Comparison of Double-Antibody Radioimmunoassay with Farr-Technique Radioimmunoassay and Double-Antibody Enzyme Immunoassay for $\alpha$-Fetoprotein

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We describe double-antibody procedures for determining $\alpha$-fetoprotein in human serum. An equilibrium procedure can be done in 24 h with a sensitivity of at least 4 µg/liter and coefficient of variation of 5.5%. There are no interferences from normal human sera or sera with certain commonly seen chemical abnormalities. We also describe and discuss sequential procedures that range in sensitivity from 250 ng to 1 µg/liter and require 24–48 h incubation. The precise (mid-range) portion of the dose/response curve for sequential procedures can be shifted to higher or lower values by an adjustment of the time of preliminary incubation of antibody with unlabeled antigen. With a 37°C incubation, a sequential procedure can be completed in 7 h. Sensitivity is 1 µg/liter, and coefficient of variation 8.0%. The relative merits of the above assay procedures are discussed. The double-antibody radioimmunoassay is twice as sensitive as the Farr procedure [J. Infect. Dis. 103, 239 (1958)], and it is free of the large and variable nonspecific precipitation that accompanies the precipitation of bound antigen with sodium sulfate solution. Double-antibody radioimmunoassay is superior to enzyme immunoassay in both sensitivity and precision.

Additional Keyphrases: normal values • concentrations in serum during pregnancy, and in amniotic fluid • anencephaly • spina bifida • diagnostic aids • radioassay • inter-method comparison

Radioimmunoassay techniques have been useful in establishing the diagnostic value of serum $\alpha$-fetoprotein determination. In 1964, Tatarinov (2) detected this fetal protein in the serum of a patient with hepatocellular carcinoma. Using gel techniques similar to Tatarinov's, other investigators subsequently detected it in serum of patients with embryonal germ cell malignancies (3), hepatitis (4), or cirrhosis (4).

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procedure precise and reproducible enough for such studies that the efforts described here were primarily directed. However, determination of serum \( \alpha \)-fetoprotein has useful clinical application other than in studies of pregnancy, and in these areas the requirements for the assay with respect to its range, precision, and speed may be quite different. We demonstrate with manipulation of the sequential procedures that by using identical antigen and antibody preparations throughout we can alter preincubation times, postincubation times, and incubation temperatures to work out procedures adaptable to various clinical problems.

We also compare the performance of the double-antibody radioimmunoassay to that of two other assay procedures now being used to estimate \( \alpha \)-fetoprotein in human serum: another radioimmunoassay in which \( \alpha \)-fetoprotein is precipitated by a salting-out procedure, and a double-antibody enzyme immunoassay.

Materials and Methods

Purification of Human \( \alpha \)-Fetoprotein and Preparation of Rabbit Antiserum to It

We used a modification of the procedure of Ruoslahti et al. (21) to isolate \( \alpha \)-fetoprotein from ascitic fluid from a hepatoma patient; it contained about 75 mg of \( \alpha \)-fetoprotein per liter:

Dilute the ascites fluid to a concentration of 2 g of total protein per liter and incubate a volume fourfold the matrix volume of the immunoabsorbent with agarose (Sephrose 4B; Pharmacia, Piscataway, N.J. 08854) to which goat anti-human \( \alpha \)-fetoprotein has been conjugated. Stir the slurry overnight at 4 °C, then filter through a coarse sintered-glass filter and wash the Sepharose with 10 times its volume of phosphate-buffered saline (sodium phosphate buffer, 10 mmol/liter, pH 7.5; NaCl, 150 mmol/liter; and NaN\(_3\), 10 mg/liter). Elute the \( \alpha \)-fetoprotein from the Sepharose with two washes each of 4 and 8 mol/liter urea, the volume used for each wash being twice the matrix volume. Combine these eluates and remove urea by passage through a dialysis unit (C-dak artificial kidney, Model 5; Cordis-Dow, Miami, Fla. 33137). Concentrate the eluate in a membrane concentration unit (Amicon, Lexington, Mass. 02173) and then incubate 16 h at 4 °C successively with Sepharose-conjugated anti-normal human serum and anti-goat serum to remove traces of these materials. Concentrate the \( \alpha \)-fetoprotein preparation to a volume of 3 to 5 ml for fractionation on a column of Sephadex G-200 (Pharmacia), using a 1.5 \( \times \) 100.0 cm column with a bed volume of 200 ml. Elute with phosphate-buffered saline and collect 10-ml fractions. Pool the fractions corresponding to the major peak and again concentrate to about 2 g of protein per liter.

