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Production and characterization of monoclonal antibodies with specificity for the S100β polypeptide of brain S100 fractions

(calcium-modulated protein/brain-specific protein/hybridoma/immunohistochemistry/pathology)

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ABSTRACT **S100 refers to a heterogeneous fraction of low-molecular-weight acidic calcium-binding proteins. We report here production and characterization of two mouse hybridomas that secrete monoclonal antibodies that appear to be specific for the S100β polypeptide of brain S100 preparations. By ELISA, RIA, and immunoblotting analysis, the monoclonal antibodies react specifically with S100β and show little or no reactivity with any S100α-like polypeptides. In addition, there is no reactivity with the structurally homologous proteins calmodulin and troponin C. The utility of these monoclonal antibodies for immunocytochemical studies of clinical pathology specimens has been demonstrated by examination of S100β localization in human autopsy brain and anaplastic astrocytoma sections. S100β is localized primarily in glial cell cytoplasm and processes, with no specific staining observed in glial cell nuclei, erythrocytes, or neuronal cells. These monoclonal antibodies may have important applications in pathological examination of surgical specimens as a specific marker for tumors containing S100β, will allow a more precise interpretation of the distribution and localization of S100β in both normal and neoplastic tissues, and may provide insight into the physiological functions of the S100 proteins.**

S100 refers to a heterogeneous fraction of low-molecular-weight acidic calcium-binding proteins (for recent review, see ref. 1). This protein fraction was first isolated by Moore (2) and termed S100 to signify its partial solubility in saturated ammonium sulfate at neutral pH. The S100 fraction is a mixture of polypeptides, only some of which have been isolated and characterized. Two S100 components, termed S100α and S100β, have been purified from the S100 fraction of bovine brain and their amino acid sequences have been elucidated (3, 4). S100α (3) and S100β (4) are polypeptides of 93 and 91 amino acids, respectively, that share 54 identical amino acids. It has been reported (4–6) that in solution S100α and S100β can exist as dimers of the form αα ($S100a_0$), αβ (S100a), and ββ (S100b). However, the physiological significance of this dimerization is not clear. There have been recent reports (7, 8) of the detection of other S100 protein species, but their relationship to the well-characterized S100α and S100β proteins is not known.

Until recently, S100 proteins have been considered nervous system-specific antigens (for reviews, see refs. 8–10), primarily localized to glial cells in the central nervous system and to Schwann cells in the periphery. However, S100 proteins have been detected in non-neuronal tissues, such as adipose tissue (11, 12), T lymphocytes (13), and skin (14–16), and in extracts of a planarian (17). In addition, S100β has recently been purified from bovine adipose tissue and shown to be indistinguishable from bovine brain S100β (12). These studies show that the S100β protein is more widely distributed than originally thought and that it should no longer be considered a nervous system-specific protein.

Although the physiological functions of the S100 proteins are not known, immunochemical studies of S100 localization may have important clinical applications. Antibodies to S100 proteins have been described as "essential" reagents for the diagnostic pathology laboratory (18). Assay of immunoreactive S100 has been used to diagnose and classify tumors of neural origin and as a marker for various types of tumors, including melanomas, granular cell tumors, schwannomas, chondroid tumors, and histiocytosis X cells (see refs. 19–26 for selected examples). However, studies on localization of S100 proteins by immunochemical methods are difficult to evaluate at present. Most such studies have used antisera prepared against a heterogeneous mixture of S100 proteins (for review, see ref. 1). Because most of these antisera react with more than one protein in the S100 fraction, precise localization of specific S100 proteins by immunochemical methods has not been possible. Even the currently available monoclonal antibodies against S100 (27–30) do not appear to measure individual S100α and S100β components. Thus, the spectrum of S100 immunoreactivity in various tissues and the immunohistochemical distribution in clinical specimens may depend on the source of the antibody and the degree of cross-reactivity among the multiple S100 proteins. To unequivocally interpret studies on the localization of S100 proteins and their potential alterations in various disease states and to assess the validity of S100 immunoreactivity as a diagnostic tool in human pathology, it would be useful to have antibodies that discriminate among the individual S100 components. We report here the production and characterization of monoclonal antibodies that appear to be specific for S100β and are applicable for immunohistochemical localization studies.

