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Letters to the Editor

Sir:

A publication from Peng, Calton and Burnett (*1*) suggests that immobilization of proteins (antibodies) to supports with a constantly high retention of immunoreactivity involving the formation of a Schiff-base and subsequent reduction to a secondary amine with sodium borohydride or sodium cyanoborohydride has been problematical. However, the references cited for supporting the authors' point (Refs. *7–10*) are irrelevant as none of the referred papers mentioned diminished immunoreactivity of proteins upon immobilization except the authors' own, still unknown, unpublished results (Ref. *10*). Even Ref. *9* does not mention a Schiff-base type coupling chemistry at all as immobilization of anti-interferon monoclonal antibody was performed via *N*-hydroxysuccinimide-ester coupling.

The authors also suggest that catalytic amounts of Zn^{2+} or Al^{3+} are commonly used with sodium cyanoborohydride to reduce undesired side reactions and increase the reaction rate. The cited reference (Ref. *12*), however, is also irrelevant, as it does not mention at all the use of either Zn^{2+} or Al^{3+}. None of the papers published on cyanoborohydride coupling of proteins (*2–7*) suggest the utilization of Zn^{2+} or Al^{3+} as catalyst. In fact, heavy metal ions are frequently detrimental to proteins including monoclonal antibodies. When testing the coincident effect of Zn^{2+} or Al^{3+} and sodium cyanoborohydride on the stability of monoclonal antibodies (Fig. 3), the authors failed to perform adequate controls as to the inactivating effects of the ions.

The authors performed their experiments in an ELISA format in which antigen was first adsorbed to wells of microtiter plates. The authors assumed without any control that proteins adsorbed to microtiter plates are fully active. However, it has been reported that adsorption of proteins to plastic surfaces does induce conformational changes or leads to the denaturation of protein (*8,9*). Denaturation increases protein susceptibility to reducing agents. This is an important point to consider as the authors failed to remove the borohydride reagents from their antibody preparations prior to adding the treated antibodies to the antigen-

coated wells. The apparent enhancement or loss of antibody activities (Figs. 1–4) may well derive from the rearrangement of epitopes on plated antigens due to the combined distortional effect of adsorption to the plastic surface and the presence of reducing agents. The authors' observations are more consistent with alterations of antigen immunoreactivity as reduction of antibodies diminishes rather than increases antibody activity. Obvious experimental errors also question the validity of the authors' data as, e.g., on Fig. 3 anti-PA antibody activity at 80 μmol of sodium cyanoborohydride concentration is almost 120% while on Fig. 2 the same antibody activity under identical conditions is approx. 60–70% of the control. Similar experimental error can be found on the other antigen studied. The figures also show that lost immunological activity is sometimes restored at elevated borohydride concentrations (Figs. 1–4). This is very difficult to comprehend and may well be due to the very same inconsistency of the assay method.

The authors also claim that antibody immobilized to agarose or cellulose beads is only 10–20% active. This proposal is clearly unfounded, as immunoreactivity of immobilized antibodies may well reach 70–90% depending on the method of immobilization, the antibody concentration, and the nature of antigen (*10,11*). I agree with the authors proposal that monoclonal antibodies selected for immobilization should be characterized for stability to the immobilization reagents. However, the technique they selected is unsuitable for this purpose. Immobilization of antibody to solid support and subsequent determination of immobilized antibody reactivity is the simplest test to attain this goal. The evaluation of monoclonal antibodies under the actual conditions of immobilization is very important, as the conditions of ELISA differ significantly from that of covalent immobilization.

Sodium cyanoborohydride has been demonstrated to be a weak reducing agent (*12*) as it is even unable to reduce Ellman's reagent (*5*) and has also been found not to reduce disulfides of native proteins (*5,7*) at a concentration 1000 times higher than that shown by the authors. The reducing capacity of sodium cyanoborohydride is lower than that of glutathion, which maintains reducing intracellular milieu without being able to hydrolyze protein disulphides. Glutathion readily reduces Ellman's reagent while sodium cyanoborohydride does not.

In fact, a Schiff-base type chemistry in combination with sodium cyanoborohydride reduction offers numerous advantages over conventional self-coupling activated resins. The coupling pH range is very wide (between pH 3 and pH 10), that is to say, an optimal coupling pH is easy to select. Monoclonal antibodies vary greatly in their isoelectric point (*13*) which is a consideration for immobilization. Reduced antibody activities observed upon immobilization may also result from the alkaline coupling conditions imposed by the conventional self-coupling chemistries. It has also been shown that numerous monoclonal antibodies poorly tolerate

alkaline conditions (*14*) which certainly affect retained immunoreactivity when using protracted, alkaline coupling schemes. A coupling method that can be optimized for the individual proteins can greatly facilitate mild and efficient immobilization of monoclonal antibodies.

