MEASUREMENT OF THE STAPHYLOCOCCAL EPIDERMOLYTIC TOXIN: A COMPARISON OF BIOASSAY, RADIAL IMMUNODIFFUSION, AND RADIOIMMUNOASSAY

Kirk D. Wuepper, M.D., Diane Haas Baker, M.D., and Robert L. Dimond, M.D.

Department of Dermatology, School of Medicine, University of Oregon Health Sciences Center, Portland, Oregon, U.S.A.

Three methods of measuring the epidermolytic toxin of Staphylococcus aureus—bioassay in newborn mice, radial immunodiffusion, and radioimmunoassay—were compared for reproducibility, specificity, and sensitivity. The bioassay is highly specific and remains the only functional assay. It is reproducible only if newborn mice of the same age are used. The time required for epidermolysis follows a dose–response relationship only if concentrations of toxin large enough to cause peeling in 90 min or less are used. This limits the sensitivity of the bioassay to about 5 µg per ml.

Single radial immunodiffusion in agar is a specific and reproducible assay method, but its sensitivity is also about 5 µg per ml.

A radioimmunoassay was established by the Farr technique using purified epidermolyisin radiolabeled with 125I-iodine. This assay was highly reproducible and specific. The staphylococcal products, α-toxin and enterotoxins A and B, did not cross-react with anti-epidermolyisin antibodies. The sensitivity of the radioimmunoassay is 20 ng per ml.

In 1970 Melish and Glasgow proposed that an epidermolytic toxin was responsible for the staphylococcal scalded-skin syndrome [1]. Since then this toxin, (called by various authors epidermolyisin, epidermolytic toxin, exfoliatin, exfoliating toxin), has been purified and partially characterized by several investigators [2–6]. Accurate measurement of toxin is necessary to further characterize it and to delineate its role in Staphylococcus aureus-associated scalded-skin syndrome.

Measurement of epidermolyisin has been limited to a functional bioassay in newborn mice. In our laboratories, bioassay results were found to be quite variable when the same amount of toxin was injected into several mice from different litters. Because of this observation, the following study was undertaken to examine the factors producing variability in bioassay results. We have also developed other more sensitive and reproducible ways to measure the epidermolytic toxin, radial immunodiffusion, and radioimmunoassay.

MATERIALS AND METHODS

Epidermolytic toxin was obtained and purified as previously described [6] from Strain EV, a phage group II, type 55/71, coagulase-positive S. aureus, isolated from a case of staphylococcal scalded-skin syndrome. Antiserum to epidermolyisin was produced in rabbits as previously described [6].

Bioassay

Newborn Webster/Swiss mice (Simonson Laboratories, Gilroy, Calif.) of known age (1 to 5 days) were injected subcutaneously, in duplicate, into the nape of the neck. We used 25-gauge needles, and 0.02-ml volumes of material were assayed for epidermolytic toxin activity. The time required for production of a localized Nikolsky sign in the skin overlying the site of injection was recorded as the end point.

Radial Immunodiffusion

Radial immunodiffusion in agar was carried out according to the method of Mancini et al [7]. In brief, a 1.5% agar gel plate (Difco, Detroit, Mich.) was prepared with 7.5% rabbit antisera to epidermolyisin incorporated into the agar. Circular wells were cut into the agar to allow introduction of 7-µl amounts of the material to be assayed for epidermolyisin. Radial diffusion took place over a 7- to 10-day period at room temperature in humidity chambers. The diameters of the circular precipitin bands that formed were measured using a microscope ocular equipped with a micrometer, and the areas of the circles were calculated. Known amounts of epidermolyisin were introduced into wells in the same agar gel plate in which unknowns were mea...
sured, allowing a standard curve to be prepared for each assay run.

**Radioiodination of Epidermolysin**

Purified epidermolysin was radioiodinated with $^{125}$Iodine (1 mCi/50 μl, New England Nuclear, Boston, Mass.) and 50 μl of chloramine T (1 mg/ml) were added to purified epidermolysin (1200 μg in 0.5 ml). Fifty μl of sodium metabisulfite (1 mg/ml) were added to terminate the reaction. Bovine serum albumin (BSA) (Schwarz/Man, Orangeburg, N.Y.), 0.2% in saline, was added as carrier. Free $^{125}$I was separated from radioiodinated epidermolysin by extensive dialysis and passage over a Sephadex G-50 column [8].