MATERIALS AND METHODS

Protein Purification. Vertebrate calmodulin (31) and rabbit skeletal muscle troponin C (32) were purified as described. S100 proteins were purified from bovine, human, rat, and chicken brain fractions as described (33). Individual S100 polypeptides were homogeneous by several criteria, including amino acid composition and limited amino acid sequence analysis.

Production of Monoclonal Antibodies. Purified bovine brain S100β was used as the immunogen. Female BALB/c mice were immunized with S100β at 50 μg per mouse per injection. Antigen was emulsified in complete Freund's adjuvant (initial injection) or incomplete Freund's adjuvant (second injection) and injected subcutaneously and intraperitoneally on days 1 and 14. Four of four mice produced a positive serum response at the first bleed (day 21). Three days before fusion was carried out, 70 μg of S100β in phosphate-buffered saline (P_i/NaCl) was injected intravenously.

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Abbreviation: P_i/NaCl, phosphate-buffered saline.

Mouse myeloma cells (P3X63-Ag8.653) in logarithmic growth were fused with spleen cells from the immunized mice essentially as described (34, 35). Briefly, spleens were removed, minced through stainless steel mesh, and treated with ammonium chloride to lyse erythrocytes. Spleen cells (10^8) were fused with 8-azaguanine-resistant myeloma cells (10^7) by using 50% (vol/vol) polyethylene glycol (M_r, 1300–1600) in serum-free DME:F12 medium (1:1 mixture of Dulbecco's modified Eagle's and Ham's F12 media). Cells were plated into 24-well tissue culture dishes (Falcon) in DME:F12 medium containing 20% (vol/vol) fetal calf serum and 0.1 mM hypoxanthine/0.4 μM aminopterin/16 μM thymidine. After 2–3 weeks, supernatants were analyzed for the presence of antibodies to S100 by using the enzyme-linked immunosorbent assay (ELISA) described below.

Antibody-producing hybridomas were cloned by limiting dilution or in soft agar until stable clones were achieved. Limiting-dilution cloning was done in 96-well tissue culture dishes (Falcon). Cells were plated at a concentration of ≈20 cells per well into dishes containing a 1:1 mixture of conditioned myeloma supernatant and DME:F12 medium/20% (vol/vol) fetal calf serum. Conditioned myeloma supernatant was prepared from cultures of mouse myeloma cells (P3X63-Ag8.653). Myeloma cells were grown for 2–3 days, at which time the culture supernatant was removed and clarified by centrifugation at 500 × *g* for 15 min. This conditioned myeloma supernatant was usually prepared fresh for each use but could be stored at 4°C for 1–2 weeks. Soft agar cloning was done in 60 × 15 mm tissue culture dishes with low-melting-point agarose (Bethesda Research Laboratories). Soft agar medium [DME:F12 medium/25% (vol/vol) fetal calf serum and 17% (vol/vol) conditioned myeloma supernatant] was mixed with a 1.25% (wt/vol) agarose stock to give a final agarose concentration of 0.5% (vol/vol). This 0.5% agarose medium was used as the bottom agarose layer (7 ml per dish). The top agarose layer (1.5 ml) was prepared by mixing 1 vol of cell suspension in DME:F12 medium/20% (vol/vol) fetal calf serum with 2 vol of 0.5% agarose medium. Cells were plated at a concentration of 200–350 cells per dish.

For some experiments, ascites fluid was prepared by injection of hybridoma cells into pristane-primed mice. BALB/c mice were injected intraperitoneally with 0.3–0.5 ml of pristane (2,6,10,14-tetramethyl-pentadecane; Aldrich) 1–2 weeks prior to injecting them intraperitoneally with 1 × 10^6 hybridoma cells.

ELISA. Antibody production was monitored by using an ELISA and the Vectastain ABC kit for detection of mouse IgG (Vector Laboratories, Burlingame, CA). To effect efficient adsorption of S100 to the E.I.A. microtitration plates (Flow Laboratories), plates were incubated with poly(lysine) at 10 μg/ml and $CaCl_2$ at 100 μg/ml at room temperature for 15 min, rinsed twice with P_i/NaCl, and then incubated overnight at 4°C with antigen in 50 mM sodium bicarbonate/100 μM $CaCl_2$, pH 9.5. Examination of the ability of various concentrations of S100 proteins to adsorb to the plates showed that a concentration of at least 2 μg/ml was required for adsorption. In all the studies reported here, S100 was used at 4 μg/ml for adsorption. After overnight adsorption and washing with P_i/NaCl, plates were incubated with 1% (vol/vol) horse serum in P_i/NaCl for 15 min at room temperature, washed three times with P_i/NaCl, and then incubated with test supernatants for 1–2 hr at 37°C. Bound antibody was detected by using the Vectastain mouse ABC kit as recommended by the manufacturer and 2,2′-azinodi(3-ethylbenzthiazoline sulfonic acid). Antibody reactivity was quantitated with a Multiskan MC plate reader (Flow Laboratories).