The purified \( \alpha \)-fetoprotein at a concentration of 2.5 g/liter gave no precipitation on double diffusion in agar, immunoelectrophoresis, or counterimmunoelectrophoresis with goat anti-normal human serum or burro anti-goat serum.

Electrophoresis of the purified protein on polyacrylamide gel produced a single major band and a slower moving minor band. Chromatography of \( ^{125}I \)-labeled \( \alpha \)-fetoprotein on a column containing Sephadex G-200 also gave evidence of minor heterogeneity, a leading shoulder appearing on the excluded peak. Although equivalent samples of radiolabeled \( \alpha \)-fetoprotein from both the shoulder and central regions of the peak produced similar antibody dilution and dose-response curves in the double-antibody radioimmunoassay, nonspecific precipitation by sodium sulfate solution in the Farr procedure (1) was twice as great in the case of protein represented by the shoulder region (16% vs. 8%). The minor component of the purified preparation may be an isoprotein of \( \alpha \)-fetoprotein, as described by Alpert and Perencevich (22); however, the behavior of the labeled protein is more compatible with the presence of a small amount of dimer.

The protein concentration of the purified material was determined by a micro-Kjeldahl procedure (23) and \( \alpha \)-fetoprotein concentration was determined by radioimmunoassay with respect to the World Health Organization international reference standard 72/225, supplied by Dr. Ph. Sizaret, International Agency for Research in Cancer, Lyon, France. This standard preparation, at a nominal concentration of 50,000 International Units (int. units)/ml was found to be 0.70 ng/int. unit with respect to our preparation. 4

The purified \( \alpha \)-fetoprotein was used to raise antisera in rabbits. For the primary immunization, 100 \( \mu \)g of \( \alpha \)-fetoprotein in complete Freund adjuvant was injected intradermally at multiple sites on the back. Six weeks later, injections of 100 \( \mu \)g each, in incomplete Freund adjuvant, were made on two consecutive days, the first subcutaneously and the second intramuscularly. At intervals of six to eight weeks, the animals received booster injections of 25 \( \mu \)g of \( \alpha \)-fetoprotein in incomplete adjuvant, administered subcutaneously.

In most of this study we used an antiserum produced in rabbits by an immunization procedure similar to that above, except that the primary injection was 200 \( \mu \)g of \( \alpha \)-fetoprotein and the secondary doses were 0.2 to 1.0 mg. These antisera reacted with cord blood, amniotic fluid, and the ascites source when tested by double diffusion and counterimmunoelectrophoresis. They did not react with normal human or goat serum. Both antisera were identical in their performance in radioimmunoassay procedures, except for titer. The first antiserum was used at a final dilution of 60,000, the second at 240,000.

\( ^{125}I \)-labeled \( \alpha \)-Fetoprotein

Pure \( \alpha \)-fetoprotein was labeled with \( ^{125}I \) (Amer- sham-Searle, Arlington Heights, Ill. 60005) by the Chloramine T procedure of Hunter and Greenwood (25). Usually 20 \( \mu \)g of \( \alpha \)-fetoprotein was labeled with 1.0 to 1.5 mCi of \( ^{125}I \). Efficiency of labeling ranged from 50 to 65%, and the specific activity of the labeled protein

4 The range of values for the WHO standard was found by 11 expert laboratories to be 0.54 to 1.81 ng/int. unit (24).
was 20 to 30 Ci/g. The \(^{125}\)I-labeled \(\alpha\)-fetoprotein was
separated from the reactants, including free \(^{125}\)I, by
passing the reaction mixture through a column contain-
ing Sephadex G50 (Pharmacia), which had been conditioned with 10 mmol/liter phosphate-buffered saline containing 20 ml of normal goat serum per liter. About 98% of the \(^{125}\)I-labeled \(\alpha\)-fetoprotein preparation
was precipitated by trichloroacetic acid immediately
after the labeling procedure. This fraction decreased
with aging of the labeled protein, and when it had
reached 85%, we cleared the solution of free \(^{125}\)I by
repeating the separation on Sephadex G-50.

\(\alpha\)-Fetoprotein Standards

Standard doses for radioimmunoassay were prepared
in the above-described goat serum/phosphate-buffered
saline, either from purified \(\alpha\)-fetoprotein or by serial
dilution of a pool of cord blood serum whose \(\alpha\)-fetoprotein
content was determined by radioimmunoassay in
reference to the World Health Organization reference
preparation 72/225 (\(\alpha\)-fetoprotein, 50 mg/liter).

