in IL-1 β -treated C6 glioma cells analyzed using micro in situ hybridization. There is a significant increase in S100 β mRNA following 24 h of IL-1 β treatment (* $p < 0.05$).

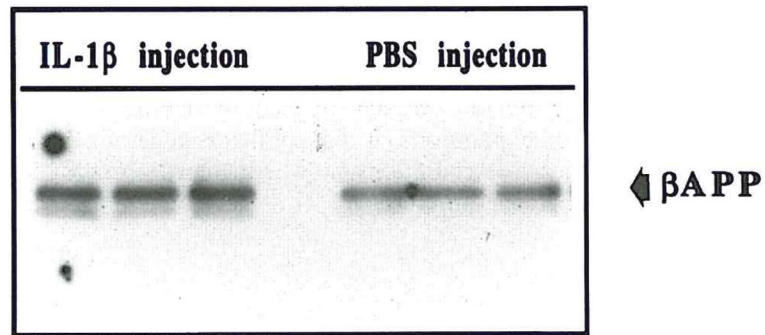


FIG. 6. Example of Western immunoblot of two β -APP-immunoreactive bands, at 130 kDa and 135 kDa, showing increased expression of both β -APP isoforms in IL-1 β -injected brain (IL-1 β injection) compared to PBS-injected (PBS-injection) brain. Results from three IL-1 β -injected and three PBS-injected animals are shown.

TABLE 1

DENSITIES OF IMMUNOREACTIVE S100 β PROTEIN BANDS IN WESTERN IMMUNOBLOTS FROM IL-1 β -INJECTED, PBS-INJECTED AND UNINJECTED RAT CEREBRAL HEMISPHERES

Group	<i>n</i>	S100 β
No injection	4	0.07 \pm 0.01
PBS injection	6	0.07 \pm 0.01
IL-1 β injection	8	0.19 \pm 0.01*

n = number of animals. Values expressed as optical density units of scanned immunoblotted gels (mean \pm SEM).

* Value is significantly different from that of either PBS-injected or uninjected control animals ($p < 0.001$).

TABLE 2

DENSITIES OF IMMUNOREACTIVE β -AMYLOID PRECURSOR PROTEIN (β -APP) BANDS IN WESTERN IMMUNOBLOTS FROM IL-1 β -INJECTED, PBS-INJECTED, AND UNINJECTED RAT CEREBRAL HEMISPHERES

Group	<i>n</i>	β -APP (130 kDa)	β -APP (135 kDa)
No injection	4	0.08 \pm 0.01	0.44 \pm 0.22
PBS injection	6	0.12 \pm 0.02	0.57 \pm 0.10
IL-1 β injection	8	0.22 \pm 0.03*	0.80 \pm 0.04*

n = number of animals. Values expressed as optical density units of scanned immunoblotted gels (mean \pm SEM).

* Value is significantly different from that of either PBS-injected or uninjected control animals ($p < 0.05$).