Enhanced Performance of Immunoassays with Affinity-Purified Analyte-Enzyme Conjugates as Signal Generators

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In a competitive enzyme immunoassay, the performance was tested with different analyte-enzyme conjugates (signal generators) in their binding constants to antibody. Analyte (progesterone)-enzyme (glucose oxidase; GO) conjugates were chemically synthesized and purified by using a gel column with an immobilized antibody to progesterone. In an elution range from the column, four peaks were detected by measuring total enzyme activities. Results from further analysis indicated that the first peak contained mainly unreacted GO while the next three peaks conjugated GO with progesterone. These three conjugate preparations were compared in dose-response curves along with the unpurified mixture. The purified conjugates showed higher detection capabilities than did the mixture. Especially, the preparation in the second peak next to the free GO peak improved the detection limit five times. This performance was comparable to that of a progesterone-horseradish peroxidase conjugate that has been identified to have one progesterone ligand.

Introduction

The performance of competitive immunoassay can be enhanced by using well-characterized components of the assay system. Two components whose qualities are of great consequence are the binding protein, *i.e.,* antibody, and the labeled analyte (tracer) which acts as a signal generator. Soon after their advent[1] monoclonal antibodies have been used instead of traditional hyperimmune sera for analytical purposes. Polyclonal antibodies in hyperimmune serum typically vary from one bleed to the next and are of limited supply. A principal advantage of the use of monoclinal antibodies is that they are, in general, homogeneous chemical species. Likewise, for radioimmunoassays there is relatively little difficulty in producing well-defined radiolabeled tracers for most small analytes, since most radiolabeled analyte derivatives can be separated from non-reacted analyte molecules by using HPLC. However, the purification of analyte-enzyme conjugates as tracers for enzyme immunoassay is not readily achieved by using conventional phase- or size-based separation methods. The author has used immunoaffinity chromatography method to purify conjugates of analyte (progesterone) and horseradish peroxidase (HRP) on which lysine residues were utilized as functional groups.[2] In this communication, the previous investigations are extended to include the affinity purification of conjugates of progesterone and glucose oxidase (GO).

There are several advantages to separating enzyme conjugates. One of these is to obtain reproducibly a conjugate of high purity and of known composition. These separation methods would minimize lot-to-lot variations in the chemical synthesis of the conjugate. A second advantage is the selection of conjugates yielding standard curves which have different detection limits. This might be useful, for example, in studies where the expected concentration range for the analyte in a sample extends over several orders of magnitude, and could eliminate the need to dilute or concentrate the sample.

The utilization of GO-based conjugates as tracers is limited by the great number of potential products of the conjugation reaction, since the enzyme has at least 13 available lysine residues[3] as functional groups for the conjugation. This is in sharp contrast to HRP, which has only two available lysine residues[4,5] and whose reaction products may readily be separated.[2] In the latter case, adequate separation is predicated on the availability of a low-affinity monoclonal antibody for the separation step. In the current communication I have used the same low-affinity column for the purification of progesterone-GO conjugates.

Materials and Methods

Materials

Glucose oxidase (GO, type V from Aspergillus niger, 224000 units/g protein under non-oxygenated condition; EC. 1.1.3.4), Horseradish peroxidase (HRP, type VI, 300 units/mg solid; EC.1.11.1.7), 1,5-diaminopentane (cadaverine), poly-L-lysine hydrobromide (MW 421,000 by viscosity, test), and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma (St. Louis, MO, U.S.A.). Disuccinimidyl suberate (DSS), CNBr-activated Sepharose 4B, Bio Gel P-30, Immulon II microtiter wells, and a monoclinal antibody to GO (GO-Ab) were purchased from Pierce (Rockford, IL, U.S.A.), Pharmacia Fine Chemicals, Inc. (Piscataway, NJ, U.S.A.), Bio-Rad (Richmond, CA, U.S.A.), Dynatech Inc. (Alexandria, VA, U.S.A.), and Zymed Laboratories, Inc. (San Francisco, CA, U.S.A.), respectively. Progesterone-11a-hemisuccinyl-1,5 diaminopentane (P-CAD) was synthesized,[2] and the following monoclinal antibodies were produced as described elsewhere:[6] two antibodies to progesterone (P-Ab), one with a high affinity constant (BQ.1) and the other with a low affinity constant (4C10); one antibody to urease as non-specific antibody (NS-Ab) to progesterone, GO, and HRP.