Anti-Rabbit IgG (Second Antibody)

Precipitating second antibody was raised in sheep by
using commercial rabbit IgG, purified by chromatog-
raphy on diethylaminoethyl cellulose (Miles Labora-
tories, Elkhart, Ind. 46514). Primary injection was 5 mg
of rabbit IgG in complete Freund adjuvant, with
monthly boosters of 1 mg of IgG in incomplete Freund
adjuvant until an acceptable titer was reached. On
immunoelectrophoresis, normal rabbit serum reacted with
this antiserum to produce a single arc.

Assay System and Procedures

The general procedure is that used in other double-
antibody radioimmunoassays. \(^{125}\)I-labeled \(\alpha\)-fetoprotein
and unlabeled \(\alpha\)-fetoprotein from samples and standard
doses are incubated with rabbit-anti \(\alpha\)-fetoprotein for
a period of time to produce antigen–antibody com-
plexes. These complexes are precipitated with sheep
anti-rabbit IgG, with use of normal rabbit serum as a
carrier. Bound and free radioactive fractions are sepa-
rated and activities determined.

Tubes in which zero doses, standard doses of \(\alpha\)- fetoprotein,
and samples of material for dose estimate are
incubated are made up with 800 \(\mu\)l of normal goat serum
in phosphate-buffered saline (250 ml/liter), 200 \(\mu\)l of
dose or sample, 250 \(\mu\)l of \(^{125}\)I-labeled \(\alpha\)-fetoprotein, and
250 \(\mu\)l of rabbit anti-\(\alpha\)-fetoprotein serum of appropriate
dilution. Both the labeled \(\alpha\)-fetoprotein and the anti-
\(\alpha\)-fetoprotein (first antibody) are diluted in normal
rabbit serum, 25 ml/liter. For the zero-dose tube,
the dose aliquot is replaced with buffer.

Control tubes: (a) No-antibody control. This solution
has the same composition as that in the zero-dose tube,
extcept that the first antibody aliquot is replaced by an
equivalent volume of 25 ml/liter normal rabbit serum.
When precipitated with second antibody, the bound
activity of this control gives a measure of the nonspecific
precipitation of labeled \(\alpha\)-fetoprotein. (b) Antigen alone.

This solution has the same composition as that for the
no-antibody control; however, second antibody is re-
placed by an equivalent volume of the 25 ml/liter nor-
mal rabbit serum. Bound activity in this control is a
measure of nonspecific precipitation in the absence
of any precipitating agent. (c) Protein-bound \(^{125}\)I. This
solution also has the same composition as that for the
no-antibody control. The second antibody addition
is replaced by the addition of an equivalent volume
of trichloroacetic acid solution (400 g/liter). The protein-
bound radioactivity in the labeled \(\alpha\)-protein preparation
is determined from the activity in either the free or
bound fraction as a fraction of total activity.

Incubation: Schedules for incubation are as follows.
All incubation can be at either room temperature or 4
\(^\circ\)C, except where otherwise indicated.

- 24-h equilibrium procedure: Standard and test
doses of \(\alpha\)-fetoprotein are incubated together with la-
beled antigen and first antibody for 16 to 20 h.

- 40-h sequential procedure: Standard and test doses
are preincubated with first antibody for 16 h before la-
beled antigen is added. Incubation is then continued for
an additional 16 to 20 h.

- 24-h sequential procedure: Standard and test doses
are preincubated with first antibody for 6 h at room
temperature before labeled antigen is added. Incubation
is continued overnight or for 16 h.

- 7-h sequential procedure: Standard and test doses
are preincubated for 4 h at 37 \(^\circ\)C before labeled antigen
is added. Incubation is continued for an additional 2 h
at the same temperature.

Precipitation with second antibody: Sheep anti-
rabbit IgG, used as the precipitating second antibody,
is diluted in phosphate-buffered saline to a concentra-
tion such that 500 \(\mu\)l of diluted antibody will optimally
precipitate the normal rabbit IgG, which serves as car-
ier in the assay, and with it the \(\alpha\)-fetoprotein/anti-\(\alpha-
\)fetoprotein complexes formed. The second-antibody
dilution is determined by a preliminary equivalence
zone test against the volume and concentrations of
normal rabbit serum used. To terminate the assays, 500
\(\mu\)l of dilute second antibody is added to all tubes of the
assay system except the protein-bound \(^{125}\)I and antigen
clon tubes. Contents of tubes are vortex-mixed and
allowed to stand for 6 h at either room temperature or
4 \(^\circ\)C, except for the 37 \(^\circ\)C sequential procedure, in which
the incubation is also at 37 \(^\circ\)C and for a period of 1 h.