Saturated ammonium sulfate precipitation was investigated as a means of separating free from antibody-bound epidermolysin [9]. Radioiodinated epidermolysin was incubated overnight at 4°C with phosphate-buffered saline, rabbit serum, human serum, or rabbit antiserum to epidermolysin. Cold saturated ammonium sulfate was then added in amounts sufficient to obtain final concentrations of ammonium sulfate from 20 to 90%. The precipitate which formed was centrifuged, separated from the supernatant, and counted in a well-type gamma counter.

**Characterization of the Rabbit Antiserum Used in the Radioimmunoassay**

Rabbit antiserum to epidermolysin was tested for its ability to bind radioiodinated epidermolysin as follows: 0.5 ml of doubling dilutions of anti-epidermolysin was incubated overnight at 4°C with constant amounts of $^{125}$Iepidermolysin (100 ng in 0.5 ml). Following incubation, 1.0 ml of saturated ammonium sulfate was added. Precipitates were centrifuged at 4°C at 3000 g for 20 min, washed with 50% saturated ammonium sulfate, re-centrifuged, and radioactivity in precipitates was counted in a well-type gamma counter.

**Assay System**

To assay unknown samples for epidermolysin the following system was used: 0.5 ml of rabbit anti-epidermolysin (1:750) was added to 0.5 ml of $^{125}$Iepidermolysin and 0.5 ml of varying dilutions of sample to be assayed. Following incubation overnight at 4°C, 1.5 ml of saturated ammonium sulfate was added to effect precipitation of antibody-bound epidermolysin. Precipitates were centrifuged, washed with 50% saturated ammonium sulfate, re-centrifuged, and radioactivity was determined. A standard curve was constructed with known quantities of epidermolysin, measured by the method of Lowry et al [10] with BSA as standard.

To determine whether other staphylococcal products—α-toxin, enterotoxin A, or enterotoxin B—could inhibit binding of $^{125}$Iepidermolysin to rabbit anti-epidermolysin, $^{125}$Iepidermolysin (100 ng in 0.5 ml), rabbit anti-epidermolysin (1:750, 0.5 ml), and amounts of α-toxin, enterotoxin A, or enterotoxin B from 0.5 to 100 μg were incubated together overnight at 4°C. Saturated ammonium sulfate precipitation, washing, and gamma counting of precipitates were carried out as described.

**RESULTS**

**Bioassay of Epidermolysin**

The results of bioassay of a single batch of toxin in mice of varying age are given in Figure 1. The end point of the assay, loosening of the epidermis overlying the injection site in response to rubbing with a finger (Nikolsky sign), was clear-cut and could be measured accurately within a 2- to 5-min interval. There was close agreement between duplicate animals.

The relationship between the amount of epidermolysin injected (dose) and the time required for epidermolysin to develop (response) was linear only if the response occurred in less than 60 to 90 min. The results became erratic and nonlinear thereafter. As shown in Figure 1, the dose—response varied with the age of the mice injected; the assay was more sensitive in mice less than 24 hr old.

To exclude the possibility that deterioration of the toxin was responsible for the delayed responses observed in older animals, experiments were repeated in 1-day-old mice. The results coincided with the first determination, confirming that the toxin was stable. The minimum time required for epidermolysis was about 15 min. This was presumably due to the time required for passive diffusion from the intradermal injection site to the overlying epidermis. Apparently, systemic adsorption also takes place, since animals which received higher doses would develop a positive Nikolsky sign at any skin site several hours after the initial injection.

**Radial Immunodiffusion Assay (Mancini) for Epidermolysin**

Single radial diffusion of antigen into agar containing antibody to epidermolysin provided a reproducible, convenient assay (Fig. 2). The single, sharp rings of precipitation developed fully in 7 to 10 days and were suitable for measurement of toxin concentrations from 5 to 75 μg per ml.

**Radioimmunoassay (Farr) for Epidermolysin**

Radioiodination of epidermolysin with $^{125}$I did not cause loss of activity in the newborn mouse bioassay. The minimum detectable concentration of
obtained when known amounts of unlabeled epidermolysin were allowed to compete with $^{125}$I epidermolysin for binding to antiserum in the radioimmunoassay system (Fig. 5 left panel). Using the logit method for linearization of data [11], the graph in Figure 5 right panel was obtained. Using this assay we are able to measure epidermolysin in con-

$[^{125}]$ epidermolysin by bioassay was the same as that of unlabeled toxin, about 5 $\mu$g/ml. The label was indeed bound to the toxin, since radioactivity and toxin comigrated in disc gel electrophoresis and the precipitin rings which formed in Mancini radial diffusion plates contained the radioactivity. The specific activity of the $[^{125}]$ epidermolysin was 0.078 Ci/gm.