Substrate for GO. The substrate solution for GO contained: 1.5 mL of a 0.12 g/mL glucose solution; 10 μL of 2 mg/mL HRP in 0.01 mol/L phosphate buffer, pH 7.0, containing 0.14 mol/L NaCl (PBS); 100 μL of 10 mg/mL TMB

in dimethyl sulfoxide; and 8.5 mL of 0.05 mol/L acetate buffer, pH 5.1.

Immobiliition of antibody in microtiter wells

Three monoclinal antibodies were immobilized separately on the surface of microtiter wells: P-Ab (BQ.1), GO-Ab, and NS-Ab. The immobilization was accomplished by a modified procedure of the sodium periodate method.[7,8] The surface of microwells was modified by poly-L-lysine, the lysine was reacted with antibody (10 μg/mL) activated by $NaIO_4$, and the resulting imide bonds were reduced with $NaCNBH_3$.[2] The treated wells were dried under vacuum and stored in the presence of silica gel as desiccant at 4 °C.

Conjugation of progesterone to GO

The progesterone derivative, P-CAD, was chemically reacted with the enzyme, GO, via DSS as a cross-linking reagent.[2] The reaction mixture of progesterone-GO conjugate was dialyzed for 1 d at 4 °C in PBS containing 0.02% (w/v) NaN_3 (Buffer 1). The dialyzed mixture was then purified by exclusion chromatography on Bio Gel P-30. The enzyme product was eluted with Buffer 1. Each elution fraction was analyzed for progesterone and for enzyme by a calorimetric assay (see below). The fractions containing the analyte-enzyme conjugate were pooled and stored at − 20 °C after adding an equal volume of glycerol until used for immuno-affinity purification.

Immuno-affinity chromatography of progesterone-GO conjugates

Immuno-affinity gel column was prepared by immobilizing the low affinity monoclinal antibody to progesterone, 4C10, on CNBr-activated Sepharose 4B.[2]

Chromatography. Before the conjugation mixture was applied to the immunoaffinity gel column (1.1 cm x 37 cm, 20 mL bed volume), the gel was equilibrated with Buffer 1. Then, the mixture containing about 4 nmol protein was loaded, and subsequently washed with Buffer 1 to separate the unbound enzyme from the bound. The bound conjugate was eluted with 0.1 mol/L sodium acetate buffer, pH 4.5, containing 0.5 mol/L NaCl, 5% (v/v) Tween-20, and 0.02% (w/v) NaN_3 (Buffer 2). The eluate from the column was delivered to a fraction collector (Model 1200 Pup, ISCO, Lincoln, Nebraska, U.S.A.) by a peristaltic pump (Type 4912A, LKB, Stockholm, Sweden). The elution rate was 2.3 mL/h and the fraction volume was 3.5 mL. The elution fractions containing Buffer 2 were dialyzed in Buffer 1.

After purification, the column was extensively washed with Buffer 2 until no progesterone eluted from the column. The elution fractions were analyzed for total enzyme and conjugated enzyme by calorimetric assays (see below). Fractions containing the conjugate were diluted with the same volume of Buffer 1 containing 0.5% (w/v) bovine serum albumin (Buffer 1 w/BSA), and stored at 4 °C. The enzymatic activity of the conjugates was preserved without loss for more than 1 year.

Determination of purified conjugate concentrations. The concentration of the affinity purified GO conjugate in solution was determined by comparing its activity in a solid-phase assay with standard concentrations of free GO as described elsewhere.[2] Solid-phase assays were performed with the immobilized GO-Ab.

Assays

The following assays were used for the analyses of the elution fractions from the chromatography columns. If not otherwise mentioned, all assays were performed in duplicate, and the means were used for the plots. Non-specific binding measured by NS-Ab was subtracted from total binding.