The immunoglobulin subclass of the monoclonal antibodies was determined by an ELISA using rabbit antisera against the various mouse immunoglobulin subclasses (Miles) and the Vectastain rabbit ABC kit.

RIA. RIAs were done as described (36) except that normal mouse serum was used as the carrier serum and goat anti-mouse serum was used as the second antibody. Iodinations were done with chloramine-T (36), Bolton–Hunter reagent (37), or lactoperoxidase (33) as described.

Immunoblots. Immunoblotting experiments were done as described (38) except that transfer was for 30–60 min at 4°C. In some experiments, the nitrocellulose was incubated with 0.2% glutaraldehyde in P_i/NaCl for 1 hr at room temperature before incubation in blocking buffer and peroxidase-labeled goat anti-mouse IgG was used as the second antibody.

Immunocytochemistry. Formalin-fixed paraffin-embedded tissue sections of human anaplastic astrocytoma and normal autopsy brain were analyzed for S100 immunoreactivity. Tissue sections were deparaffinized and hydrated through xylene and graded alcohol series, rinsed with water, and incubated for 60 min with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity. Samples were washed with P_i/NaCl and incubated for 20 min with 2% (vol/vol) horse serum in P_i/NaCl, and then overnight at room temperature with a 1:300 or a 1:600 dilution of primary antibody in P_i/NaCl containing 1% (vol/vol) horse serum. The sections were washed with P_i/NaCl and incubated sequentially with (*i*) biotinylated second antibody in P_i/NaCl, (*ii*) Vectastain ABC reagent in P_i/NaCl containing 0.1% Tween 20, and (*iii*) diaminobenzidine tetrahydrochloride peroxidase substrate in 50 mM Tris·HCl, pH 7.2. Sections were washed with P_i/NaCl between incubation steps.

Materials. Chemicals were obtained as follows: polyethylene glycol (M_r, 1300–1600) and myeloma cells from the American Type Culture Collection; hypoxanthine, thymidine, aminopterin, diaminobenzidine·HCl, and 2,2′-azinodi(3-ethylbenzthiazoline sulfonic acid) from Sigma; Dulbecco's modified Eagle's and Ham's F12 media from GIBCO or Flow Laboratories; radiochemicals from Amersham or New England Nuclear; fetal calf serum from HyClone (Logan, UT); normal mouse serum and goat anti-mouse serum from Cappel Laboratories (Cochranville, PA); nitrocellulose paper from Sartorius. BALB/c mice were from Harlan–Sprague–Dawley Industries (Indianapolis, IN).

RESULTS

The S100β polypeptide of bovine S100 fractions is a logical first choice in attempts to develop immunochemical reagents specific for the individual S100 polypeptides. Bovine brain S100β is a well-characterized chemical structure (4). S100β has also been characterized from tissues other than brain (12), suggesting a more widespread distribution than nervous tissue alone. Therefore, homogeneous bovine brain S100β was isolated and used as the immunogen to prepare mouse monoclonal antibodies. Two clones were produced that secrete monoclonal antibodies (designated 1A1 and 4D4) that appear to be specific for S100β. Both monoclonal antibodies are of the IgG1κ isotype. In all characterizations done to date, the two monoclonal antibodies are indistinguishable in their reactivities. ELISA of the reactivity of tissue culture supernatants from one of the two clones is shown in Fig. 1. The antibodies react well with bovine S100β, with half-maximal binding occurring at a supernatant dilution of approximately 1:1000. The ability of the antibodies to bind to S100 proteins from other species was also examined by ELISA. The antibodies cross-react with S100β from human, rat, and chicken brain. In contrast, there is no reactivity with any S100α-like proteins examined (bovine and human).

