Particle Counting Immunoassay of S100 Protein in Serum. Possible Relevance in Tumors and Ischemic Disorders of the Central Nervous System

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S100 protein (S100) was assayed by particle counting immunoassay in serum samples from 50 healthy individuals, 325 patients with various neurological disorders, and 20 patients with malignant melanoma. The detection limit for this protein was 0.3 µg/L. We detected none in healthy individuals or in 50 patients with multiple sclerosis, 23 with dementia, or 20 with meningitis. S100 was detectable in serum of only a few patients with meningoaracnoidalitis (2/20), peripheral neuropathy (2/30), encephalitis (1/14), Guillain–Barré syndrome (1/25), or AIDS (2/20). In contrast, we observed high concentrations in 29 of 75 patients with tumors of the central nervous system, especially in meningioma (6/9), glioblastoma (9/23), and neurinoma (5/6). Values for S100 were mainly abnormally high (>0.3 µg/L) in serum from patients with cerebrovascular disorders (43/48) or with metastases of melanoma (9/11).

Additional Keyphrases: stroke · melanoma · diseases of the nervous system · cancer · marker of brain damage

S100 protein, an acidic protein of 21 000 Da (1), was originally discovered by Moore (2), in the brain. It is now known to occur in three forms—S100a, S100a, and S100b—all dimers with the respective subunit composition of αα, αβ, and ββ (3–5). S100a (αα) is mainly distributed in striated muscles, heart, and kidney (6, 7). The α subunit of S100 (β S100) is very abundant in the brain but is not brain-specific (8). Some species differences exist, however, the relative amounts of subunits α S100 and β S100 being 47:53 (by wt.) in bovine brain and 4:96 in human brain (9). S100 protein is located mainly in astrocytes (10) and Schwann cells, but it has also been detected in other tissues such as melanocytes (11, 12), adipocytes (8, 13), chondrocytes (14), interdigiting reticulum cells of the lymph node (15), epidermal Langerhans cells (11, 16), and a few human T-lymphocytes (17, 18). In addition, S100 has been demonstrated by immunohistochemical methods to be present in malignant melanomas (19–22), acoustic neurinomas (23), salivary gland neoplasms (24), skin tumors (25, 26), and in astrocytoma, glioblastoma, Schwannoma, epidermoida, and mixed glioma (27, 28).

Here we describe the assay of S100 in serum by immunoassay by particle counting and discuss the results of a retrospective study involving patients with various neurological disorders (n = 325) or melanoma (n = 20). This preliminary study shows the prevalence of increased S100 in serum in ischemic disorders, in tumors of the central nervous system, and in metastases of melanoma.

Materials and Methods

Samples

Blood samples from 325 patients of the Department of Neurology (Cliniques Saint Luc, Brussels) were centrifuged (10 min, 4000 x g) and the serum was collected and stored at −20 °C until sample treatment. After retrospective review of the clinical records of these patients, patients without firm diagnoses were excluded from the study.

The control group (n = 50) comprised non-neurological patients with minor neurosis or tension headache but with no clinical signs of neurological disorders.

In addition, we studied blood samples from 20 patients with melanoma, 16 patients with coronary thrombosis, and a patient suffering from Duchenne's muscular dystrophy.

Purification of S100 from Bovine or Human Brains

We isolated S100 from bovine or human brains as described previously (29–31). For the last step we used an anion-exchange Mono Q HR 10/10 column (Pharmacia, Uppsala, Sweden) in an FPLC system ("Fast Protein Liquid Chromatography"; Pharmacia) and a linear gradient of 110 mL of potassium phosphate buffer (10 mmol/L, pH 7.2, containing 50 mmol of NaCl and 1 mmol each of EDTA and 2-mercaptoethanol) and 110 mL of 0.6 mol/L NaCl. The main immunoreactive fraction (Figure 1) was collected,

![Fig. 1. Elution profile from an anion-exchange column MonoQ HR 10/10 in FPLC system of brain preparation supernate after precipitation with 100% saturated ammonium sulfate](image_url)

The supernate applied to the column contained about 7 mg of protein; the column had been equilibrated and was eluted with 20 mL of potassium phosphate buffer (10 mmol/L, pH 7.2) containing 1 mmol of EDTA, 1 mmol of 2-mercaptoethanol, and 50 mmol of NaCl per liter. The agglutination of the effluent (rate: 2 mL/min, 2-mL fractions) was monitored by immunoassay by particle counting (---), protein by its absorbance at 280 nm (----).