Internal standards: All assays were monitored by
including an internal standard of normal human serum
adjusted to an \(\alpha\)-fetoprotein concentration of 100 \(\mu\)g/
liter and another 10 \(\mu\)g/liter by the addition of pure
\(\alpha\)-fetoprotein or cord blood standard. On assays run
repeatedly, running charts were kept on zero-dose
binding, 50% intercept, and within- and between-assay
variance, as suggested by Rodbard et al. (26).

Calculations and Selection of
the Dose/Response Curve

After centrifugation of an assay mixture, 1 ml of the
supernate is removed for counting. The remainder of
the supernate is aspirated from the precipitate and the activity of both fractions is counted to an accuracy of 1%. Total counts for $^{125}$I-labeled $\alpha$-fetoprotein are determined from either (a) precipitate count of the protein-bound $^{125}$I tube, (b) the mean total count of the control tubes corrected for free $^{125}$I in the protein-bound $^{125}$I supernate, or (c) the individual total counts of each sample tube calculated as follows:

$$\text{Total} = (P_1 - P_0) + 2(S_i - S_0)$$

where: $P_1 =$ activity in the precipitate  
$P_0 =$ bound activity in the no-antibody control tube  
$S_i =$ activity in 1 ml of supernate  
$S_0 =$ activity in 1 ml of the protein-bound $^{125}$I supernate

In cases where variation in total counts for the separate samples exceeded the error in the count of the separate fractions, totals for individual samples were used in calculations; otherwise either the mean control count of the mean protein-bound $^{125}$I bound count, each appropriately corrected, was used as the total count.

Dose/response curves were plotted as the ratio of the fraction of $^{125}$I-labeled $\alpha$-fetoprotein bound at a given dose to the fraction bound at zero dose:

$$\frac{B/T}{B_0/T} = B/B_0$$

against log dose (micrograms of $\alpha$-fetoprotein per liter).

**Results**

**Sensitivity of Antibody and Immunoreactivity of $^{125}$I-labeled $\alpha$-Fetoprotein**

To determine that the antibody used had the sensitivity to detect the minimum $\alpha$-fetoprotein concentrations of normal human serum (2 to 4 $\mu$g/liter), we ran antibody dilution curves with concentrations of $^{125}$I-labeled $\alpha$-fetoprotein bracketing these values, 0.5 and 5.0 $\mu$g/liter (Figure 1). The shift in binding at the same antibody dilution indicates that the required sensitivity is indeed present.

Further, to compare the affinity of the antibody for unlabeled $\alpha$-fetoprotein as compared to $^{125}$I-labeled $\alpha$-fetoprotein, we added doses of 5.0 $\mu$g of $\alpha$-fetoprotein per liter, made up of 0.5 $\mu$g of labeled antigen and 4.5 $\mu$g of unlabeled antigen per liter, to a series of tubes and ran an antibody dilution curve. This curve was essentially coincident with that run for 5 $\mu$g of labeled $\alpha$-fetoprotein per liter alone, indicating comparable affinity of the antibody for both species of the antigen.

**Selection of $^{125}$I-labeled $\alpha$-Fetoprotein Concentration and Sensitivity of Dose/Response Curve**

Based on the study of Hunter (27), the minimum sensitivity of radioimmunoassay is generally equal to about twice the dose of labeled antigen, and at this minimum dose, the labeled antigen is bound at 90% of the maximum, or zero-dose, binding. Accordingly, we chose a concentration of $^{125}$I-labeled $\alpha$-fetoprotein of 1.5 $\mu$g/liter for the assay, and the antibody dilution curve indicated that this amount of $^{125}$I-labeled $\alpha$-fetoprotein is 50% bound by an antibody dilution of 1/80 000 in the antibody aliquot of the assay system (final dilution 1/480 000). An equilibrium dose/response curve run with these concentrations of $^{125}$I-labeled $\alpha$-fetoprotein and antibody indicates that $^{125}$I-labeled $\alpha$-fetoprotein is bound at 90% of maximum at 4 $\mu$g/liter of dose $\alpha$-fetoprotein. This 10% fall from zero-dose binding compares with the mean slope of the standard curve of 12% from 5 to 160 $\mu$g/liter (five doublings of dose), thus meeting another of Hunter’s criteria for minimum sensitivity. In all standard curves run at these concentrations, binding for a 4 $\mu$g/liter dose did not overlap with any of those for the next lowest dose of 2 $\mu$g/liter and, according to this practical criterion, could be reliably detected.