The solubility of the toxin in ammonium sulfate is shown in Figure 3. With a 50% saturated solution of ammonium sulfate, when $[^{125}]$ epidermolysin was incubated with immune rabbit serum (1:100), 80% of the radioactivity added was precipitated; whereas, when $[^{125}]$ epidermolysin was incubated with selected human sera (1:5), normal rabbit serum (1:5), or phosphate-buffered saline, less than 5% of the radioactivity added was precipitated. That is, $[^{125}]$ epidermolysin remains soluble in 50% saturated ammonium sulfate unless it is bound to specific antiserum. This allows distinct separation of free from antibody-bound epidermolysin in the radioimmunoassay.

Figure 4 illustrates the characterization of the rabbit antiserum selected for use in the radioimmunoassay. Antigen-binding capacity, expressed as ABC-33, is calculated from the reciprocal of the serum dilution which precipitates 33% of the antigen added (in this case 2150, as indicated in Figure 4). The ABC-33 of this antiserum was 27.7 $\mu$g antigen nitrogen per ml [9]. In the radioimmunoassay itself, a dilution of antiserum (1:750) capable of precipitating 60% of the antigen added was used.

A standard curve for comparison when measuring unknown amounts of epidermolysin was

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**Fig. 2.** Standard curve for measurement of epidermolysin by single radial immunodiffusion in agar. Inset shows typical precipitin rings that develop 7 to 10 days after 7-$\mu$l sample is introduced into wells cut in agar containing 7.5% antibody.

**Fig. 3.** Solubility of $[^{125}]$ epidermolysin in ammonium sulfate following preincubation with phosphate-buffered saline - - - , normal rabbit serum (1:5) O --- O, “normal” human serum (1:5) $\triangle$ - - $\triangle$, or dilute rabbit antiserum (1:100) $\Delta$ - - $\Delta$.

**Fig. 4.** Characterization of the rabbit antiserum used in the radioimmunoassay for epidermolysin. The antigen-binding capacity of the antiserum was determined from the ABC-33 end point which is indicated on the graph.
centrations from 20 to 250 ng per ml. Staphylococcal α-toxin, enterotoxin A, or enterotoxin B did not significantly inhibit binding of \(^{125}\text{I}\)epidermolysin to rabbit anti-epidermolysin.

**Measurement of \(^{125}\text{I}\)Epidermolysin in Vivo**

After injection of 20 μg of \(^{125}\text{I}\)epidermolysin intraperitoneally in 6 1-day-old mice, blood was obtained at 30-min intervals from the tail vein in 10-μl heparinized micropipets. The whole blood levels of epidermolysin up to the time of generalized peeling or death are shown in Figure 6. Four animals survived the experiment and experienced generalized positive Nikolsky phenomena. Two animals died, possibly due to excessive blood loss, and did not develop epidermolysis.

Epidermolysin was also measured by direct gamma radiation counting of weighed whole skin or peeled epidermis obtained at the time of generalized peeling in the four surviving animals as shown in Figure 7. The concentration of epidermolysin in peeled epidermis seems to be increased compared to the concentration in whole skin or blood at the time of generalized peeling.

**DISCUSSION**

A systematic evaluation of the factors that influence the measurement of the staphylococcal epidermolytic toxin by bioassay revealed that the age of mice used in the assay has a critical influence on the results obtained (Fig. 1). The variability we experienced in day-to-day testing of the toxin was eliminated if mice of identical age were employed and if we used the linear portion of the dose–response curve. For the functional assay, we recommend that a unit be established which falls within the linear portion of the curve. For example, we presently define one unit as that quantity of epidermolysin in 0.02 ml which will result in epidermal separation 60 min after intracutaneous injection in a 1-day-old mouse. In our hands, this corresponds to approximately 500 ng of highly purified epidermolysin.