In attempts to develop a RIA using these monoclonal antibodies, we examined the ability of the antibodies to react with iodinated bovine S100β. S100β was iodinated by three different procedures: Bolton–Hunter reagent, lactoperoxidase, or a mild chloramine-T method. The antibodies bind to

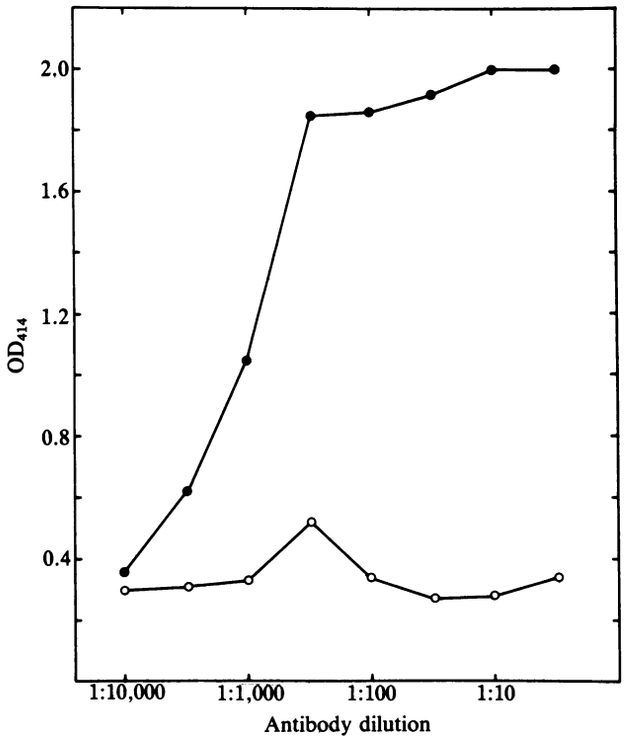

FIG. 1. Reactivity of a monoclonal antibody supernatant in the ELISA. Various dilutions of antibody supernatant were incubated with a fixed concentration of bovine S100β (●) or S100α (○) that had been adsorbed to a microplate.

bovine S100β that has been iodinated by any of these three methods, although some variability in binding to S100β iodinated using the chloramine-T procedure was observed. We routinely use Bolton–Hunter reagent for iodination of S100β. The ability of the antibodies to bind to iodinated S100β allowed the establishment of a RIA using ^{125}I-labeled bovine S100β as the tracer. In competition RIA (Fig. 2), unlabeled S100β competes with ^{125}I-labeled S100β for binding to the antibody whereas unlabeled S100α does not. In addition, there is no reactivity with calmodulin or troponin C, calcium-modulated proteins that are structurally homologous to S100β.

Another method available to demonstrate the reactivity of

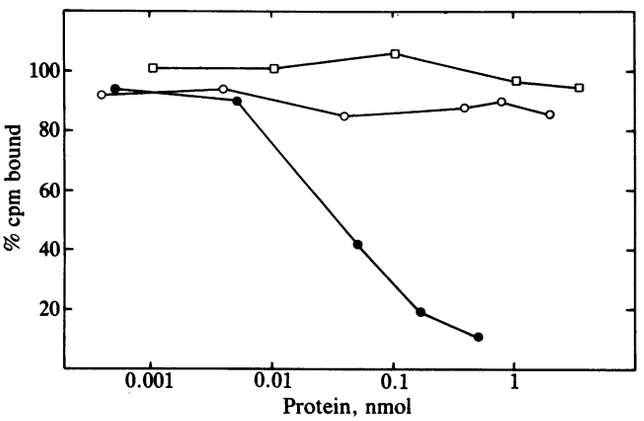

FIG. 2. Characterization of the monoclonal antibody by competition RIA with ^{125}I-labeled S100β (Bolton–Hunter reagent) as tracer. Various concentrations of human brain S100β (●), bovine brain S100α (○), and chicken gizzard calmodulin (□) were mixed with antibody and analyzed for competition with ^{125}I-labeled S100β. Degree of competition is expressed as percentage of the radioactivity bound in the absence of competing protein.