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concentrated to 5 g/L (Lowry method), and stored at -20 °C. The purity of the preparation was checked by sodium dodecyl sulfate/polyacrylamide (150 g/L) gel electrophoresis. To prepare the standard stock solution, we diluted the concentrated preparation 1000-fold in glycine-buffered isotonic saline (GBS; per liter, 0.17 mol of NaCl, 0.1 mol of glycine, and 40 mg of NaN₃, adjusted to pH 9.2 with NaOH) containing 50 mmol of CaCl₂ per liter, then further diluted this with a mixture of normal human serum in glycine-buffered isotonic saline, 400 mL/L, to the desired concentration.

Preparation of F(ab')₂ Fragment-Coated Latex

After washing 1 mL of 0.8-μm-diameter particles of carboxylated latex ("Estapor K150," containing 100 g of latex per liter; Rhône-Poulec, Courbevoie, France), with 5 mL of 1 mol/L NaCl solution buffered with borate (20 mmol/L, pH 8.2), we resuspended the latex particles in 2 mL of saline-buffered borate containing, per liter, 250 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (Sigma Chemical Co., St. Louis, MO) by shaking for 40 min at room temperature. To stop the activation, we added 2 mL more of saline-buffered borate, centrifuged the suspension for 10 min at 9000 × g, and suspended the latex in 2 mL of a 4 mg/mL solution of F(ab')₂ fragments from anti-S100 IgG (Dakopatts, Copenhagen, Denmark; we used lots 025 and 026, with similar results) in saline-buffered borate. We sonicated the mixture for a few seconds in a Model B12 sonifier (Branson, Danbury, CT 06810), then shook it for 16 h at 4 °C. To stop the conjugation, we added 8 mL of glycine-buffered isotonic saline containing 10 g of bovine serum albumin per liter (Calbiochem, La Jolla, CA). The latex suspension was then sonicated and washed with 4 mL of glycine-buffered isotonic saline containing 1 mL of Tween 20 [polyoxyethylene (20) sorbitan monolaurate] surfactant per liter, followed by two 4-mL portions of glycine-buffered isotonic saline/bovine serum albumin. Finally, we resuspended the latex particles in 8 mL of glycine-buffered isotonic saline/bovine serum albumin, as the stock preparation, which can be stored at -20 °C for six months without loss of activity. For working use, we diluted this preparation 10-fold with glycine-buffered isotonic saline/bovine serum albumin.

Assay of S100

We assayed S100 by immunoassay by particle counting, using a commercial instrument (IMPACT; Acafe Diagnostic Systems, Brussels, Belgium). The reliability of this technique is described elsewhere (32). For the assay of serum S100, the various automated steps were as follows: dilution of 30 μL of sample with 200 μL of glycine-buffered isotonic saline containing 1.8 g of dithiothreitol per liter; incubation with vortex-mixing for 10 min at room temperature; addition of 30 μL of 6.6 mL/L hydrogen peroxide solution; reaction of 30 μL from this mixture with 30 μL of latex particles and 30 μL of the additive containing aggregated rabbit F(ab')₂ fragments, 5 g/L (33), Dextran T500 (20 g/L; Pharmacia), and Tween 20, 20 mL/L. After incubation for 43 min the reaction was stopped by addition of 1200 μL of glycine-buffered isotonic saline. Then 500 μL was re-aspirated into the flowcell for the nonagglutinated particles to be counted.

Results

Analytical Variables

Calibration curve. A plot of the number of nonagglutinated particles, expressed as peak height vs S100 concentration, formed a sigmoidal curve extending from 0.3 to 80 μg/L (Figure 2). The curve was identical whether material of bovine or human source was used. Excess antigen decreased the agglutination for analyte concentrations exceeding 320 μg/L, which would have been read as 80 μg/L on the calibration curve. The minimum detectable concentration of bovine or human S100, defined as the concentration giving a peak height 2 SD lower than the mean of 20 peak heights measured in the absence of S100, was estimated to be 0.3 μg/L. Despite the use of aggregated rabbit F(ab')₂ to prevent nonspecific agglutination by putative anti-F(ab')₂ antibodies present in human serum and the use of Tween 20 to avoid protein interaction, latex coated with F(ab')₂ fragments of anti-S100 IgG was agglutinated by 18 of the 50 serum samples from the control group. This agglutination was nonspecific, and was no longer observed when the samples were automatically reduced with dithiothreitol before oxidation with hydrogen peroxide (see Materials and Methods). In contrast, the standard curve was unaffected by such treatment.