We prefer to inject a small volume, 20 μl, since appreciable leakage of the injected fluid occurs with larger volumes (such as 50 or 100 μl). We also favor intracutaneous injections and testing for

![Fig. 5. Standard curve for measurement of epidermolysin by radioimmunoassay. The left panel shows raw data obtained when known amounts of "cold" epidermolysin were allowed to compete for binding to anti-serum with \(^{125}\text{I}\)epidermolysin in the assay system. The right panel represents the same data plotted by the logit method for linearization of radioimmunoassay data.](image)

![Fig. 6. Whole venous blood levels of \(^{125}\text{I}\)epidermolysin following injection of 20 μg of \(^{125}\text{I}\)epidermolysin intraperitoneally into each of 6 newborn mice. Blood levels rose up to the time when a generalized positive Nikolsky sign could be elicited, 3 to 4 hr post injection. Two animals died before peeling occurred.](image)

![Fig. 7. \(^{125}\text{I}\)Epidermolysin levels in blood, whole skin, and peeled epidermis of four newborn mice at the time of generalized peeling 3 to 4 hr post intraperitoneal injection of 20 μg per mouse. The toxin seemed to be concentrated in peeled epidermis on a μg per gm basis as compared to whole skin or blood.](image)
peeling in the skin overlaying the injection site, because the time required for assay is short by this method and the assay has a crisp end point. The animals need not be returned to their mothers in such short-term experiments and this prevents loss due to cannibalism.

The minimum detectable concentration of epidermolysin in the bioassay is 5 μg per ml (100 ng in 0.02 ml), although with this dose peeling is usually noted 3 to 4 hr after injection and occurs in the nonlinear portion of the dose–response curve. To our knowledge, the only functional assay of epidermolysin is the bioassay.

Immunoadsorbs offer an advantage of precision and reproducibility but do not measure function. Radial immunodiffusion is readily adapted to precise measurement of the toxin. The sensitivity of this assay was similar to bioassay, since the minimum concentration that could be measured accurately by this assay was also about 5 μg per ml. Radial immunodiffusion is useful for preparative studies and toxin purification, since it does not require the use of laboratory animals. However, for measurement of toxin or antibody to toxin in serum or body fluids in a clinical setting, a still more sensitive assay method, the radioimmunoassay, was required. Bioassay or radial immunodiffusion did not detect toxin in serum of children experiencing the staphylococcal scalded-skin syndrome.

Since the toxin was soluble in half-saturated ammonium sulfate, a single antibody radioimmunoassay was readily developed using the Farr technique [9]. Its sensitivity is as low as 20 ng per ml and its specificity was established by addition of known quantities of cold epidermolysin to serum. The staphylococcal products α-toxin, enterotoxins A, or enterotoxin B did not inhibit binding of [125I]epidermolysin to specific antibody in the radioimmunoassay, nor did they form precipitin bands against rabbit anti-epidermolysin by double immunodiffusion in agar (method of Ouchterlony).

Kondo and associates have recently reported the existence of two serotypes of epidermolysin [12, 13]. Phage group II S. aureus strains usually produce the heat-stable epidermolysin; non-phage group II organisms produce a less stable toxin which is antigenically distinct. Some organisms produce both types. The epidermolysin from the S. aureus isolate we studied was heat stable and gave a single precipitin band in Ouchterlony double immunodiffusion studies [6]. Therefore, our radioimmunoassay may not detect the less stable epidermolysin. Wiley and associates have reported the development of a primary binding assay for human antibody to epidermolysin [14], but gave no data on measurement of epidermolysin itself.

We detected radioactivity in the blood, whole skin, and peeling epidermis 3 hr after intraperitoneal injection of [125I]epidermolysin in newborn mice. [125I]epidermolysin was concentrated 2- to 3-fold in the epidermis as compared to whole skin or blood. Although no conclusions about mechanism of action can be drawn from this study, it does suggest that epidermolysin binds preferentially to a site or sites in the epidermis.

S. aureus-associated scalded-skin syndrome is a dramatic but rarely fatal form of epidermal injury. Toxic epidermal necrolysis of nonstaphylococcal origin, usually caused by drugs, is more often fatal and is difficult to distinguish clinically from the S. aureus-associated disease. The treatment of these two diseases differs, so early distinction between them is important. We plan to use the radioimmunoassay to study this problem, and to study the other forms of the staphylococcal scalded-skin syndrome, staphylococcal scarlatiniform eruption and bullous impetigo.

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