these antibodies with purified S100 polypeptides is the immunoblot or "Western blot" technique. We examined the ability of the monoclonal anti-S100β antibodies to react with bovine brain S100α or S100β after electrophoresis and electrophoretic transfer of the S100 proteins to nitrocellulose paper (Fig. 3). One nitrocellulose section was stained with amido black, showing that the molecular weight standards and both S100 polypeptides were transferred to the nitrocellulose paper (Fig. 3*A*). Incubation of a nitrocellulose section (Fig. 3*B*) with the monoclonal antibody supernatant resulted in specific reactivity with S100β with no staining observed for S100α. Nitrocellulose sections incubated with conditioned myeloma supernatant (Fig. 3*C*) or with antibody adsorbed with excess S100β (data not shown) showed no reactivity with S100α or S100β. The specificity of the antibody for S100β was further demonstrated by immunoblotting analysis of a crude brain extract. The antibody reacts specifically with a single band that co-migrates with purified S100β (Fig. 4). Thus, by three different criteria (ELISA, RIA, and immunoblotting), these monoclonal antibodies appear to react specifically with S100β and show little or no reactivity with S100α.

We examined the utility of the monoclonal antibodies for immunocytochemical procedures using human tissue sections. The analysis of the localization of S100β in human anaplastic astrocytoma and autopsy brain specimens is shown in Fig. 5. In the anaplastic astrocytoma sections (Fig. 5 *A–H*), the large neoplastic astrocytes show intense staining of the cytoplasm and processes, with very little staining of the nuclei. For example, a section of anaplastic astrocytoma incubated with antibody supernatant (*A*) shows several neoplastic astrocytes with dense cytoplasmic staining. A similar section incubated with conditioned myeloma supernatant (*B*) shows no cytoplasmic staining. The nuclear staining seen in *B* (and *J*) is not reaction product but is due to the hematoxylin counterstain. The lack of specific nuclear staining is more evident in *C* and *D*, where the sections were not counterstained with hematoxylin. In *C*, the section incubated with antibody supernatant shows staining in the astrocyte cytoplasm but not in the nuclei (arrow). A control section incubated with antibody supernatant that had been absorbed with excess bovine S100β (*D*) shows little or no staining. The

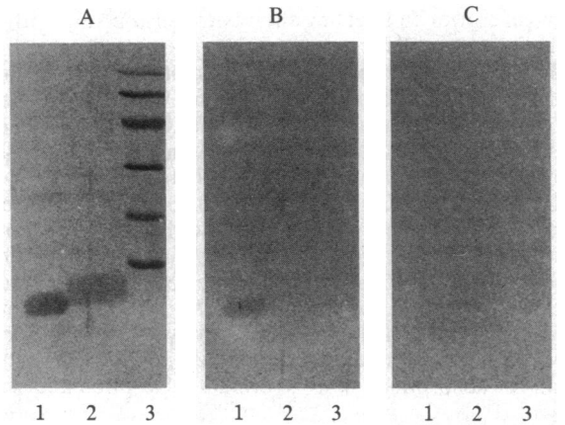

FIG. 3. Immunoblotting analysis of monoclonal antibody reactivity. Bovine S100β (lanes 1) and S100α (lanes 2) and molecular weight standards [phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,000; α-lactalbumin, 14,000 (lanes 3)] were subjected to NaDodSO_4/polyacrylamide gel electrophoresis on a 15% (wt/vol) acrylamide gel, transferred to nitrocellulose, and analyzed for ability to bind antibody. (*A*) Amido black staining of the proteins after transfer to nitrocellulose. (*B*) Nitrocellulose was incubated with monoclonal antibody supernatant. (*C*) Nitrocellulose was incubated with conditioned myeloma supernatant.

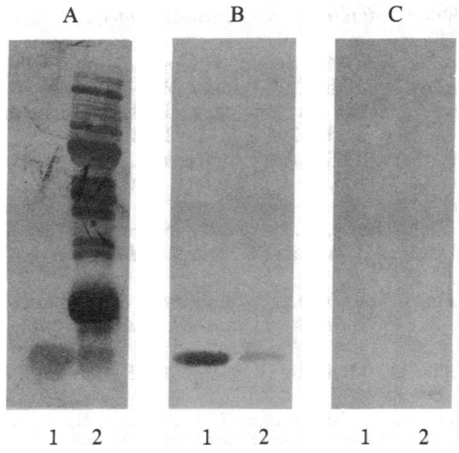

FIG. 4. Immunoblotting analysis of crude brain extract. Purified S100β (lanes 1) and a crude rat brain extract prepared as described (31) through the pH 4.0 precipitation step (lanes 2) were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis on a 15% (wt/vol) acrylamide gel. The proteins were transferred to nitrocellulose, fixed with glutaraldehyde, and analyzed for ability to bind antibody. (*A*) Amido black staining of the proteins after transfer to nitrocellulose. (*B*) Nitrocellulose was incubated with monoclonal antibody supernatant. (*C*) Nitrocellulose was incubated with conditioned myeloma supernatant.