**Optimization of Specific Activity of the Labeled Antigen**

A target specific activity for the $^{125}$I-labeled $\alpha$-fetoprotein was selected so that on the average, the precipitate counts and those of 1 ml of the supernate could be counted to an accuracy of 1% in 1 min or less (10 000 counts). In an assay which binds 50% at maximum, the mean dose binds 25% of the counts, and the total counts in the tube should be about 40 000 to be counted to the desired accuracy in this time. We estimated that labeling could be conveniently performed every six to eight weeks, and that our laboratory demand for $^{125}$I-labeled $\alpha$-fetoprotein (allowing for waste and manipulative loss) would be 20 $\mu$g. At the time that the mean assay would be performed, the $^{125}$I-labeled $\alpha$-fetoprotein would have decayed to 0.75 of its original level.

Ideal specific activity should then be:

$$4.0 \times 10^4 \text{cpm} \times 10^3 \mu g/g \times 10^6 \text{dpm}/\mu Ci = 20 \text{Ci/g}$$

where 1.5 ng = dose/tube; 0.75 = decay factor at mean time; 0.80 = counter efficiency.
Effect of Normal Human Serum on Dose/Response Curve

To detect any interference with the determination of α-fetoprotein in normal human serum, we made up parallel standards from purified α-fetoprotein in both normal goat serum and normal human serum from which α-fetoprotein had been removed by absorption. We ran dose/response curves, using identical 125I-labeled α-fetoprotein and antiserum concentrations and the two sets of standards. The two curves were coincident (Figure 2).

Nonspecific Precipitation and Completeness of Precipitation of Bound 125I-labeled α-Fetoprotein with Second Antibody

The amount of nonspecific precipitation in the absence of both first and second antibody ranged from 1 to 2%; nonspecific precipitation in the absence of first antibody and presence of second antibody ranged from 2 to 3%.

The completeness of precipitation of bound 125I-labeled α-fetoprotein by second antibody was evaluated by removing 1 ml of the supernate from all tubes of a precipitated assay set, adding 0.5 ml of 20 ml/liter normal rabbit serum and 0.5 ml of the dilute second antibody solution. After 6 h of incubation, bound and free fractions were separated and counted in the usual manner. The amount of 125I-labeled α-fetoprotein in the bound fraction in no case exceeded that found in the no-antibody control tube in the original assay.

Specificity and Accuracy

Determination of α-fetoprotein in materials from normal subjects. α-Fetoprotein values of normal human sera ranged from <4 to 9 μg/liter. Thirty amniotic fluid samples from women between 13 and 20 weeks of gestation gave values ranging from 2.0 to 28.0 mg/liter. Maternal serum from these patients ranged from 20 to 87 ng α-fetoprotein/ml. The values are in good agreement with normal values for these materials as published by Seppala and Rouslahti (13), Belanger et al. (28), Leighton et al. (20) and Masseyeff et al. (8).

α-Fetoprotein in serum from patients having common serum abnormalities. To determine whether α-fetoprotein could be reliably detected in sera from patients with certain diseases, we analyzed 20 sera with abnormally high enzyme (aspartate aminotransferase, creatine kinase, or alkaline phosphatase) activity or abnormally high concentrations of glucose, lipids, or bilirubin, as well as sera with high titers for rheumatoid factor, and hemolyzed samples for their content of native α-fetoprotein, and then analyzed again after adding known amounts of α-fetoprotein from amniotic fluid, cord blood, or solutions of pure α-fetoprotein. Target values were plotted against the measured values and the results compared (Figure 3). The correlation (r) was 0.99, indicating good agreement. The mean analytical recovery was 95%. The coefficient of variation for the analytical recovery was 10.4%, calculated as:

$$\sqrt{\frac{\sum (x_i - \bar{x})^2}{(n - 1)}}$$

where $x_i$ = sample recovery, %; $\bar{x}$ = mean recovery, %; $n$ = number of samples.

Precision and Reproducibility

We evaluated within-run variance by determining α-fetoprotein concentrations in 15 different samples of sera from women in the second trimester of pregnancy. Duplicate determinations of each sample were run as non-adjacent pairs in the same run. The coefficient of variation was calculated as:

$$\sqrt{\frac{\sum (x_i - \bar{x})^2}{2n \bar{x}}}$$

where $\bar{x}$ is the mean of each duplicate pair, and $n$ is the number of samples. For the range 28 to 235 μg/liter, the value of the coefficient of variation was 4.5%.

The between-run variance was estimated by running 15 such maternal sera five times each in five separate runs and reading the α-fetoprotein values from five different standard curves. The coefficient of variation was calculated as:

$$\sqrt{\frac{\sum (x_i - \bar{x})^2}{(n - 1) \bar{x}}}$$

where $\bar{x}$ is the mean value found for each sample and $n$ is the number of determinations. For the range 9 to 265 μg/liter, the coefficient of variation was 5.5%.
The coefficient of variation for the percent binding of standard samples, calculated for the same runs and with use of the same procedure, was 3.4%. These values compare favorably with similar values published for radioimmunoassay procedures by Seppala and Rouslahti (13), Leighton et al. (20), and Masseyeff et al. (8).