In conclusion, the problem the authors attempted to address is not supported by any legitimate reference as the papers cited in support to their argument are irrelevant to the subject. The authors are apparently unaware of key publications on sodium cyanoborohydride, all of them emphasizing the mild reducing capacity of this compound. Other statements they make are either untraceable from their references or run counter to well-established facts of immunoaffinity chromatography. The experimental data supporting their claim lack fundamental control experiments which then led the authors to erroneous conclusions. The data also show other significant inconsistencies suggesting methodological problems. It is particularly disturbing that data reported on different figures differ by as much as 100%, even though the experiments were performed under identical conditions. In light of these facts, I suggest that the authors retract and redo this communication.

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In response to the letter to the editor by P. J. Grandics:
Sir:

We regret that Grandics did not carefully consider our paper, its references, and its conclusions.

Grandics charges that our references do not indicate that Schiff base formation followed by borohydride reduction is problematic; however, two papers (our refs. *8,9*) by authors with the same company show that immobilization of a monoclonal antibody to interferon by *N*-hydroxysuccinimide ester gave a 30.9% retention of activity of the antibody (based on 30 mg human leukocyte interferon (IFLrA) being purified from 408 mg MAb), whereas Schiff base chemistry with the same antibody gave a 2.6% retention of activity (based on isolation of 550 μg IFLrA from 88 mg MAb). Since this process is utilized on an industrial scale, the method and yields involved are quite significant and are indicative of problems with the Schiff base/borohydride process.

We presumed that it was common knowledge that Zn^{2+}, Al^{3+}, or Ni^{2+} have been used in organic reactions with borohydrides due to the availability of reference works which document these uses over the last 25 years.[1] Our Ref. *12* used Ni^{2+}. However, due to potential color problems in the ELISA, we used Zn^{2+} and Al^{3+}. Since neither ion affected the activity of the monoclonal antibodies under the experimental conditions, the data was not inserted in the paper in order to decrease the size of Fig. 3.

The activity of immobilized divalent antibody, according to Eveleigh and Levy (Grandics' Ref. *10*), was no higher than 60% with purified antibodies while activities of 8.9–23% were reported in their Table 1. Grandics' Ref. *11* states that the "loss of immunoreactivity varies in extent (from 90% to less than 10%)." These statements would not lead one to believe that 70–90% immunoreactivities are routinely obtained, as implied by Grandics. In addition, it should be noted that most authors do not calculate their immunoreactivity on a molar basis nor report data which make it possible to do so. As in most papers, one does not normally begin a study of this type without first having encountered a problem in an experimental area. In our case, this problem arose in attempting to immobilize monoclonal antibodies to *Bacillus anthracis* which is referenced as our unpublished work (our Ref. *10*). Based on a poor retention of antibody activity, we attempted to vary the conditions of the experimental procedure to obtain high-level binding through a Schiff base type chemistry. This is due to the fact that we did not want to have contamination from murine antibody which is known to leak when cyanogen bromide activation is used (*see* Refs. *5,6*). Thus, the initial lack of retained antibody activity was encountered upon immobilization on aldehyde activated beads as well as in our ELISA assay. The use of the ELISA format provides a preliminary screen to characterize the chemical stability of antibodies. It is time-consuming to immobilize each clone followed by immunosorbent chromatography in order to determine the re-

activity of the antibody. We believe that the ELISA screen proposed in our paper is effective.

It should be pointed out that a number of different epitopes are always exposed due to variation in the hydrophobic domains of the immunoglobulins. It is unfortunate that Grandics did not notice that we had 100 μg of antibodies in Fig. 3, whereas 10 μg were employed in Fig. 2. Thus the effects of sodium cyanoborohydride in these two figures do not involve experimental error as Grandics asserts. Although there is a variation within the data, there is no comprehensible way that this could be interpreted as "lost immunological activity restored at elevated borohydrides" based on our Figs. 1–4. Dr. Grandics also misses the main point of our paper, which is that there is a difference between antibodies, which is exhibited in our Figs. 1–4.

As Grandics points out, sodium cyanoborohydride is a weaker reducing agent than sodium borohydride is also illustrated in our paper (Fig. 1 vs Fig. 2). We obviously agree that Schiff base type chemistry in combination with sodium cyanoborohydride reduction does offer numerous advantages over conventional self-coupling activated resins. This is certainly true when proper attention is paid to selection of the monoclonal antibody, thus Grandics' final paragraph is completely unfounded and we emphasize that our data do agree since the experiments were not performed under identical conditions.

Our conclusion remains that antibodies to be used for immunosorbent purification must be evaluated individually to determine whether their structure is stable to immobilization reagents.

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