intense labeling of the astrocytic cytoplasm and cellular processes is evident in *E–G*. Erythrocytes in the vessels are not stained (*H*).

A localization pattern similar to that of the anaplastic astrocytoma sections is seen with human autopsy brain sections (Fig. 5 *I* and *J*). S100β appears to be localized primarily in the cytoplasm of the glial cells (arrow), with no significant staining of the glial cell nucleus or the surrounding larger neuronal cells (*I*). There is no glial (arrow) or neuronal staining observed using conditioned myeloma supernatant instead of antibody supernatant (*J*).

DISCUSSION

In this paper, we have described the production and characterization of monoclonal antibodies against homogeneous S100β and have reported that these antibodies can discriminate between the highly related S100α and S100β polypeptides.

As far as we know, this is the first demonstration of monoclonal antibodies against S100 that are specific for an individual S100 polypeptide. Other reports (27–30) have appeared on the production of monoclonal antibodies against S100 proteins. In two reports (27, 30), the antibody reacted with two major proteins in the S100 preparation. The other reports (28, 29) give no data on the specificity of the antibodies for individual S100 proteins. We have reported here the preparation of monoclonal antibodies that appear to react specifically with S100β.

Because S100α and S100β are so highly related structurally, it has been extremely difficult to prepare antibodies that show specificity for one of these S100 proteins. We have previously produced rabbit antisera against S100α and S100β by using either homogeneous bovine brain S100α or S100β