Colorimetric assays. Two calorimetric assays were performed to measure enzyme activities for the conjugated GO and for the total enzyme activity in eluates from the columns. For the quantitative determination of the enzyme components containing analyte, microwells were prepared with the immobilized high affinity P-Ah (coat with 200 μL per well of a solution of 10 μg/mL of antibody). Aliquots of eluate (10 μL) from the affinity columns and Buffer 1 w/ BSA were incubated in a total volume of 200 μL in the wells for 5 h on an orbital shaker. The wells were washed and the enzyme activities remaining in the wells were measured by a modification of the method described by Bos *et al.* :[9] (a) add 200 μL of the substrate solution prepared just before use, (b) develop the colored TMB derivative, (c) add 50 μL of 0.5 mol/L of sulfuric acid to stop the enzymatic reaction, and (d) measure the color intensity at the absorbance of 450 nm with a spectrophotometer (Titertek Multiscan, Type 310C; Eflab Oy, Finland). For the evaluation of the total enzyme activity, a liquid-phase assay was used, For this determination, 10 μL of eluate diluted to 1 : 10 with Buffer 1 w/BSA was transferred into non-treated microwells, and the enzyme activities were then measured as described above.

Dose-response curves Dose-response curves were obtained by competitive immunoassay with the immobilized high affinity P-Ah. A constant concentration of the affinity-purified progesterone-GO conjugate (20 μL of 7.4 pmol/mL) and different concentrations of progesterone in Buffer 1 w/BSA were added to the wells (total volume 200 μL), and incubated for 5 h on a shaker. After washing the wells, the signal from the labeled progesterone was measured as described above. The same procedure was repeated with a HRP conjugate with mono-liganded progesterone molecule (20 μL of 3.5 pmol/mL).[2] The dose-response curves were analyzed by the log-logit transformation.[10]

Results and Discussion

As demonstrated in a previous report,[2] the conjugation of small analyte molecules to an enzyme can yield the following two undesired results with regard to the quality control of the enzyme tracer for immunoassays: (1) multiple number of analyte ligands on the enzyme and (2) heterogeneous binding constant of the individual ligand. The conjugate with more than one analyte molecule may have a potential to bind to the binding sites of antibody by multi-attachment. Even if this is not the case, the binding constant of the conjugate is higher than that of the monovalent.[2] In either case, the multisubstituted conjugate binds more strongly than does the monosubstituted, and thus yields a lower detection capability. Further, the analyte ligands on the same enzyme may have different affinity constants according to the conjugation sites available on the enzyme.[2] Along with different numbers of ligands on each enzyme molecule, this factor results in the heterogeneity for the binding constants of the synthesized conjugate product. This

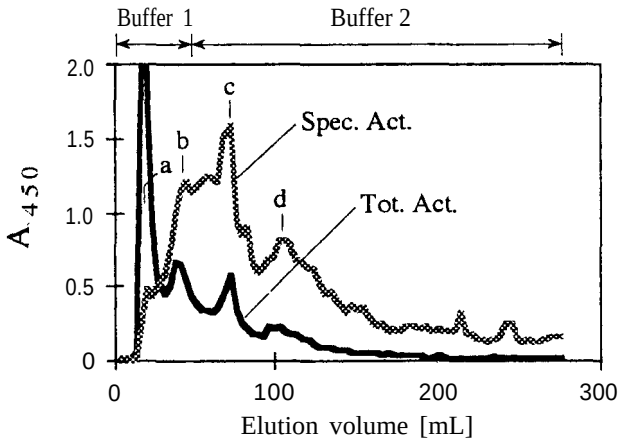

Figure 1. Purification of progesterone-glucose oxidase (GO) conjugates by immuno-affinity chromatography. Buffer 1 was a neutral pH buffer, and buffer 2 was an acidic buffer containing Tween. The dark curve represented the total activity (Tot. Act.) of GO and the gray curve the specific activity (Spec. Act.). Peak a contained free GO as major component, and peaks b to d represented different preparations of progesterone-GO conjugates.

