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Lack of Blindness Procedures in Alzheimer's Study

TO THE EDITOR: The recent article by Fukuyama et al. (*1*) raises the intriguing possibility that relative preponderance of synaptic versus cellular damage in Alzheimer's disease (AD) could be detected by the metabolic ratio between oxygen and glucose consumption. It is unclear, however, that the sample consisted of typical AD patients, considering their young age (58 ± 8 yr). Furthermore, the authors' use of the term "age-matched" is highly questionable. The controls were over 10 yr younger than the patients (47 ± 16 yr) and only the small sample size and high variance prevented this difference from being highly significant ($p < 0.07$). Finally, it is unfortunate that Dr. Hachinski's name was consistently misspelled and a citation to his scale was not provided. Readers who want to follow-up on this vague and misleading reference would be unable to find it as spelled.

My greatest concern, however, is with the apparent lack of any blindness procedures or other efforts to reduce bias. In this instance, the most interesting finding of this paper consists of a mismatch between oxygen and glucose utilization in specific cortical regions derived by placing small, circular ROIs in relevant areas by visual examination. If the investigators knew which scans belonged to patients and which were controls (and, in the case of AD, this is not a difficult distinction), it is hard to ensure the absence of bias in the manual placement of these ROIs. Furthermore, 4/9 patients and all controls had the two PET procedures on separate days, so even the choice of ROIs for CMRgl and CMRo2 determinations within a subject cannot be considered bias-free. Finally, were we to try and reproduce this finding, in the absence of rigorous definitions of these ROIs any lack of reproducibility would be hard to interpret. In other words, other investigators trying to replicate this study would be hard-pressed to place the ROIs in the same regions as used here, due to the lack of rigorous criteria for ROI placement.

The findings are intriguing, and sufficiently important to require replication and follow-up. However their interpretation, and eventual confirmation, would benefit from greater clarity.

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Ascorbate Plays a Major Role in Labeling Proteins with Technetium-99m

TO THE EDITOR: I read with interest the recent article by Hnatowich et al. (*1*) in which the authors studied the role of ascorbate in the reduction of antibody disulfide groups to sulfhydryls which provide binding sites for reduced ^{99m}Tc. The method, developed in our laboratory several years ago (*2,3*), showed that certain concentrations of sodium ascorbate (AA) was preferred for controlled generation of antibody sulfhydryls. Dithionite-reduced ^{99m}Tc was then incubated with AA-treated protein at pH 7.8–9 for subsequent radiolabeling in high yields. We also observed that under these experimental conditions, AA stabilized reduced ^{99m}Tc which bound to protein and prevented colloid formation.

In 1990, we determined the number of cysteine residues generated by the variety of reducing agents used in direct methods of labeling monoclonal antibodies with ^{99m}Tc (*4*). Although Ellman's reagent was readily available and commonly used to determine cysteine residues in protein, we chose not to use it in this assay because both AA and dithionite seriously interfered with the assay (*4,5*) and it was insensitive ($E_{412} = 1.36 \times 10^4\ cm^{-1}\ M^{-1}$) to accurately determine the low levels of cysteine residues generated in reasonable quantities of expensive antibodies. We considered several other spectrophotometric methods and chose the Ninhydrin method (*5*), for it had less interference from the reagents in question and was more sensitive ($E_{520} = 3.025 \times 10^4\ cm^{-1}\ M^{-1}$).

Using different chemical conditions, higher concentrations of protein, AA and dithionite, Hnatowich et al. (*1*) have determined the quantity of cysteine residues generated by Ellman's assay and ^{99m}Tc labeling efficiency by gel and paper chromatography. The basic findings of their investigations were that only 7% of the available cysteine residues were generated which were much less than we had observed with Ellman's assay of protein, incubated with 10 times higher concentrations of AA, (*4*) and that using protein incubated with 5 times the recommended concentration of dithionite, 5% of the cysteine residues were generated. In both cases, following incubation with protein, the reducing agents were removed since they interfere with Ellman's assays. Furthermore, Hnatowich et al. observed that when protein was treated with AA and labeled with ^{99m}Tc, after removal of AA, the labeling efficiency was much lower than we had reported; and that when protein was not treated with AA and incubated with ^{99m}Tc reduced with 5 times the dithionite concentrations than we had recommended, the labeling efficiency was 90%–95%. Based on these results, they have basically concluded that in reduction of protein disulfides, AA plays a minor role and dithionite a major one.