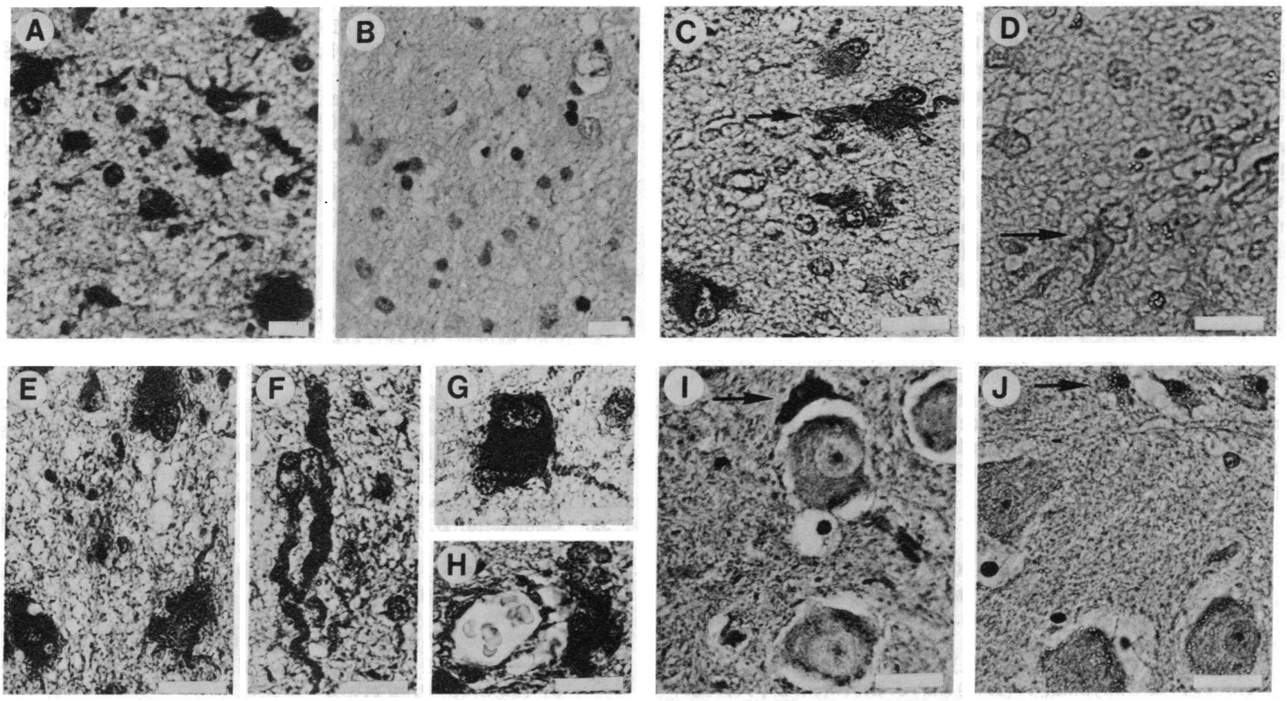

FIG. 5. Immunocytochemical analysis of S100β localization in human anaplastic astrocytoma and normal autopsy brain. Anaplastic astrocytoma sections (*A–H*) and autopsy brain sections (*I* and *J*) were analyzed by an immunoperoxidase procedure. Sections were incubated with monoclonal antibody supernatant (*A*, *C*, and *E–I*), conditioned myeloma supernatant (*B* and *J*), and monoclonal antibody supernatant adsorbed with excess S100β (*D*). (*A*, *B*, and *E–J*, sections were counterstained with hematoxylin; *All*, bars = 20 μm.) (*A*) Anaplastic astrocytoma section showing labeling of astrocytes and cell processes. (*B*) *A* section similar to that in *A* showing no staining of the astrocytes with conditioned myeloma supernatant. Hematoxylin-counterstained nuclei are evident. (*C*) Anaplastic astrocytoma section not counterstained with hematoxylin showing labeling of the astrocytic cytoplasm (arrow) but not of the nuclei. (*D*) A section similar to that in *C* showing no staining of the astrocytes (arrow) when antibody that had been adsorbed with S100β was used. (*E* and *F*) Staining of the astrocytic processes is evident. (*G*) A large multinucleated astrocyte shows intense staining in the cytoplasm and processes. (*H*) Erythrocytes in the vessel are not stained. (*I*) Human autopsy brain section showing that the glial cell cytoplasm is heavily labeled (arrow) whereas the surrounding neuronal cells are unlabeled. (*J*) A section similar to that in *I* but incubated with conditioned myeloma supernatant. There is little or no staining of the glial cell cytoplasm (arrow) or the neuronal cells. The dense nuclear staining seen is due to the hematoxylin counterstain.

as the immunogen (39). Even though these antisera react preferentially with the appropriate S100 protein (i.e., anti-S100α sera react better with S100α than with S100β), there is substantial cross-reactivity between the S100 proteins. The only other published study (12) using a purified S100 protein as immunogen describes production of rabbit antisera against purified S100β. However, these rabbit antisera also show some cross-reactivity with S100α. Thus, it appears that previously described antibodies directed against S100 either do not define a single structure or react with amino acid regions shared by these highly related proteins.

The fact that most of the earlier studies of S100 have used or monitored a heterogeneous mixture of proteins with antisera that probably do not discriminate among the individual S100 polypeptides may be the basis for the various and sometimes conflicting reports in the literature concerning the localization and properties of S100 proteins (for reviews, see refs. 1, 8, and 9). There have also been many reports in the literature on the use of antibodies against S100 proteins as diagnostic tools in surgical pathology. An obvious limitation of such studies is that the source and specificity of the antibodies may determine the pattern of immunoreactivity obtained. The preliminary immunocytochemical analyses reported here with monoclonal antibodies against S100β have shown the feasibility of using these well-defined antibodies for localization studies in human pathology. In this regard, it is interesting to note the recent work of Isobe *et al.* (40). They isolated S100 proteins from human tumors by high performance liquid chromatography and showed that tumor cells of human schwannoma contain only S100β while those of malignant melanoma contain both S100α and S100β. These data establish the precedent of differences in the distribution of S100 proteins among tumor tissues.

Our current studies are directed toward refining immunocytochemical procedures for analysis of S100β localization and distribution in various cells and tissues and establishing immunochemical assays for quantitative measurement of individual S100 proteins. The specificity of these antibodies will allow a more unambiguous interpretation of the properties and distribution of S100β and its potential alterations in various disease states and may provide new insight into the possible roles of S100β in cell function.

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