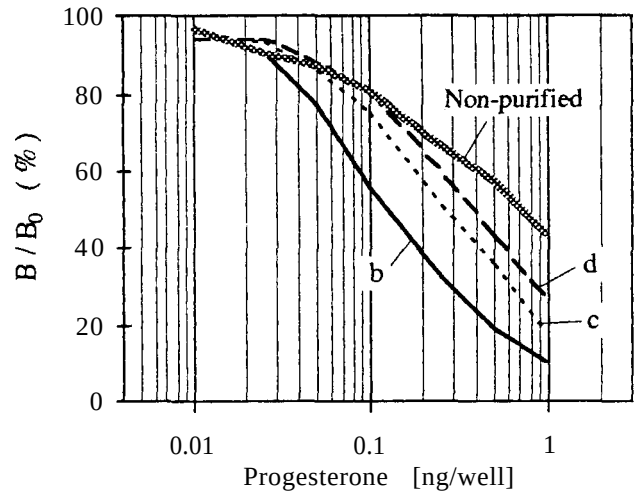

Figure 2. Dose-response curves of three affinity-purified progesterone-GO conjugates (b to d from Figure 1) and non-purified GO conjugate mixture. The non-purified mixture required more progesterone to obtain 50% of B/B_0 than did any purified conjugates.

feature also effects negatively on the standard curves of immunoassay with regard to the slope and the position.[11]

The enzyme, horseradish peroxidase (HRP), I previously used was comparatively small molecule and has only two available functional groups (lysine residues) per molecule for the conjugation.[4,5] These made an easy separation of the conjugates in homogeneous species with regard to the binding constant to antibody. A purified HRP conjugate with two analyte ligands had a low possibility of multiple binding to antibody on a solid matrix, and did not result in a large difference in the binding constants from a purified monovalent conjugate.[2] As a result, the purified monovalent HRP conjugate decreases the effective dose of analyte at 50% displacement of the bound conjugate (ED50) only about 1.5 times as compared to that of the non-purified under constant conditions.

However, the situation with glucose oxidase (GO) is different from that with HRP as to at least two regards: (a) the molecular size of the enzyme and (b) the number of functional groups (lysine residues). GO (M_r=160,000) is four times larger than HRP (M_r=40,000), and has more than 13 lysine residues.[3] First, due to the larger number of the functional groups, the synthesized GO conjugate mixture would have a wider range of heterogeneity for the binding constant and a higher average number of analyte ligands per enzyme molecule. If compared with a monosubstituted conjugate as tracer in immunoassays, the GO mixture would result in a far higher ED^{50}. Thus, the purification of this conjugate by the immunoaffinity chromatography is very meaningful for the reproducible preparation and the utilization in immunoassays for a better detection limit.

Separation of conjugates

Immunoaffinity purification. The progesterone-GO conjugates were purified on an affinity gel column with an immobilized antibody of low affinity to progesterone. The affinity chromatography was carried out by eluting first with a neutral pH buffer (Buffer 1 in Figure 1), and then with an acidic buffer containing Tween (Buffer 2). After dialyzing the elution fractions containing the acidic buffer in the neutral pH buffer, all fractions were then analyzed by two calorimetric assays: a liquid-phase assay and a solid-phase assay with the immobilized antibody to progesterone (P-Ab). The liquid-phase assay does not discriminate between the free and conjugated enzyme molecules, and thus reflects the total GO activity (Tot. Act., Figure 1) in the eluent. In contrast, the solid-phase assay detects only the conjugated enzyme molecules captured by the solid-phase antibody, *i.e.*, the specific GO activity related to progesterone (Spec. Act.).

At least four different fractions of GO activity (peaks a to d, Figure 1) could be separated by immunoaffinity chromatography. Peak a contained the free GO as major component since the total activity was much higher than the specific activity. The last three preparations of conjugate in peaks b to d were retained by the progesterone affinity column, and also showed significant specific GO activities related to progesterone. Therefore, these preparations contained the GO-progesterone conjugates.

Dose-response curves. After pooling fractions from each peak, the three conjugate preparations (b to d from Figure 1) were evaluated and compared with the non-purified mixture in dose-response curves (Figure 2). The curves were obtained from solid-phase calorimetric assays in which the amounts of the conjugates per well were controlled to generate an approximately equal amount of signal from the oxidized chromogen (TMB) within a defined time. The conjugates b to d gave parallel dose-response curves with an increasing ED_{50} (effective dose of progesterone at 50% displacement of the bound conjugate, *i.e.*, 50% of B/B_0) of the sigmoidal curve in the same order (Table 1). The assay performed with conjugate b gave about five times lower ED_{50} than did the non-purified preparation, *i.e.*, responded five times more sensitively to the analyte concentration.