**Accelerated Sequential Procedures**

The success of any sequential procedure depends on the rate of association between antigen and antibody being substantially greater than its rate of dissociation, so that the increased sensitivity or "shift to the left" produced by the binding of unlabeled antigen and antibody during a preliminary incubation is not eliminated by its dissociation and replacement with 125I-labeled α-fetoprotein during the second incubation step.

Association studies were done at 4, 22, and 37 °C by using 1.5 ng of 125I-labeled α-fetoprotein and a 480,000-fold dilution of antibody (Figure 4). Maximum binding occurred at 24, 20, and 10 h, respectively, for these incubation temperatures. Dissociation was followed by allowing the same concentrations of 125I-labeled α-fetoprotein and antibody to bind to equilibrium, at which time 400 ng of unlabeled α-fetoprotein was added to each tube. The reaction was followed by terminating the incubations at selected intervals. At 4 and 22 °C, no dissociation was seen after 24 h of incubation with unlabeled α-fetoprotein. At 37 °C, dissociation at 8 h was 3%.

We explored the flexibility of the assay system with respect to sensitivity, range, and duration within the restrictions of the same concentrations of antibody and labeled antigen by manipulating the basic sequential procedure as follows:

In an attempt to shorten the incubation time necessary for a sensitive sequential procedure, we allowed standard doses of unlabeled α-fetoprotein and antibody to incubate at 4 °C for 16 h, a time at which association studies indicate that more than 85% of the maximum binding of 1.5 ng of 125I-labeled α-fetoprotein is reached. Post-incubation with 125I-labeled α-fetoprotein was terminated at 4, 8, 24, and 32 h. A comparative plot of B/T vs. dose for these incubation times (Figure 5A) indicates that there is a sufficient range in the binding of 125I-labeled α-fetoprotein from low to high doses even at 4 h post-incubation to produce a useful dose/response curve (Figure 5B). Extending the post-incubation time to equilibrium results in a slight increase in slope and better resolution of values of less than 1 ng/liter. Midpoint sensitivity was the same for all dose/response curves, no matter how long the period of post-incubation.

To demonstrate that the linear position of the dose/response curve in which dose estimates are made most precisely can be shifted in range, the sequential procedure was altered by shortening the time of the preincubation to 6 h at room temperature, a point at which a 1.5-ng dose was shown to be bound to 70% of maximum. At this time 125I-labeled α-fetoprotein was added and allowed to incubate overnight, after which the assay was terminated. This manipulation shifted the linear portion of the dose/response curve to a range intermediate between that of the 24-h equilibrium and 40-h sequential procedures. The sensitivity of this assay is also intermediate. The pre- and post-incubation times were selected because they conform to normal 8-h working day schedules, but experience with the assay described previously indicates that the dose/response curve would still be usable if the post-incubation period were shorter.

The time required to perform the assay can be considerably shortened by incubating at 37 °C. The conditions of the rapid 37 °C sequential assay are similar to the 40-h sequential procedure in that the preliminary incubation is for a period of time (4 h) during which the α-fetoprotein dose as determined by previous studies with labeled antigen binds to 85% of maximum. However, since dissociation of the antigen-antibody complex is significant under the conditions of the assay, binding of 125I-labeled α-fetoprotein presumably occurs through this mechanism as well as through the binding of excess available antibody.

A comparison of the dose/response curves for the equilibrium procedure and the various sequential procedures is shown in Figure 6 and Table 1.

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Comparison to Other Assays

As an assessment of the suitability of this double-antibody radioimmunoassay for the determination of α-fetoprotein in human sera, it was compared to two other assay procedures.

Comparison with another radioimmunoassay procedure. The double-antibody radioimmunoassay was compared to a similar assay developed by Wespic. The latter procedure was one terminated by a salting-out of the antigen–antibody complex with Na2SO4 (160 g/liter) solution, rather than by immunoprecipitation. Dose/response curves for the two assays were run under identical conditions, using the same standards and dose of 125I-labeled α-fetoprotein. The first antibody used in the salting-out procedure was raised in a goat by using purified α-fetoprotein prepared by another laboratory. The two dose/response curves were similar in shape and slope, the only difference being one of sensitivity (Figure 7). The double-antibody procedure was free of the significant amount of nonspecific precipitation of 125I-labeled α-fetoprotein that is inherent in the salt-precipitated technique—which ranges from a minimum of 6% to as much as 20%, and seriously affects precision, especially in the high-dose region of the standard curve. It is presumably caused by the presence of dimers and protein aggregates of comparable size present in the labeled antigen. Molecules of high molecular weight would be precipitated with the antigen–antibody complexes in appropriate concentration of sulfate salts but remain in solution during the immunoprecipitation of a double-antibody procedure.