Analytical recovery and parallelism. We supplemented 20 sera to give various concentrations of S100, and assayed them. The mean percentages of added S100 accounted for were 94 (SD 2.55%) for 1.5 μg/L, 96 (SD 2.7%) for 6 μg/L, and 104 (SD 2.5%) for 24 μg/L. Six samples with S100 concentrations ranging from 1.4 to 24 μg/L were assayed at twofold to fivefold dilutions in normal human serum. Results for the diluted samples paralleled the calibration curve, and the CVs calculated for the results obtained for each sample at the various dilutions ranged from 2.5% to 9.9%.

Precision. To evaluate intra-assay precision, we assayed three supplemented serum samples containing 5, 10, or 50 μg of S100 per liter, repeating the assay 10 times on the same day. The respective CVs were 1.5%, 1.9%, and 6%. Interassay precision was assessed by assaying the same serum samples once each day for 15 days. The respective CVs were 8.5%, 9.1%, and 5.5%.

Inhibition test. For inhibition experiments, we added to the incubation mixture in-house-prepared rabbit anti-S100 IgG (30, 31). Under these conditions, inhibition was com-

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*Fig. 2. Calibration curve for the assay of bovine (—) or human (—) S100 by immunoassay by particle counting. The peak heights are directly proportional to the number of non-agglutinated particles. Bars indicate SE of daily determinations over a period of 15 days.*
plete for concentrations of S100 as great as 100 μg/L. In the absence of treatment by dithiothreitol, the agglutinations observed in the control samples were not inhibited by added rabbit anti-S100 IgG, indicating that these agglutinations were not specific.

**Specificity.** To check the putative cross-reaction of our anti-S100 antibody with the α subunit of S100, we assayed a few samples from patients with disorders characterized by increased serum S100α (αα) (6). The S100 concentration in serum from a patient with Duchenne’s muscular dystrophy was <0.3 μg/L. Of serum samples from 16 patients with coronary thrombosis, 12 had an S100 concentration <0.3 μg/L. In the other four, three of which were from patients who presented with cardiac arrest as a complication of coronary thrombosis, S100 concentrations were 2.1, 1.8, 1.5, and 0.6 μg/L. We found no correlation between the concentrations of S100 and creatine kinase in serum. We therefore consider that the anti-S100 antiserum we used is mainly directed against the β subunit of S100.

**Clinical Findings**

**Concentrations of S100 in serum in neurological disorders.** In the control group (n = 50) and in patients with multiple sclerosis (n = 50), dementia (n = 23), or meningitis (n = 20), the S100 value was <0.3 μg/L. Values exceeding 0.3 μg/L were found in two of 20 patients with meningoradiculitis (1.6 and 3.6 μg/L) caused by Borrelia burgdorferi, in one of 25 with Guillain–Barre syndrome (2.1 μg/L), in two of 20 with AIDS (2.3 and 6.5 μg/L), in two of 30 patients with peripheral neuropathy (0.6 and 5.8 μg/L), and in one of 14 patients with encephalitis (1.3 μg/L). The last-named group comprised three cases with subacute sclerosing panencephalitis, one infected with Epstein–Barr virus, and 10 with herpes simplex virus. In contrast, about 40% of patients with tumors of the central nervous system (39/75), and more than 90% of patients with vascular disorders (43/48), had

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Fig. 3. Distribution of S100 concentrations measured in sera of 65 patients with primitive tumors of the central nervous system, 10 with cerebral metastases, and 20 with melanoma.

The “miscellaneous” group included hemangioblastoma (n = 3), olfactory glioma (n = 2), ependymoma (n = 2), medulloblastoma (n = 2), and chordoma (n = 1). The data at top give the numbers of the patients in each group whose serum S100 exceeded 0.3 μg/L. Broken line represents the detection limit of the assay.

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Fig. 4. Distribution of S100 concentrations measured in sera of 29 patients with ischemic stroke (ο: stroke in the vertebral-basilar area), seven with intracerebral hematoma, and 12 with subarachnoid hemorrhage.

Broken line represents the detection limit of the assay, 0.3 μg/L.

S100 concentrations >0.3 μg/L.

Figure 3 further details results for the tumor group. All the patients with neurinoma, and some of the patients with glioblastoma (9/23), astrocytoma (4/12), or meningioma (6/9) had high values for S100. However, S100 was not detected in the serum from six patients with primitive lymphoma of the brain.

In the 10 patients with cerebral metastases, serum S100 was substantially increased in the three cases whose primitive tumors were small-cell lung cancer. No increase in S100 was found for metastases from other primary tumors, except in melanoma (see below).