As shown in the previous report the dose-response curve

Table 1. Comparison of ED50 from dose-response curves (Figure 2).

GO-progesterone	ED_{50} [ng/well]
b	0.144
c	0.261
d	0.368
Non-purified	0.712

with a non-purified HRP preparation located in about average position of those with the homogeneous conjugated constituents.[2] It is noteworthy that the dose-response curve with the non-purified GO mixture was not placed in an average location of those curves with purified conjugates b to d (Figure 2). This suggests that GO conjugates which were highly substituted with progesterone ligands strongly bound to the immobilized antibody in the affinity gel column, and did not subsequently elute out from the column in a monitored range.

Comparison of conjugation ratios

In affinity chromatography, the retention time of the conjugate in the affinity column depends on the number of ligands on the enzyme, provided that each ligand has an identical affinity constant to the antibody immobilized in the affinity column. It may be possible to determine the conjugation ratio if the conjugates are separated into homogeneous populations. However, the chromatography results (Figure 1) showed that the neighboring peaks seemed to overlap, and each peak may contain heterogeneous populations of conjugates. Although the characterization of all conjugate preparations is difficult, I attempted to assess the quality of conjugate b (Figure 1) which, due to its low ED_{50}, was of interest for immunoassays.

The quality of labeled analyte, *e.g.*, binding constant and homogeneity, affects the location and slope of a dose-response curve. Other major factors are the concentrations of the labeled analyte and the immobilized antibody. If a constant antibody concentration is used, variations of the binding constant and concentration of the labeled analyte do not alter the slopes of dose-response curves at each inflection point, *i.e.*, only position is varied.[12] This is true only if the labeled analyte is homogeneous with respect to the binding constant.[2] It is conceivable that a mixture of the labeled analytes with different binding constants shows a broader slope of the curve than does a homogeneous tracer.[13]

The quality of the purified GO conjugate b (Figure 1) as signal generator in immunoassay was compared in dose-response curves with that of a progesterone-horseradish peroxidase (HRP) conjugate that has one progesterone ligand.[2] The curves were obtained using the same batch of microwells with the immobilized P-Ah (Figure 3). The concentration of the HRP conjugate was two times lower than that of the GO conjugate since the signal from the bound HRP developed very rapidly. Although these different conditions for the two tracers were relatively unfavorable to the GO conjugate *(i.e.*, higher concentration), the dose-response curve with this enzyme conjugate showed a higher detection capability. This can result from a lower binding constant of the GO preparation. However, the slope of the sigmoidal curve with the GO conjugate was significantly broader than that with the HRP conjugate, and the two curves were eventually crossed at a high concentration range of analyte. From these results, I conclude that the GO preparation contained heterogeneous species of conjugates of which intrinsic binding constants (binding constant measured from the interaction of an enzyme with one analyte ligand with one binding site of antibody) could be lower than that of the HRP conjugate.

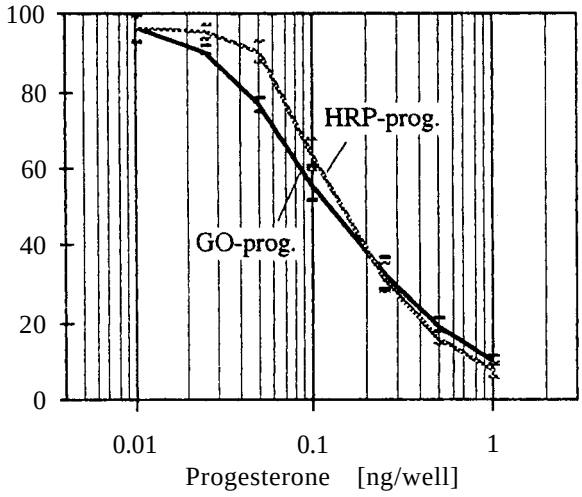

Figure 3. Comparison of dose-response curves of an affinity-purified progesterone-GO conjugate (from peak b in Figure 1) and a defined progesterone-HRP with mono-ligand.

It is noted that GO conjugate b in Figure 1 is eluted with a retention volume (subtracted the elution volume from that of peak a) of 17 mL which is lower than that of the HRP conjugate (33 mL)[2] under constant conditions. This is not unexpected that the analyte ligand on the larger enzyme, GO, would have a lower interaction frequency with the immobilized antibody on the gel column than has that on the smaller enzyme, HRP.