With the use of a relatively less sensitive Ellman's assay, with strong interference from the reagents involved, the different chemical conditions, the higher concentrations of dithionite (that may not have been fully eliminated by dialysis) and the low optical density readings, such results are plausible but not convincing. Furthermore, Mather et al. (*6*) and Pimm et al. (*7*) have independently observed that when protein was incubated with 2-mercaptoethanol, the other prominent agent used for reduction of protein disulfides, and removed, Ellman's assay showed no increase in cysteine residues and that the labeling efficiency with ^{99m}Tc was poor. The authors have attributed these results to reoxidation of reduced sulfhydryl groups and postulated that, because of the close proximity of adjacent thiol groups, reoxidation occurred too rapidly and prevented detection of sulfhydryls and ^{99m}Tc binding

in high quantities. Results of the AA incubation and its elimination experiments obtained by Hnatowich et al. appear only to be consistent with their findings.

In their dithionite experiment, not only did Hnatowich et al. use 5 times the recommended concentration, but, unlike our experimental conditions (pH 7.8–9) also used a low pH for incubation media which we measured to be 5.5–6. There is ample evidence in the literature (*8,9*) that dithionite quickly decomposes and forms a colloid that may be hard to detect at a low pH. Having no dithionite left to reduce protein disulfides, the reduced ^{99m}Tc will readily bind to the large quantity of colloid produced. This may not be separated from the protein, either by paper or gel chromatography and may be easily misunderstood as the radioactivity bound to the protein. The authors have neither performed HPLC nor have they confirmed that the radioactivity was bound to cysteine residues and not to other functional groups of the protein.

We realize that like the evidence provided by Mather et al. (*6*) and Pimm et al. (*7*) for 2-ME, and as discussed in various ways by Eckelman and Steigman (*10*) for other reducing agents, our testimony that AA reduces protein disulfides and not the dithionite is only indirect. However, additional independent support exists in the literature that AA-treated protein can be efficiently labeled with ^{99m}Tc by a transchelation technique in which no dithionite is involved (*11*).

Three separate clinical trials were performed in a series of patients in which two antibodies and one peptide labeled with ^{99m}Tc by the AA technique (*2,3*) were evaluated. These trials gave highly encouraging results (*12–14*) and the preparations appeared to be stable as indicated by in vivo distribution 3–4 hr postadministration.

Encouraged by these data, we performed additional experiments that are also indirect but more elaborate than just the paper and gel chromatography and, we believe, more convincing. We have shown that when dithionite-reduced ^{99m}Tc is added to 100–500 μg/ml of protein not treated with AA (final pH 7.8–9), only approximately 20% of the radioactivity binds to protein (*2*). When the Σ-amino groups on the unreduced protein are first blocked with fluorescein/isothiocyanate (FITC) and the protein is then incubated with dithionite-reduced ^{99m}Tc, this binding is significantly reduced. When protein was reduced with AA, amino groups were blocked with FITC and incubated with dithionite-reduced ^{99m}Tc, approximately 80% of the activity was bound to the protein. When protein was treated with AA and the sulfhydryls generated were subsequently blocked with iodoacetate (the excess of which was eliminated) and further treated with dithionite-reduced ^{99m}Tc, only about 15%–20% radioactivity was bound to protein. This is equivalent to nonspecific binding to Σ-amino groups. When protein is reduced with AA and none of the groups are blocked, labeling efficiency is 90%–95% (*15,16*).

These experiments indicate that when protein is not treated with AA but incubated with dithionite-reduced ^{99m}Tc (at pH 7.8–9) only approximately 20% of the activity is associated with the protein, most of which is bound to Σ-amino groups. These experiments, together with our spectrophotometric assay (*4*), show that the AA treatment of the protein generates cysteine residues to which approximately 80% of reduced ^{99m}Tc binds. We believe that these functional group blocking experiments provide additional evidence that it is AA that plays a major role in disulfide reduction and not dithionite. Our data further indicate that such nonspecific binding also occurs in some bifunctional chelating agent methods and that the nonspecific binding can be eliminated by FITC blocking (*15*). Thus, with our several years of experience with this method and with published and unpublished data generated by several such definitive experiments, we remain confident that under our experimental conditions, AA plays the major role in the generation of cysteine residues in protein molecules.

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REPLY: Regarding our recent article in the *Journal* (*1*), it may be worthwhile to stress again that we had no difficulty in radiolabeling two antibodies (Sandoglobulin IgG and B72.3 IgG) following the ascorbate/dithionite method as described (*2*), although it was necessary to increase the dithionite concentration by a factor of five. The issue is therefore not whether the labeling method works but rather the role that ascorbate plays in the process and, in particular, whether ascorbate is capable of reducing antibody disulfides. Furthermore, our conclusions were based on only two antibodies and should not be generalized to others.

We were aware that Ellman's reagent is slowly reduced by ascorbate and, therefore, unless precautions are taken, an erroneously *high* reading for antibody thiols may result. In our inves-