Comparison with an enzyme immunoassay procedure. The double-antibody radioimmunoassay was also compared to an enzyme immunoassay developed by Belanger et al. (28). In this study, identical reagents—125I-labeled α-fetoprotein, antiserum, and standards—were used, the only difference being the methodology and the concentration of antigen and antiserum [125I]-labeled α-fetoprotein: 1.5 ng for radioimmunoassay, 10 ng for enzyme immunoassay; antiserum: (rabbit anti-α-fetoprotein) 1/480 000 for radioimmunoassay, 1/80 000 for enzyme immunoassay.] The assays were run under identical conditions, both by sequential procedures. These dose/response curves were also similar in shape and slope (Figure 8) and differed in sensitivity by a factor of 10, the radioimmunoassay having a minimum sensitivity of about 500 pg, as compared to 6 ng for the enzyme immunoassay, and a comparable difference in the dose bound at mid-range. These differences are due

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Table 1. Comparison of Data for Various Radioimmunoassay Procedures for α-Fetoprotein

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<tr>
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<td>500 pg</td>
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<td>50% intercept</td>
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<td>11.5 ng</td>
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<td>8.0%</td>
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Fig. 6. Dose/response functions for various assay procedures. A, 24-h equilibrium; B, 40-h sequential (16/24); C, 24-h sequential (6/16); D, 7-h, 37 °C sequential. 125I-labeled α-fetoprotein = 1.5 ng; rabbit anti-α-fetoprotein, 1/480 000. Dose/response functions for assays A and B were similar whether the procedures were done at 4 or 22 °C. Assay C was preincubated at room temperature, and incubated with 125I-labeled α-fetoprotein at either 22 or 4 °C.

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Fig. 7. Comparison of dose/response function from double-antibody radioimmunoassay with that of radioimmunoassay terminated by Na2SO4 precipitation. 125I-labeled α-fetoprotein dose: 1.5 ng, both assays; antiserum for double-antibody radioimmunoassay, rabbit anti-α-fetoprotein, 1/480 000; for Na2SO4 radioimmunoassay, goat anti-α-fetoprotein, 1/24 000

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Fig. 8. Comparison of dose/response function of double-antibody radioimmunoassay with that of double-antibody enzyme immunoassay. 125I-labeled α-fetoprotein dose: radioimmunoassay, 1.5 ng; enzyme immunoassay, 10 ng. Antiserum (rabbit anti-α-fetoprotein): radioimmunoassay, 1/480 000; enzyme immunoassay, 1/80 000

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to the requirement of the enzyme immunoassay for higher concentrations of both antigen and antibody, which reduces sensitivity.

A set of 25 samples was run on both assays and their α-fetoprotein values compared (Figure 9). These were found to be in excellent agreement, having a correlation coefficient (r) of 0.99.

In connection with another study of Belanger et al. (28), 25 samples were run under identical conditions with the Na2SO4 radioimmunoassay and their enzyme immunoassay, with use of the same 125I-labeled α-fetoprotein, α-fetoprotein standards, and the same respective antisera described here. The correlation of values from these assays also proved to be excellent (r = 0.99).

Clinical Assessment: α-Fetoprotein in Pregnancy

Serial measurements of α-fetoprotein in serum were made during two pregnancies by taking serum samples at least biweekly, in one case from the 8th week of pregnancy until two weeks after delivery, and in the other from the 10th through the 27th week (Figure 10). In the first case, α-fetoprotein concentrations in serum increased above the normal at about 13 weeks of pregnancy, and advanced to 60 μg/liter by the end of the first trimester. They rose to 200 μg/liter by the end of the second trimester, and reached a peak of 295 μg/liter at the 33rd week. After this, there was a decline in α-fetoprotein concentration to 110 μg/liter on the day of delivery. Two weeks postpartum, the value was 50 μg/liter. In the case of the second pregnancy, a similar pattern was seen. Again, this is in good agreement with the findings of Belanger et al. (28) and Seppala and Ruoslahti (13).

Samples of amniotic fluid from two patients and the mother's serum from one of these had α-fetoprotein values above the normal published range. Patient A at 18 weeks of gestation had a concentration of α-fetoprotein in her serum of 880 μg/liter; the concentration in the amniotic fluid was 205 mg/liter. Anencephaly was confirmed by sonogram; however, the pregnancy was continued. The mother's α-fetoprotein concentrations in serum at 27, 29, and 32 weeks were 765, 545, and 440 μg/liter, respectively.