Increased concentrations of S100 in serum were detected within the three first days in 43 of 48 samples from patients with vascular disorders of the central nervous system (29 ischemic strokes, seven intracerebral hematomas, and 12 subarachnoid hemorrhages) (Figure 4). Patients with transient ischemic attacks—i.e., with neurological deficits lasting <24 h—were not considered in this study.

**Serum S100 concentrations in melanoma.** The finding of increased concentrations of S100 in serum from two cases of cerebral metastases from melanoma prompted us to screen this kind of malignancy. S100 concentrations >0.3 μg/L were found in serum of nine of 11 cases with metastases, whereas no increase in S100 was detected in malignant melanoma at stage 1 (0/9).

**Discussion**

Because commercial kits for assay of S100 in serum were not available, we did not compare our technique with others. However, the analytical recovery and linearity, and the complete inhibition obtained by the use of another source of rabbit anti-S100 IgG, collectively showed the reliability of our immunoassay for S100 in serum. Despite the addition of aggregated F(ab')2 fragments from nonimmunized rabbit
serum to the incubation mixture to prevent interferences, latex particles were agglutinated by 18 of the 50 serum samples from the control group, even in the presence of rabbit anti-S100 IgG, which suggested that the reaction was nonspecific. Treatment of the samples with diethiothreitol eliminated these false agglutinations.

Latex was coated with Fab(\')2 fragments of rabbit IgG directed to bovine S100, which is a mixture of S100a and S100b with a respective subunit composition of \(\alpha\beta\) and \(\beta\beta\) (4). In contrast, S100 from human brain would be almost exclusively a dimer of \(\beta\) subunits (9). Recent studies described the presence of S100ao, a dimer of \(\alpha\) subunits in the human heart and skeletal muscles (5, 6), and increased concentrations (>12 \(\mu\)g/L) of this protein in serum samples from patients with coronary thrombosis (6). We observed identical curves when our assay was calibrated with either human or bovine brain S100 standards, and we failed to detect increased concentrations of S100 in sera of most patients with coronary thrombosis or Duchenne's muscular dystrophy. These data indicated that the anti-bovine S100 Fab(\')2 fragments of IgG we used were directed mainly towards the \(\beta\) subunit of S100. Therefore the protein detected in our assay probably originated in the brain.

The increase of S100 in serum in cases of primitive tumors of the central nervous system could have been expected, because immunohistochemical studies had shown this protein to be present in astrocytoma, glioblastoma, and neurinoma (23, 27).

It should be noted that the staining characteristics obtained with the use of a monoclonal antibody specific for the \(\beta\) subunit of S100 were similar to those observed with the commercially available polyclonal anti-S100 antibody that we used in this study (34, 35). However, increased concentrations of S100 were found with the same frequency in benign astrocytoma and in highly proliferating glioblastoma. Thus, serum S100 is not a marker of malignancy. In addition, S100 is not detected by immunohistochemistry in meningioma cells (12), but we observed high concentrations of S100 in the serum of six of nine patients with such a tumor. It is therefore likely in these cases that S100 is released by the surrounding brain tissue, which is the seat of edema and peri-tumoral gliosis. S100 in serum should not be considered as a marker of tumors of the central nervous system.

Regarding the increase of serum S100 in cases of melanoma, Gaynor et al. (19, 20) reported the presence of S100 protein in cultured human malignant melanoma and proposed this protein as a real immunohistochemical marker of this type of tumors. To our knowledge, ours is the first study on serum S100 from patients with melanoma. Even though the number of patients was too small to establish the real diagnostic power of the assay, our preliminary results emphasize the high frequency of increased concentrations of serum S100 in cases of metastases from melanoma (nine positive cases out of 11). In contrast, no increase in S100 was found in malignant melanoma at stage 1. The S100 assay in serum could therefore be useful to grade this malignancy. In contrast, oat-cell lung carcinoma is not known to contain S100 (12, 36) and the occurrence of S100 concentrations >0.3 \(\mu\)g/L in serum of cases of brain metastases is probably the result of the injury to the nervous tissue surrounding the lesion.

The very frequent increase in S100 concentrations in serum from patients with cerebrovascular diseases underlines the potential value of this protein as a marker of brain damage. Our results must now be correlated with the extent of lesions as detected by computerized tomography and the clinical data and outcome of these patients.

In conclusion, the results of this assay of S100 in serum confirm previous observations (30, 37) on the concentrations of S100 in cerebrospinal fluid, and suggest that this protein can be considered as an index to injury of the nervous tissue.

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