In conclusion, an intensive purification of comparatively large enzyme conjugates with a potentially high number of analyte ligands has to be emphasized before the utilization as signal generators in immunoassay. This would be valuable to obtain a reproducible preparation of the signal generator and to decrease the detection limit of the analytical systems. In the sense that the larger molecular size of enzyme in the conjugate has a substantial effects on the affinity purification, several parameters must be optimized to obtain a desired purified conjugate in a large quantity: (1) the average conjugation ratio of the synthesized conjugates, (2) the binding constant of an antibody immobilized on the chromatography gel, (3) the gel column capacity, and (4) the elution conditions of the bound conjugate from the column.

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Synthesis and Ion Binding Properties of Rebek's Cleft-Type Ionophores Bearing Two Convergent Carboxylic Acid Functions

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A series of new ligands having convergent dicarboxylic acid functions, based-upon Rebek's cleft-type ionophore, have been prepared and their ion binding properties were investigated by the competitive extraction and transport experiments. The main purpose of the modification was to increase the lipophilicity of the Rebek's ionophore, which was attempted by utilizing propyl analog of Kemp's triacid or by changing the bridging unit. Ionophores **5** and **6** were found to have a pronounced ion-binding property toward Ca^{2+} ion. The selectivity in competitive extraction of ionophore **5** at pH 9 for Ca^{2+} over Mg^{2+} and Sr^{2+} is 2.0 and 59.3, respectively. The selectivity in competitive transport of ionophore **5** for Ca^{2+} over Mg^{2+} and Sr^{2+} is 29.8 and 99.3, and that of ionophore **6** is 10.0 and 23.2, respectively.

Introduction

The design of a molecular recognition system for a chemically and biologically important guest is a very attractive research area.[1~3] A great number of studies to develop selective completing agents have been performed aiming for the applications in the fields of separation and analytical sciences as well as biological and medical studies.[4~7] Recently, chemists have a strong interest in Kemp's triacid as a key motif for the design of organized structures as well as molecular recognition devices.[8] The Kemp's triacid has three axial carboxylic acid groups on a cyclohexane ring framework and the triaxial conformation of the three carboxylic acids forces a U-shaped relationship between any two of them. Rebek *et al.* [9,10] have synthesized a variety of interesting molecules having a cleft-like shape from Kemp's triacid for the recognition of many biologically interesting guests. The design principle is the formation of new structures by utilizing the more basic syn lone pairs of carboxylate, along with the convergent functionality of the carboxylate functions. Among them, they prepared a new type of ionophore based-upon the Kemp's triacid as shown in Figure 1 and found that the ionophore showed an extraordinary selectivity in binding calcium and magnesium ions.[11]

In contrast to its great potential as a specific ionophore, however, there are few reports on the application to chelating agents or carriers for metal ions.[12,13] We prepared a series of new carriers based on the Rebek's original structure, having a purpose to develop a simple but efficient ligand or carrier for calcium ion. The main strategy is to increase the lipophilicity with the molecular framework intact: that is the substitution of equatorial methyl group with more lipophilic propyl group and the utilization of methylene dianiline or more lipophilic aromatic ring instead of simple *m*-xylidine diamine of the Rebek's ionophore. The ionophoric behavior of the prepared compounds was investigated by means of the competitive extraction and competitive transport through liquid membrane of alkali and alkaline earth metal cations.

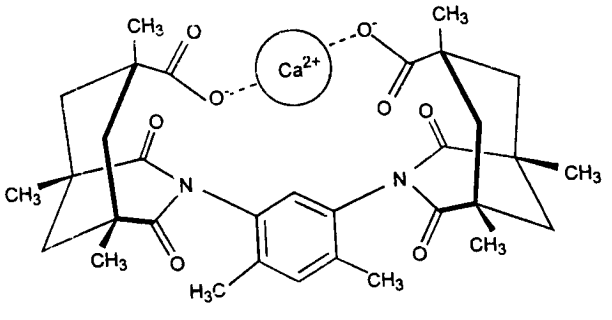

Figure 1. Kemp's triacid-based chelating agent developed by Rebek *et al.*