Patient B at 16 weeks had an amniotic fluid value of 415 μg of α-fetoprotein per liter. The blood was not sampled at this time. Anencephaly was confirmed by sonography and the pregnancy terminated at 20 weeks. The value for the mother's serum preceding abortion was 580 μg/liter, for the amniotic fluid obtained at abortion 250 mg/liter.

Discussion

The double-antibody radioimmunoassay procedures described offer a rapid, precise, and highly reproducible determination of α-fetoprotein in human serum and amniotic fluid. They are superior to radioimmunoassay procedures that require a salting-out of bound antigen for termination (Farr technique), because they are free of the nonspecific precipitation of radiolabeled antigen inherent to the latter procedure. Nonspecific precipitation results in a background count that must be subtracted as a constant from the counts of all precipitates in the assay. The background correction diminishes the performance of an assay, not only because of its magnitude, but because of its variability. The major effect of its error is to increase the relative error at those levels where counts are low, the high-dose region (26). Background error also introduces an error into the determination of the counts bound at zero dose, and because all doses are evaluated relative to zero dose, this ultimately affects all determinations of the assay and produces shifts in the dose/response curves from one assay to the next.

Although enzyme immunoassay is attractive because it avoids the problem associated with the use of radioisotopes, our data indicate that radioimmunoassay is superior in efficiency, sensitivity, and reproducibility. The equilibrium double-antibody radioimmunoassay procedure, requiring 24 h total incubation time, is highly precise and reproducible at the upper limit of normal concentration of α-fetoprotein in human serum (10 to 20 μg/liter). To bring such concentrations into a range of high reproducibility by enzyme immunoassay re-
quires a sequential procedure involving three days of incubation. In comparable equilibrium and sequential procedures radioimmunoassay is three- to 10-fold more sensitive than enzyme immunoassay. Over its equilibrium assay range, the reproducibility determined for this radioimmunoassay is 5.5%, as compared to 10% for the same range reported by Belanger et al. for their enzyme assay (28). In studies of α-fetoprotein where demands for precision are stringent, as in the identification of fetal neural-tube defects based on maternal serum α-fetoprotein concentrations, double-antibody radioimmunoassay is the method of choice.

A major advantage of the radioimmunoassay procedures described here is the flexibility in dose range, which may be exploited to optimize the sensitivity and precision of α-fetoprotein determinations required by various studies. Using not only this criteria of the anticipated concentrations of α-fetoprotein in the sample to be analyzed, but also the urgency of the need for a result, a person may select, from among the assay procedures presented, that which is most nearly accurate and appropriate to the situation.

For routine determination of α-fetoprotein in the serum of the mother, the 24-h equilibrium procedure is most applicable. The mid-point of the dose/response curve, where variance is least, occurs at 70 μg/liter, about the mean of the α-fetoprotein values in serum during the 13th to 20th weeks of pregnancy, when screening for fetal neural tube defects is usually done. The linear portion of the curve covers the entire range of values during this period of a normal pregnancy, and samples can be run without dilution. The 40-h sequential procedure is prolonged and suffers the inconvenience of an additional operation in the delayed addition of antigen. However, it has some application in making very accurate assessments of normal α-fetoprotein concentrations in serum, because the midpoint of the curve lies at about 12 μg/liter, the upper limit of normal human serum values. Terminating the procedure prematurely—that is, before the labeled antigen has reached equilibrium with antibody—preserves the precision of the assay from the standpoint of midpoint dose and range of linear dose response, while sacrificing only the ability to estimate concentrations of α-fetoprotein that are below the level of clinical usefulness.

The 24-h (6:16) accelerated sequential procedure is a manipulation that allows an increase in the sensitivity of the assay by means of delayed addition of antigen, which nonetheless can be done in the same time as the equilibrium procedure. The inconvenience of an extra operation is offset not only by the gain in sensitivity but by the greater precision common to sequential procedures (27, 28). This procedure, in which the mid-point of the dose/response curve lies at 25 μg/liter, is best applied in analyses of serum samples which may or may not have supranormal α-fetoprotein concentrations. The 37 °C, 7-h sequential procedure of course has the advantage that data can be provided the same day the sample is received. Although it is not as reproducible as the equilibrium procedure (CV, 8.0% vs. 5.5%), this is as good or better than most radioimmunoassay procedures.

Preliminary efforts to adapt these double-antibody radioimmunoassay procedures to automation by using a Micro-Medic apparatus have been highly successful.

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